siRNA Transfection Into Primary Neurons Using Fuse-It-siRNA

This Application Note describes a protocol for siRNA transfection into sensitive, primary cortical neurons using Fuse-It-siRNA. This innovative reagent perfectly combines highly efficient siRNA transfection rates with excellent biocompatibility—even in sensitive cells, such as primary neurons. In this Application Note, primary rat cortical neurons were treated with Piezo1 siRNA twice within 48 hours to generate a highly efficient and stable knockdown over time.

Keywords:
Fuse-It-siRNA, siRNA transfection, siRNA transfer, RNA interference, RNAi, knockdown, gene silencing, small non-coding RNAs, primary neurons

Table of Contents

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. [1]

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). [2]

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.

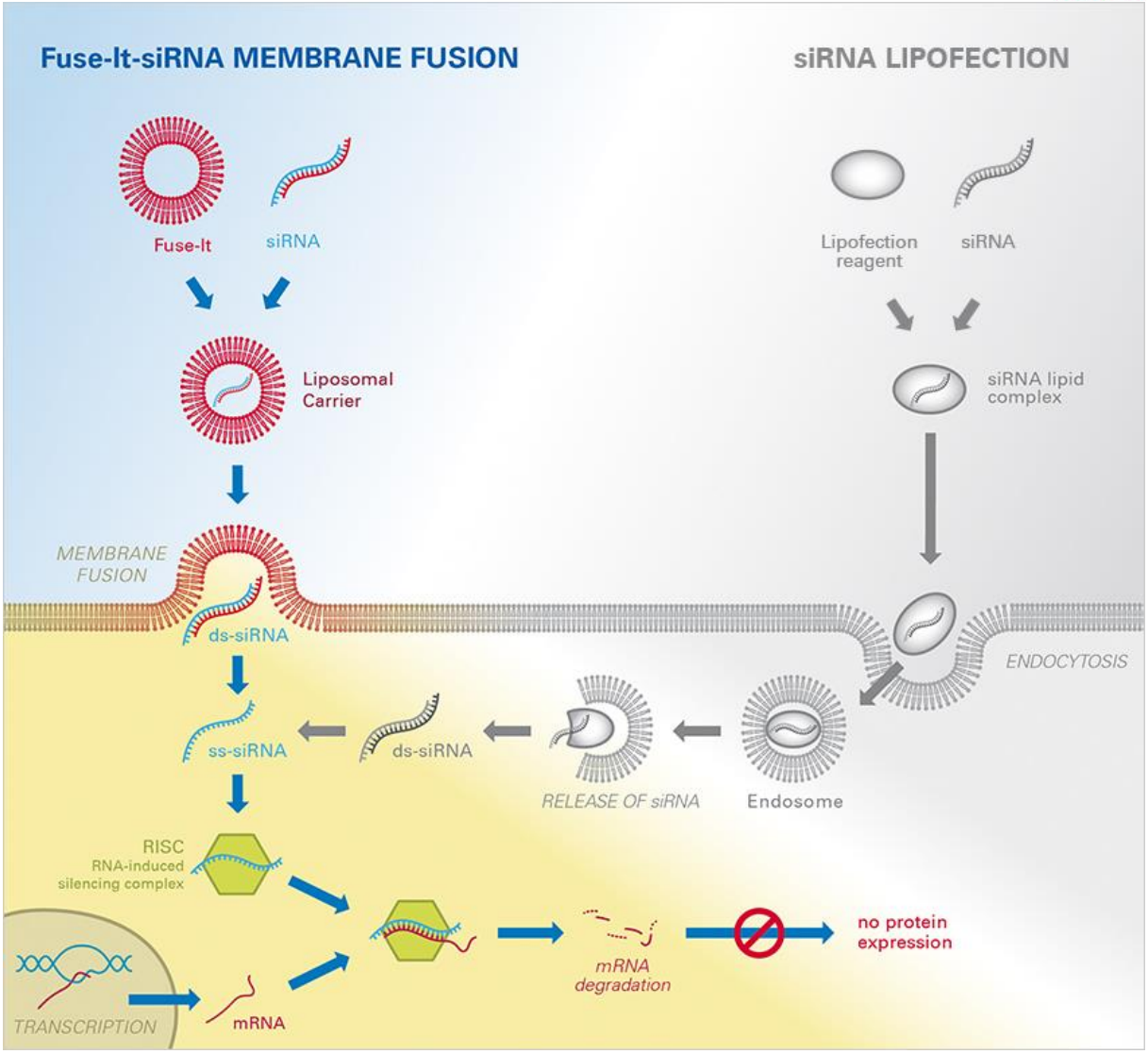

Figure 1: Simplified illustration of the membrane fusion mechanism of Fuse-It-siRNA vs. classical lipofection and the subsequent pathway of gene silencing by RNAi.

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 Piezo1 in primary neurons.

Name	Company	Order No.
Fuse-It-siRNA	ibidi GmbH	60510
6-well plate	VWR	10861-696
Rat cortical neurons	Self-isolated or various	Various
Neurobasal Medium	Thermo Fisher Scientific Inc.	21103049
B27 supplement	Thermo Fisher Scientific Inc.	17504044
Poly-L-lysine	Sigma-Aldrich Chemie GmbH	P4832
Piezo1 siRNA (FlexiTube GeneSolution)	Qiagen	GS9780 (1027416)
1x PBS	Sigma-Aldrich Chemie GmbH	D8537
Ultra-pure water	Various	Various
RNeasy Plus Mini Kit	Qiagen	74136
QuantiTect Reverse Transcription Kit	Qiagen	205313
Piezo1 rat primer	Thermo Fisher Scientific Inc.	Rn01432593_m1
GAPDH rat primer (endogenous control)	Thermo Fisher Scientific Inc.	Rn01775763_g1

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

- Ultrasonic bath (power 50 W, frequency 35 kHz)
- Quantitative real time polymerase chain reaction (qRT-PCR)–equipment

Important Note: Quantitative measurement of the knockdown efficiency can be performed on mRNA level using qRT-PCR. To measure the knockdown quantitatively on protein level, a western blot with specific antibodies, e.g. Piezo1 rat (Anti-FAM38A, Abcam plc, Ab128245) can be performed as well.

3. Experimental Procedure

In this Application Note, the knockdown of Piezo1, a mechanosensitive ion channel protein, by siRNA transfer into primary rat cortical neurons is described. Here, two technical replicates were performed of each condition: the fusion experiment and the untreated control. Therefore, four wells of the 6-Well Plate (growth area 9.5 cm² per well) were required.

1.1. Coating the 6-Well Plate with Poly-L-Lysine (PLL)

1. Calculate the coating concentration: The coating concentration of PLL is 2 µg/cm², resulting in 19 µg PLL per well.
2. For a final volume of 4 ml, mix 760 µl of the PLL stock solution (100 µg/ml) and 3240 µl of ultra-pure water. The final concentration of PLL is 19 µg/ml.
3. Add 1 ml of PLL dilution to each well.
4. Incubate 1 hour at 37°C.

5. Aspirate the PLL solution from each well.
6. Wash each well once with 1 ml of ultra-pure water.

1.2. Seeding Cells

Seed 1 ml cell suspension with a concentration of $1x10^6$ cells/ml in each well (growth area 9.5 cm^2). Incubate primary cortical neurons for 4–7 days at 37°C using standard cell culture conditions. Exchange the medium daily to ensure network formation and expression of specific Piezo1 mRNA. Be careful, only half of the medium volume should be exchanged daily to avoid network deletion. The optical cell density at the time of transfection should be 70–90 %.

1.3. Fusion Protocol with Fuse-It-siRNA

Two technical replicates were performed for the siRNA transfer by Fuse-It-siRNA. All steps were performed according to the standard protocol given in the "Fuse-It-siRNA Instructions".

1. Cool down the ultrasonic bath with an ice pack.
2. Thaw the Piezo1 siRNA on ice.
3. Thaw the Neutralization Buffer (NB) and the Fusogenic Solution (FS) at room temperature.
4. Mix 180 pmol of siRNA and 36 µl of NB thoroughly. Incubate for 10 minutes at room temperature.
5. Vortex the FS until the solution is homogeneous.
6. Transfer 10 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.
7. Add the neutralized siRNA (siRNA + NB from step 4) and mix thoroughly.
8. Sonicate the mixture in the ultrasonic bath for 5 minutes.
9. Dilute the fusogenic mixture in 1x PBS to make a final volume of 2 ml.
10. Sonicate the dilution in the ultrasonic bath for 5 minutes.
11. Remove the medium completely from the cells. Be careful to avoid network deletion.
12. Add 1 ml of the fusogenic mixture, drop-wise, onto the cells of each of the two wells.
13. Incubate for 10 minutes at 37°C.
14. Replace the fusogenic mixture of each well with 2 ml of fresh culture medium.
15. Incubate the cells for 24 hours at 37°C using standard cell culture conditions.
16. Optionally: To ensure a highly efficient knockdown on protein level repeat the Fusion Protocol (steps 1–15) 24 hours after the first fusion process.

Important Note: Fuse-It-siRNA is of highest biocompatibility, therefore, repetitive fusions for efficient protein reduction of very stable proteins do not influence cell viability, even in primary or sensitive cells.

17. Image the cells using phase contrast and fluorescence microscopy. Liposomal fusion efficiency is most easily determined by tracking the infrared control dye at 780 nm.

1.4. Quantitative Analysis by qRT-PCR

Perform all qRT-PCR steps according to the manufacturer's protocols.

1. Isolate the total RNA of the cells using RNeasy Plus Mini Kit.
2. Measure RNA-concentration using a NanoDrop.
3. Transcribe total mRNA into cDNA using QuantiTect Reverse Transcription Kit.
4. Perform the qRT-PCR by a TaqMan Assay using the Piezo1 rat primer and the GAPDH rat primer as endogenous control.
5. Analyse the qRT-PCR results using StepOne Real-Time PCR System and StepOne Software.

4. Results and Discussion

For the knockdown of Piezo1 mRNA, primary rat cortical neurons were fused twice within 48 hours using Fuse-It-siRNA. Due to the extraordinary biocompatibility and endocytosis-independent transfer of siRNA by Fuse-It-siRNA, the sensitive primary cortical neurons did not show any morphological changes, and have a similar morphology as nontreated control cells (Figure 2).

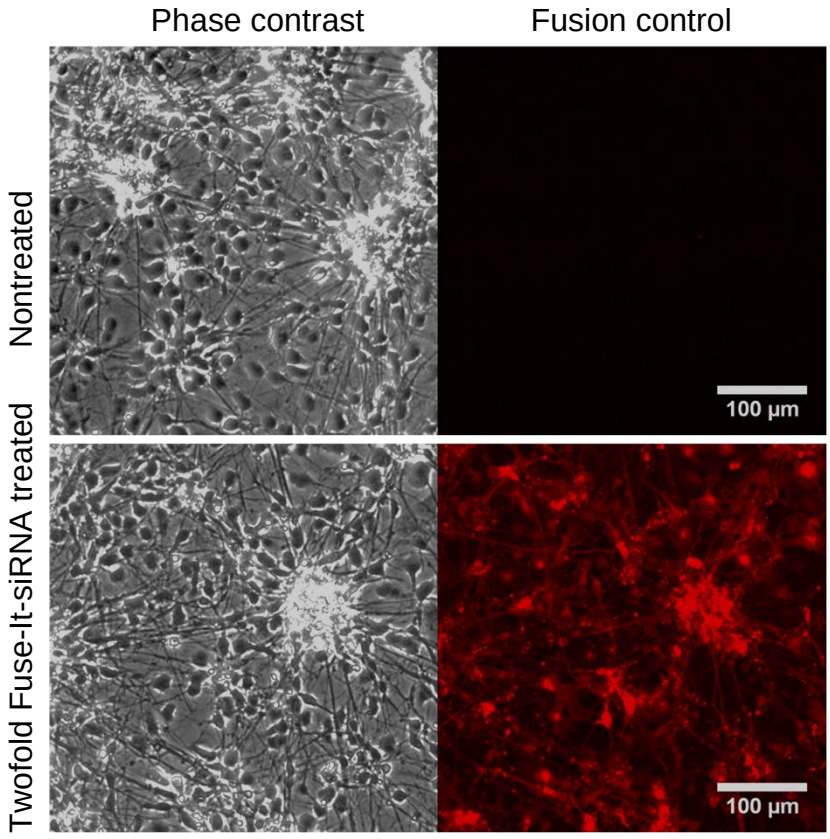

Figure 2: Live cell images of rat cortical neurons 24 hours after the second treatment with Fuse-It-siRNA. The images on the left show phase contrast images of the neuronal cells and the images on the right show the appropriate fluorescence images of the infrared fusion control dye, representing fusion efficiency.

Furthermore, Fuse-It-siRNA-treated rat cortical neurons showed high mRNA knockdown efficiencies, with a reduction of Piezo1 mRNA of up to 76 ± 5%, compared to mRNA of nontreated control cells (Figure 3).

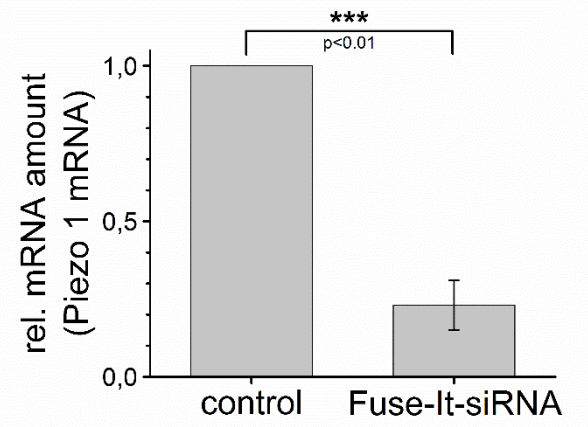

Figure 3: Knockdown Analysis by qRT-PCR 24 hours after the second treatment with Fuse-It-siRNA. The relative mRNA amount of Piezo1 mRNA in nontreated control cells and in Fuse-It-siRNA treated cells is shown. The control was set to 100%. Data is presented as mean ± SD from two technical replicates. Statistical significance was determined using ANOVA. Asterisks mark statistically significant changes and n.s. means not significant.

In addition, cell viability was evaluated by comparing the amount of total isolated mRNA. The same cell numbers were seeded for knockdown and nontreated control experiments. Therefore, it can be assumed, that a decrease of isolated mRNA represents a decrease of cell number and hence cell viability. The results are given in Figure 4. The mRNA amount isolated from nontreated control cells (180 ± 20 ng/µl) is comparable to the mRNA amount isolated from twofold Fuse-It-siRNA treated cells (170 ± 30 ng/µl). These findings indicate that cell viability was retained even after multiple treatments.

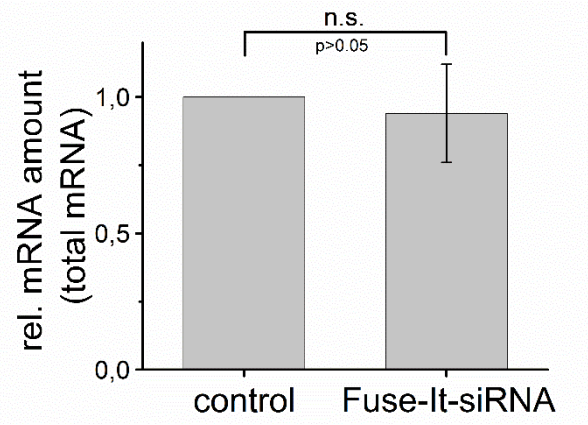

Figure 4: Viability Analysis by mRNA Isolation 24 hours after the second treatment with Fuse-It-siRNA. The graph shows the relative amount of total isolated mRNA in nontreated control cells and in Fuse-It-siRNA treated cells. The control was set to 100%. Data is presented as mean ± SD from two technical replicates. Statistical significance was determined using unpaired, two-tailed student's t-test. Asterisks mark statistically significant changes and n.s. means not significant.

In conclusion, those results demonstrate that the siRNA transfer using Fuse-It-siRNA works in a very mild fashion with extremely low cytotoxicity and yields high knockdown efficiencies, even in sensitive neuronal cells.

Important Note: The fusogenic liposomes of Fuse-It-siRNA fuse comparably efficient with other types of neuronal cells and other types of siRNAs.

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] 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.