Fluorescence Cross-Correlation Spectroscopy Reveals Mechanistic Insights into the Effect of 2′-O-Methyl Modified siRNAs in Living Cells

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Supplemental Data

Methods

Target and siRNA sequences

All RNA strands were obtained from IBA GmbH (Goettingen). The siRNA oligonucleotides were synthesized with a 5'-phosphate and a 3'-amino group on a C6-carbon linker and were labelled with Cy5 succinimidyl ester (Cy5-NHS, Amersham Biosciences) as described previously (1). Target RNA sequence is derived from the pRL-TK vector (Promega) containing the target sequence for siTK3: CCA GAA GAA UUU GCA GCA UAU CUU GAA CCA UUC AAA GAG AAA GGU GAA GU, 2'OMe modifications at positions 1-7 and 44-50 and Cy5 at the 3' end.
Antisense siTK3: UGA AUG GUU CAA GAU AUG CUG; sense siTK3: GCA UAU CUU GAA CCA UUC AUU
Antisense siTK3 bulge: UGA AUG GUC AUA GAU AUG CUG, sense siTK3 bulge: GCA UAU CUA UGA CCA UUC AUU
For luciferase experiments *Silencer*^{TM} Negative control #2 (NegsiRNA) from Ambion was used.

siRNA transfection and EGFP-AGO2 immunoprecipitation

EGFP-Ago2 expressing cells were transfected at 70% confluency in a 6-well format with 150 pmol siRNA using Lipofectamine RNAiMAX (Invitrogen) as recommended by the manufacturer. Cells were harvested 18 h post transfection and lysed in 50 mM HEPES-KOH (pH 7.4), 150 mM KCl, 2 mM EDTA, 0.5 mM DTT, 0.5% NP-40, and Complete Protease Inhibitor (Roche). EGFP-Ago2 was immunoprecipitated with anti-GFP antibody (clones 7.1 and 13.1; Roche) conjungated to Protein G-Dynabeads (Invitrogen). Immunoprecipitate was washed twice with 50 mM HEPES-KOH (pH 7.4), 250 mM KCl, 2 mM EDTA, 0.5 mM DTT, 0.5% NP-40, and Complete Protease Inhibitor (Roche) and final wash in 50 mM HEPES-KOH (pH 7.4), 100 mM KCl, 5 $MgCl_2$, 0.5 mM DTT.

Luciferase assay

Dual-luciferase assays (Promega GmbH) were performed 24-48 h after transfection according to the manufacturer's protocol for 24-well chambers and detected with a TD20/20 luminometer (Turner designs). *Pp*-luc target vector (pGL2-Control, Promega) was co-transfected with the control vector Rr-luc (pRL-TK, Promega GmbH). Lipofectamine 2000 (Invitrogen GmbH) was used for the triple transfection of the dual luciferase assay vectors pGL2-control (contains the cDNA of Firefly luciferase (FL)) and pRL-TK (contains the cDNA of Renilla luciferase (RL)) together with the siRNAs. The desired amount of each siRNA is mixed with 0.9 μg pGL2-control and 0.1 μg pRL-TK. The cells were transfected with the indicated amounts of siRNAs, 100 µl Opti-MEM and 2 μl Lipofectamine 2000. 3-5 h after transfection the medium is replaced by 500 μl fresh growth medium.

siRNA Guided Cleavage Assay

Target RNA was ^{32}P- labeled with T4 polynucleotide kinase, and cleavage was assayed in 20 µl reaction: 0.1 pmol target RNA was incubated with immuno-precipitates using 19.0 µl volume of beads in 50 mM HEPES-KOH (pH 7.4), 100 mM KCl, 5 $MgCl_2$, 0.5 mM DTT. The reaction was incubated at 37ºC for 1 hour. The reaction was stopped by addition 8 m Urea loading buffer. The RNA was separated by 12% PAGE and radioactivity was detected by phosphorimaging.

Purification of 6xHis-tagged Exportin-5 and RanQ69L

For the purification of Exportin-5 the pQE60-Exp5 construct was transformed into BL21-Codon Plus bacteria (Stratagene). On the next day the colonies of transformants were scraped from LB-Amp plates (Ampicillin: 100µg/ml, Sigma) into 1L LB-Amp/Car (Amp: 100µg/ml)/0,5X (Carbenicillin: 50µg/ml)) at 23°C until OD600=0,7-0,8. 300µM IPTG were added and the protein expression was induced overnight at 23°C. On the next day the bacteria were centrifuged for 30min at 4000rpm and 4°C, washed once with cold 0.9% NaCl and purified as has been described in (2, 3).

Ran expression was induced with 1mM IPTG for 3h and purified as has been described in (4).

Loading of Ran with GTP was performed according to the protocol of Rexach & Blobel 1995 (5): 20µM Ran are incubated in the presence of 60mM KCl; 15mM EDTA pH8; 10mM Tris, pH 7.5; 2mM DTT; 2mM MgOAc; 1mM GTP and 5%(v/v) glycerol for 1h30min at room temperature. Then 15mM MgOAc were added for at least 15min at 4°C.

RNA electrophoretic mobility shift assay (REMSA)

For RNA electrophoretic mobility shift assays (REMSA) 0.4µM Exportin-5 were incubated with 1µM RanGTP and 100nM siRNA mixed with indicated amounts of competitor siRNA (unmodified, 5'-phosphorylated, identical sequence) in 10mM Tris pH 7.5; 75mM KCl; 2mM DTT; 8µM single stranded RNA; 10%(v/v) glycerol for 20min at RT. Then 20x loading buffer (Heparin 600µg/ml; 6% (v/v) Glycerol) is added for 5min. The samples were loaded onto a 6% polyacrylamide gel and ran at 200V for 25min on a mini-gel. The gels were analyzed with a Typhoon 9410 Variable Mode Imager (Amersham Biosciences) by using the manufacturers' settings for Cy5 (Amersham Biosciences) and Alexa488 (Molecular Probes).

Laser scanning confocal fluorescence microscopy

LSM imaging was performed using a commercial Zeiss ConfoCor 2 laser scanning microscope (Zeiss) with argon ion (488nm, 25mW, at 10% of maximum power output), helium-neon (543nm, 1mW, at 20% of maximum power output) and helium-neon (633nm, 5.0mW, at 8% of maximum power) lasers. A water immersion objective 40x/1.2W (Zeiss) was used in the epidetection configuration with an adjustable pinhole set at 71µm. A band-pass filter transmitting 505–550nm (Zeiss) was used in the experiments with Alexa488, while a long-pass filter at 650nm (Zeiss) was applied to separate Cy5 fluorescence.

In order to avoid saturation of the fluorescence intensity in the scanned images the detector settings were optimized by using the feature Range Indicator provided by the Zeiss software (Operating Manual LSM510, Zeiss).

FCCS data acquisition

Fluorescence cross-correlation spectroscopy was performed in 10G cells, stably expressing EGFP-Ago2 (6). Fluorescently labeled siRNAs or targetRNAs were delivered via microinjection. The 488nm Ar-laser-line of a Confocor 3/LSM510 system (Zeiss, Jena, Germany) was used to excite EGFP and the 633nm line of a HeNe-laser was used to excite Cy5. Both laser lines were attenuated by an acousto-optical tunable filter to 3.5 and 1.05 kW/cm², respectively, in order to minimize photobleaching and cellular damage. Both excitation laser lines were directed by a 488/633 dichroic mirror (HFT) onto the back aperture of a Zeiss C-Apochromat 40x,

N.A.=1.2, water immersion objective. The fluorescence light was collected by the same objective, separated from the excitation light by a 488/633 dichroic mirror (HFT), passing a confocal pinhole (70 µm in diameter) and split into two spectral channels by a second dichroic (LP635). After removing residual laser light by a 505-610 nm bandpass or 655 nm longpass emission filter, respectively, the fluorescence light was recorded by avalanche photodiode detectors (APDs). Before each experiment the setup was adjusted using Alexa488/Cy5 double labeled siRNA, yielding cross-correlation amplitudes of 75 (±5%). The discrepancy to 100% is due to imperfect overlap of the detection volumes and/or imperfect labeling of the siRNA.
In each cell, FCCS was measured in the nucleus and the cytoplasm. For each measurement, 8 runs, each 30 seconds long, were collected. Only cells that exhibited a higher number of red than green fluorophores in the focal volume were used, to avoid, that a too low siRNA concentration limits the amount of cross-correlation. Cell measurements were performed in air-buffer (150 mM NaCl, 20 mM HEPES pH 7.4, 15 mM glucose, 150µg/ml BSA, 20 mM trehalose, 5.4 MM KCl, 0.85 mM $MgSO_4$, 0.6 mM $CaCl_2$) at RT (6).

FCCS data analysis

The fluorescence signals of each run were software correlated following the definition of auto- and cross-correlation

$$G_{ij}(\tau) = \frac{\left\langle \delta F_i(t) \cdot \delta F_j(t+\tau) \right\rangle}{\left\langle F_i(t) \right\rangle \left\langle F_j(t) \right\rangle}.$$

Runs showing significant photobleaching or intracellular movement were discarded from the data evaluation.
A model including two diffusing species and a term accounting for the photophysics was fitted to the two autocorrelation and the cross-correlation curves, using a weighted Marquardt non-linear least square fitting algorithm. The cross-correlation curve was the average of the two cross-correlation curves (red vs. green and green vs. red channel).

$$G_{ij}(\tau) = G_{ij}(0) \cdot \left(1 + \frac{T}{1-T}\right) \cdot e^{\left(-\tau/\tau_T\right)} \cdot \left(\frac{F}{\left(1+\frac{\tau}{\tau_1}\right)\sqrt{1+\frac{\tau}{S^2 \cdot \tau_1}}} + \frac{1-F}{\left(1+\frac{\tau}{\tau_2}\right)\sqrt{1+\frac{\tau}{S^2 \cdot \tau_2}}} \right)$$

T represents the fraction of molecules in the dark state, τ_T the lifetime of the dark state, τ_1 and τ_2 are the diffusion times of the two species, F is the fraction of the two species and S is the form factor, which is the ratio of axial (ω_z) over the radial radius (ω_{xy}). Using a model for anomalous diffusion (7), didn't improve the accuracy of the resolved particle numbers (8). During the fit, the lifetime of the dark state τ_T was fixed to 130 µs and 70 µs for EGFP and Cy5 respectively. These values were determined in preceding experiments of EGFP and Cy5 *in vivo*. The triplet fraction T of the cross-correlation curve was fixed to zero. The amplitudes $G_{ij}(0)$ were corrected for spurious autofluorescent background of the cells, using the following algorithm:

$$\tilde{G}_{ij}(0) = G_{ij}(0) \frac{F_i}{F_i - B_i} \cdot \frac{F_j}{F_j - B_j}$$

Here, F is the measured count rate and B the measured background count rate, which were determined in unlabeled ER293 cells. The background in the green

channel was between 5 and 10% of the measured fluorescent signal and for the red channel between 1 and 2%. In addition, background corrected amplitudes, $\tilde{G}_{ij}(0)$, were corrected for spectral crosstalk. Spectral crosstalk was only encountered from the green into the red channel, with an amount of β=0.7%. Subsequently, the green amplitude is unaffected, while the red and the cross-correlation amplitude need to be corrected for spectral crosstalk:

$$\hat{G}_g(0) = \tilde{G}_g(0)$$

$$\hat{G}_r(0) = \frac{F_r^2 \tilde{G}_r(0) + \beta^2 F_g^2 \tilde{G}_g(0) - 2\beta F_r F_g \tilde{G}_{CC}(0)}{\left(F_r - \beta F_g\right)^2}$$

$$\hat{G}_{CC}(0) = \frac{F_r F_g \tilde{G}_{CC}(0) - \beta F_g^2 \tilde{G}_g(0)}{\left(F_g F_r - \beta F_g\right)^2}$$

From the corrected amplitudes, the number of fluorescent particles in the detection volume were calculated, which are given by the following set of formulas:

$$\hat{G}_g(0) = \frac{1}{N_g + N_{gr}}; \hat{G}_r(0) = \frac{1}{N_r + N_{gr}}; \hat{G}_{CC}(0) = \frac{N_{gr}}{\left(N_g + N_{gr}\right)\left(N_r + N_{gr}\right)}$$

Here, N_g is the number of only green labeled particles, N_r the number of only red labeled particles and N_{gr} the number of particles, which carry both labels. The amount of cross-correlation was calculated by dividing the number of double labeled particles by all particles carrying a green label:

$$CC = \frac{N_{gr}}{N_g + N_{gr}}$$

Supplemental Figures

Figure S1: Sub-cellular localization of differently modified siRNAs after microinjection

A) Sub-cellular localization of siRNAs modified with 2'OMe (upper left panel), PTO (lower left panel) and 2'F (lower right panel) on the last 4 nucleotides of each 3'-end. The localization of an siRNA modified with 2'OMe on the last 8 nucleotides on both strands is shown in upper right panel. Scale bar = 10 µm.
B) Photobleaching curves of Cy5-labeled siRNAs modified with either 2'OMe (upper panel), PTO or 2'F (lower panel) after microinjection. Curves were plotted as normalized intensity versus total exposure time.

Figure S2: Binding of different modified and unmodified siRNAs to Exp5-RanGTPQ69L depending on different amounts of unmodified competitor siRNA

All siRNAs including the competitor exhibited identical sequences to exclude sequence dependent effects. For the competitor experiment the 3'4nt-2'-OMe, 3'5nt-2'-F, 3'4nt-PTO, 5'desoxy and the unmodified siTk3 duplex labelled on the 3'-end of the antisense strand with Cy5 were mixed with the unmodified competitor siTK3 duplex labelled on the 3'-end of the antisense with Alexa488.
Below, the quantification of the upper agarose gels is shown. Fluorescence intensities co-migrating with the shifted bands were quantified using a fluorescence scanner (GE Healthcare, Typhoon 9400). The values obtained without competitor

were arbitrary set at 100 and those obtained with the indicated amounts of competitor were calculated.

Figure S3: Sub-cellular localization of 2'OMe modified siRNAs.
The different modified siRNA duplexes were microinjected into ER293 cells and analyzed after 10-15 minutes by confocal microscopy. The resulting ratios of nuclear to cytoplasmic staining intensities were quantified by taking mean fluorescence intensity measurements from three 20μm^2 regions within the nucleus and the cytoplasm of injected cells. The nuclear-to-cytoplasmic fluorescence intensity ratios for each siRNA-like variant are shown in the graph, with clear nuclear exclusion corresponding to values smaller than 0.5. The plotted data were averaged from 10 different cells ± SD.

Figure S4: Obtained Cross-correlation curves for RISC loading and target RNA interaction
A) Abstract of normalized cross-correlation curves of EGFP-Ago2 with the modified duplexes labeled either on the guide (as) or passenger strand (ss) of the siTK3 duplex in the cytoplasm after 3 h in 10G cells *in vivo*. Curves are the average of at least 10 different measurements. The resulting cross-correlation amplitudes in the cytoplasm (filled bars) and nucleus (open bars) are displayed in Figure 3.
B) Abstract of normalized cross-correlation curves of EGFP-Ago2 loaded with the indicated modified guide strands with the target RNA in the cytoplasm after 1 h in 10G cells *in vivo*. Curves are the average of at least 10 different measurements. The resulting cross-correlation amplitudes in the cytoplasm (black) and nucleus (white) are displayed in Figure 4.

Figure S5: Effect of internal 2'-OMe modifications on silencing
ER293 cells were transfected with the indicated amounts of differently modified siTK3 together with the fixed concentration of the pGL2-Control and pRL-TK reporter plasmids. After 48 h the ratios of target to control luciferase concentration were normalized to the NegsiRNA control indicated in black; siTK3-3'4nt-as/ss open bar; siTK3-3nt-Int-as/ss dark grey; siTK3-as3'4nt/3nt-Int-ss lined bar; siTK3-3nt-Int-as/ss3'4nt bright grey. The plotted data were averaged from six independent experiments ± SD.

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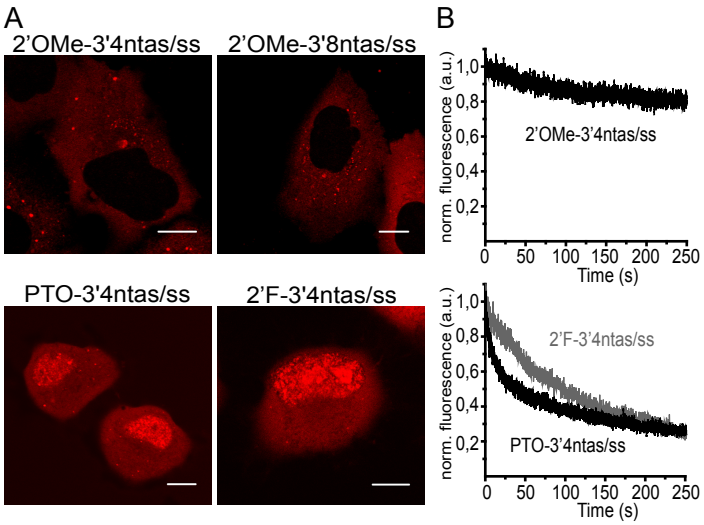
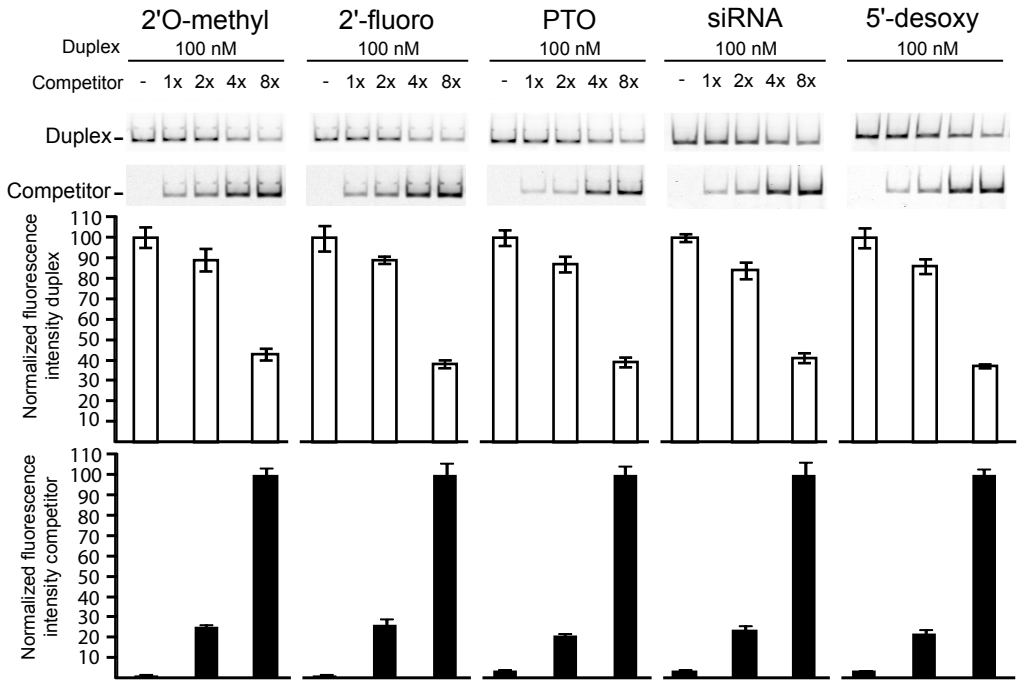
Suppl. Figure 1



Suppl. Figure 2



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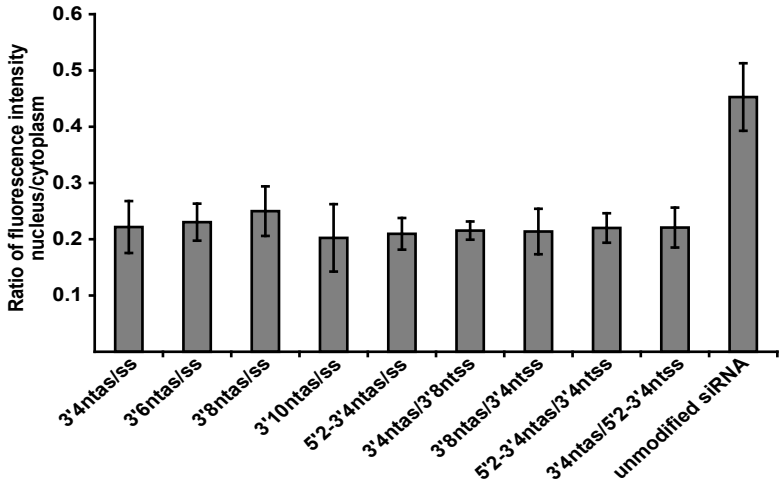
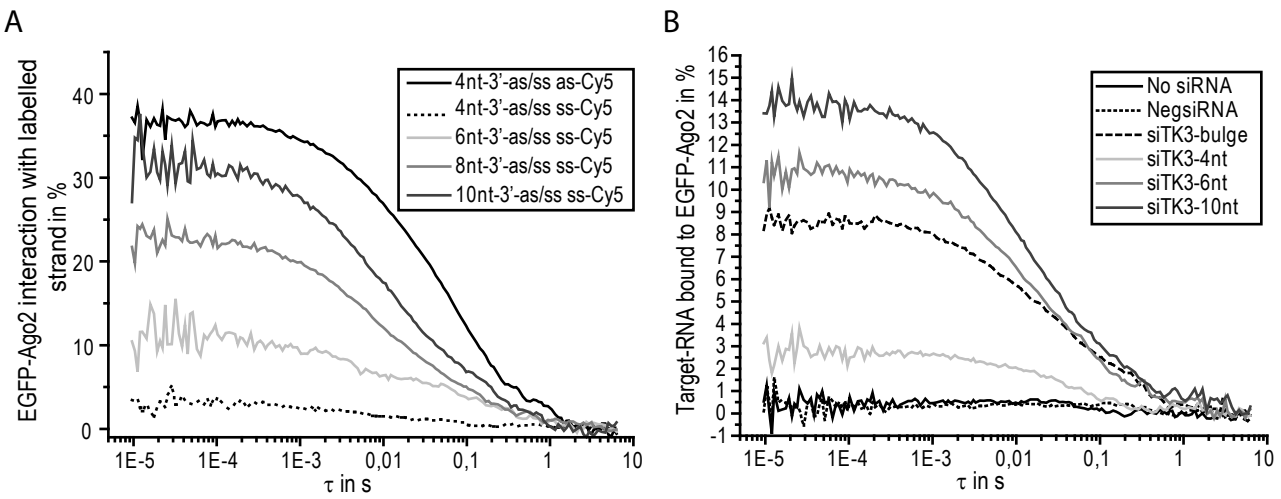


Suppl. Figure 4



Suppl. Figure 5