# „siRNA traffic lights“: Arabino-configured 2’-anchors for fluorescent dyes are key for dual color readout in cell imaging

Jeannine Steinmeyer,[a] Heidi-Kristin Walter,[a] Mathilde Bichelberger,[b] Violetta Schneider,[c] Tomáš Kubař,[c] Franziska Rönicke,[a] Bettina Olshausen,[e] Karin Nienhaus,[b] Gerd Ulrich Nienhaus,[b,d,e,f] Ute Schepers,[e] Marcus Elstner,[c] and Hans-Achim Wagenknecht*[a]

[a] Institute of Organic Chemistry, Karlsruhe Institute of Technology (KIT), Fritz-Haber-Weg 6, 76131 Karlsruhe
[b] Institute of Applied Physics, Karlsruhe Institute of Technology (KIT), Wolfgang-Gaede-Str. 1, 76131 Karlsruhe, Germany
[c] Institute of Physical Chemistry, Karlsruhe Institute of Technology (KIT), Fritz-Haber-Weg 2, 76131 Karlsruhe
[d] Department of Physics, University of Illinois at Urbana-Champaign, 1110 W. Green St, Urbana, IL 61801, USA.
[e] Institute of Toxicology and Genetics, Karlsruhe Institute of Technology (KIT), Eggenstein-Leopoldshafen, Germany
[f] Institute of Nanotechnology, Karlsruhe Institute of Technology (KIT), Eggenstein-Leopoldshafen, Germany

* To whom correspondence should be addressed. Tel: Int+49-721-8067486; Fax: Int+49-721-608-44825; Email: Wagenknecht@kit.edu

## SUPPORTING INFORMATION

**1. Preparation of RNA1 – RNA6**

All oligonucleotides were synthesized on an Expedite 8909 Synthesizer from *Applied Biosystems* (ABI) using fast deprotection phosphoramidite chemistry (2'-TBDMS protected RNA monomers, Activator 42® were used). Reagents and CPG (1 µmol) were purchased from *Glen Research* and *Sigma Aldrich*. The commercially available 2'-*O*-propargyl-uridine (**cU**) was purchased from *ChemGenes*. For the **cAraU** building blocks, a slightly extended coupling time of 10 minutes (**cAraU)** was used. For the synthesized phosphoramidite building blocks the coupling time was enhanced up to 57 min. After solid phase synthesis, the trityl-off oligonucleotides were cleaved from the resin and deprotected using conc. $NH_4OH$:EtOH 3:1 at room temperature for 4 h (**RNA1 - RNA2)** and AMA (1:1 v/v; methylamine in ethanol and conc. $NH_4OH$) (**RNA3 - RNA6**) at 65 °C for 10 minutes. The click reaction of **RNA3 - RNA6** was performed before removing of the 2'-TBDMS protection group.

Azide modified dyes and phosphoramidite building blocks were synthesized as published before.[1]

To the lyophilized alkyne-modified RNA sample were added 100 µL DEPC-treated water, 25 µL sodium ascorbate (0.4 M in water), 34 µL tris-[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (0.1 M in DMSO/t-BuOH 3:1), 114 µL azide (0.01 M in DMSO/t-BuOH 3:1) and finally 17 µL tetrakis(acetonitrile) copper(I)hexafluorophosphate (0.1 M in DMSO/t-BuOH 3:1). The reaction mixture was kept at 60 °C for 1.5 h. After cooling to room temperature, the RNA was precipitated by adding 150 µL $Na_2EDTA$ (0.05 M in water), 450 µL sodium acetate (0.3 M in water) and 3 ml ethanol and 7 mL n-butanol and stored at -32 °C for 16 h. After centrifugation the supernatant was removed and the residue washed two times with 2 mL cold n-butanol/ethanol/water (v/v/v 6:2:2) and then dried under reduced pressure.

The RNA pellet was dissolved in 300 µL DMSO and treated with 300 µL $NEt_3$·HF for 2.5 h at 60 °C. After cooling to room temperature, HF was quenched by adding 600 µL of isopropoxytrimethylsilane. The two phase were vortexed until one phase and a precipitate was formed. Then 300 µL diethyl ether were added and the mixture was kept at -32 °C for 16 h. After centrifugation the RNA pellet was washed twice with 1 mL diethyl ether, dried under reduced pressure and then further purified by HPLC chromatography.

**2. HPLC-purification and characterization of RNA1 – RNA6**

The labelled DNA strands were purified via HPLC Reversed Phase Supelcosil™ LC-C18 column (250 x 10 mm, 5 µm) on a Shimadzu HPLC system (autosampler, SIL-10AD, pump LC-10AT, controller SCL-10A, diode array detector SPD-M10A) using the following conditions:

eluent A: $NH_4HCO_3$ buffer (0.1 M in DEPC-treated water)

eluent B: acetonitrile

flow rate: 2.5 mL/mL

For gradients, see

Table **S1**. UV/Vis detection at 260 nm, 459 nm for oligonucleotides modified with donor dye, 548 nm for oligonucleotides modified with acceptor dye.

**Table S1.** HPLC-gradients for semi-preparative purification of oligonucleotides **RNA1** – **RNA6**. [a] **RNA1, 3, 5**; [b] **RNA4, 6**; [c] **RNA2**.

| **time [min]** | **eluent B [%]** |
|---|---|
| 0 | 0 |
| 45 | $15^{[a]}/17^{[b]}/20^{[c]}$ |
| 65 | $15^{[a]}/17^{[b]}/20^{[c]}$ |
| 66 | 80 |
| 75 | 80 |
| 76 | 0 |
| 85 | 0 |

Analytical HPLC of the purified DNA samples was performed using a Reversed phase Supelcosil™ LC-C18 column (250 x 4.5 mm, 5 µm) on a Shimadzu HPLC system (autosampler, SIL-10AD, pump LC-10AT, controller SCL-10A, diode array detector SPD-M10A) using the following conditions:

eluent A: $NH_4HCO_3$ buffer (0.1 M in DEPC-treated water)

eluent B: acetonitrile

flow rate: 1.0 mL/min

For gradients, see Table S2. UV/Vis detection at 260 nm, 459/462 nm for oligonucleotides modified with donor dye, 548/550 nm for oligonucleotides modified with acceptor dye.

**Table S2.** HPLC-gradients for analytical determination of purified oligonucleotides **RNA1** – **RNA6**.

| time [min] | eluent B [%] |
|---|---|
| 0 | 0 |
| 45 | 20 |
| 60 | 20 |
| 61 | 80 |
| 70 | 80 |
| 71 | 0 |
| 75 | 0 |

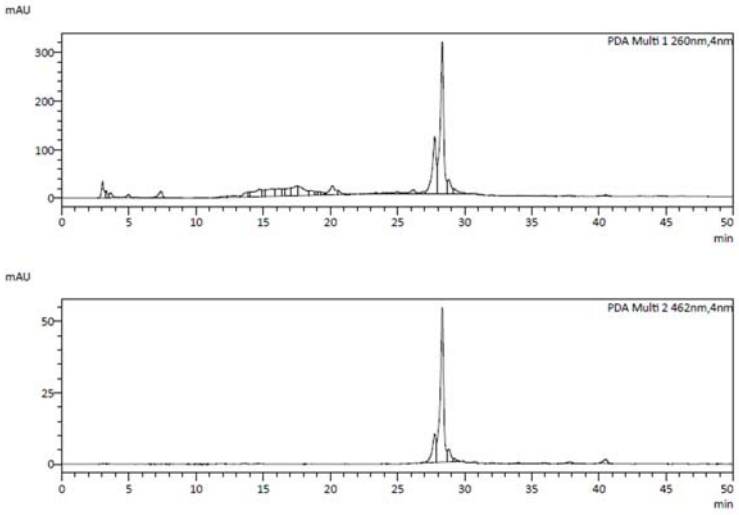


**Figure S1.** HPLC elution profile of purified **RNA1**.

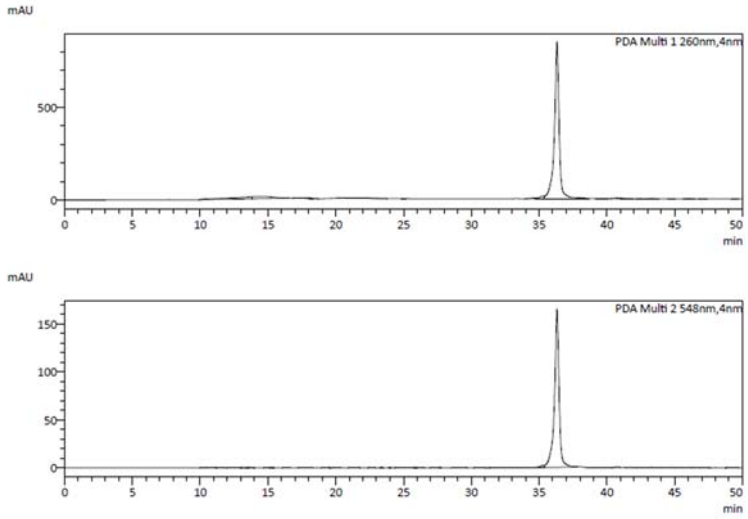


**Figure S2.** HPLC elution profile of purified **RNA2**.

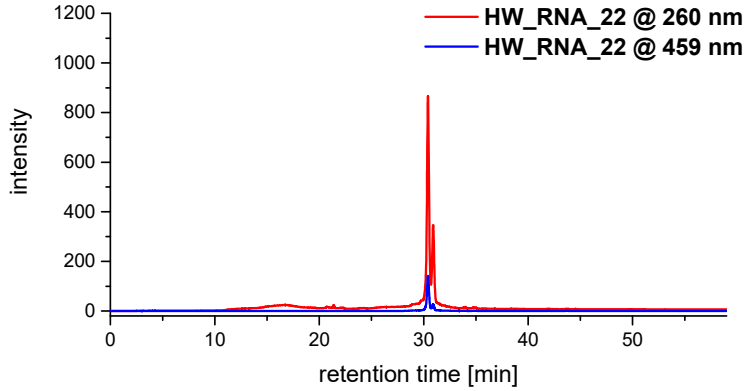


**Figure S3.** HPLC elution profile of purified **RNA3**.

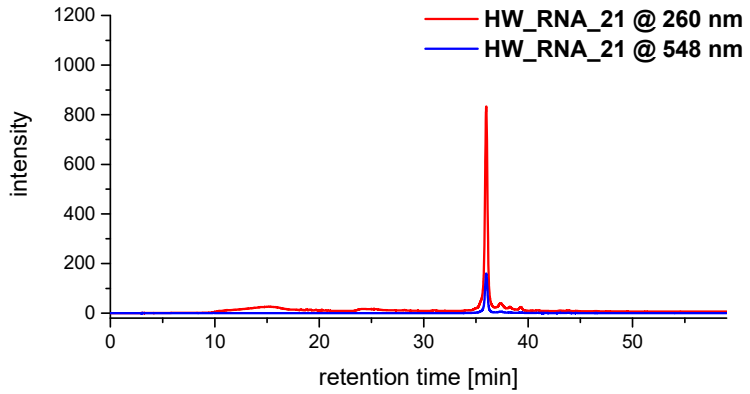


**Figure S4.** HPLC elution profile of purified **RNA4**.

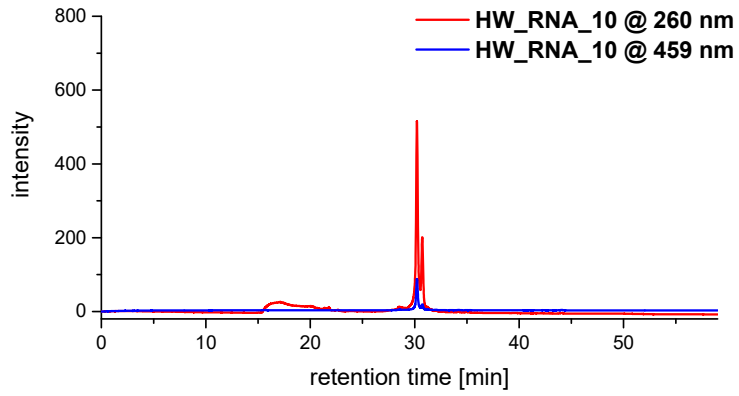


**Figure S5.** HPLC elution profile of purified **RNA5**.

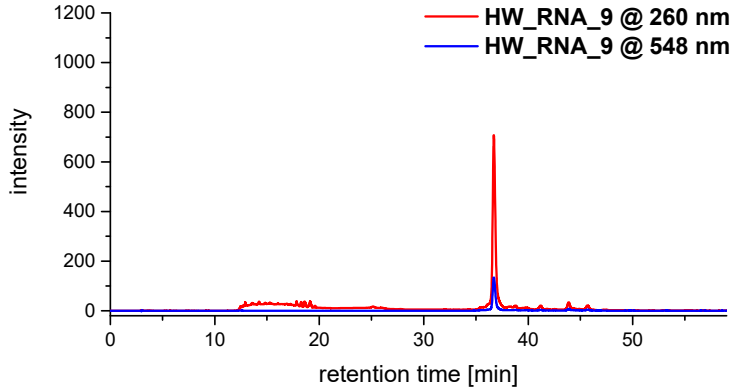


**Figure S6.** HPLC elution profile of purified **RNA6**.

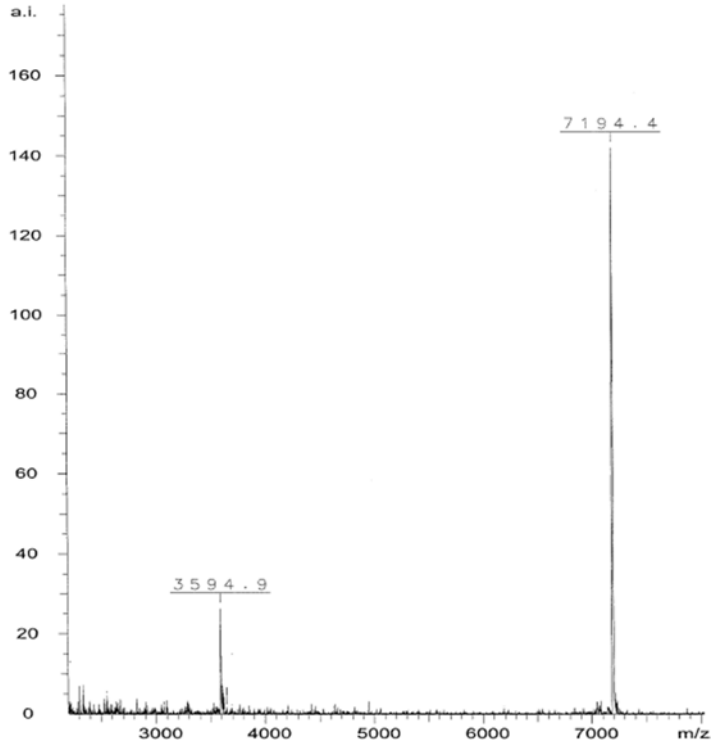


**Figure S7.** MALDI-TOF MS analysis of purified **RNA1**; calculated: 7197.1 Da, found: 7194.4 Da.

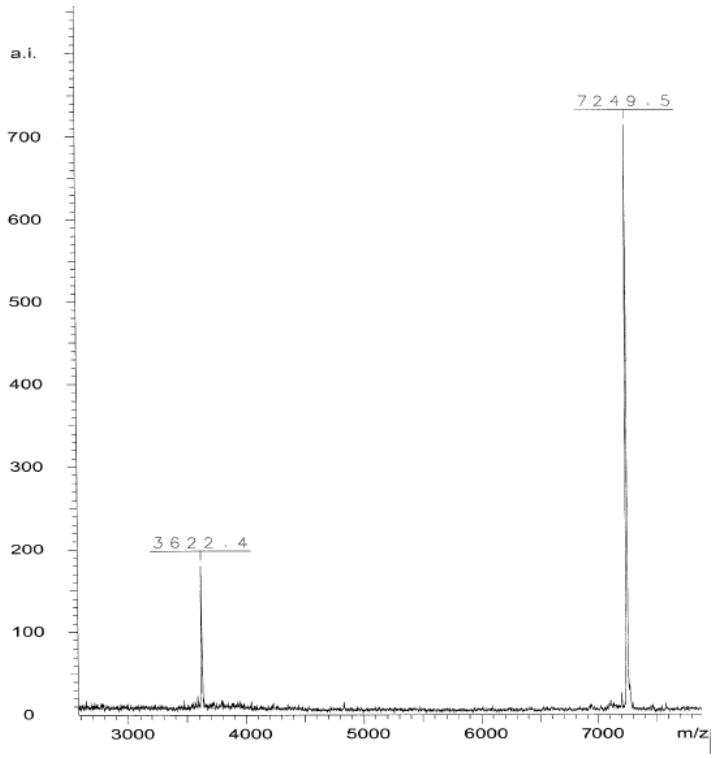


**Figure S8.** MALDI-TOF MS analysis of purified **RNA2**; calculated: 7251.1 Da, found: 7249.5 Da.

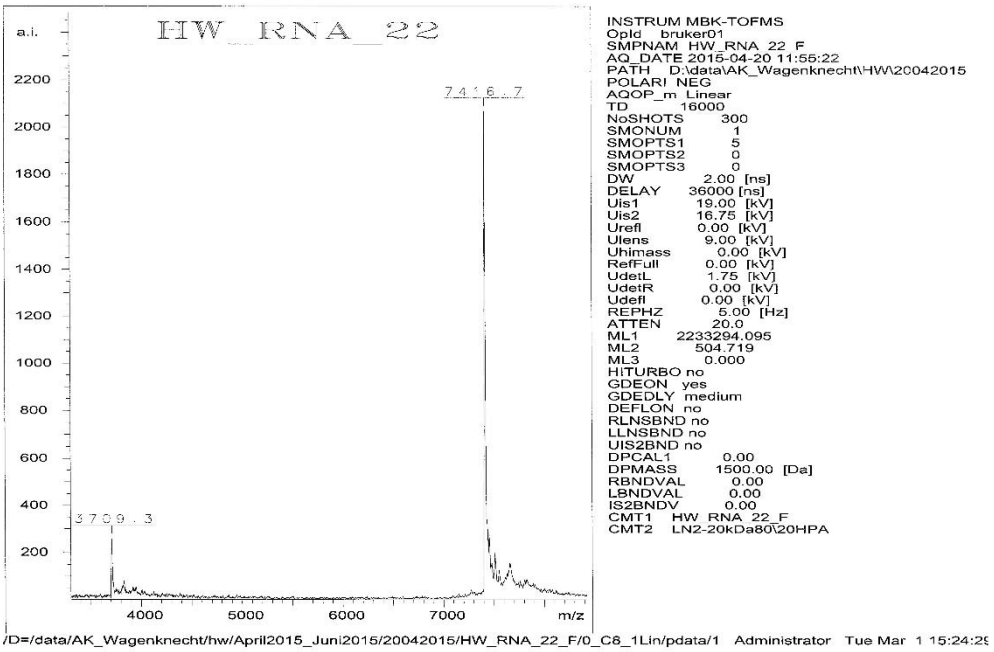


**Figure S9.** MALDI-TOF MS analysis of purified **RNA3**; calculated: 7410.2 Da, found: 7416.7 Da.

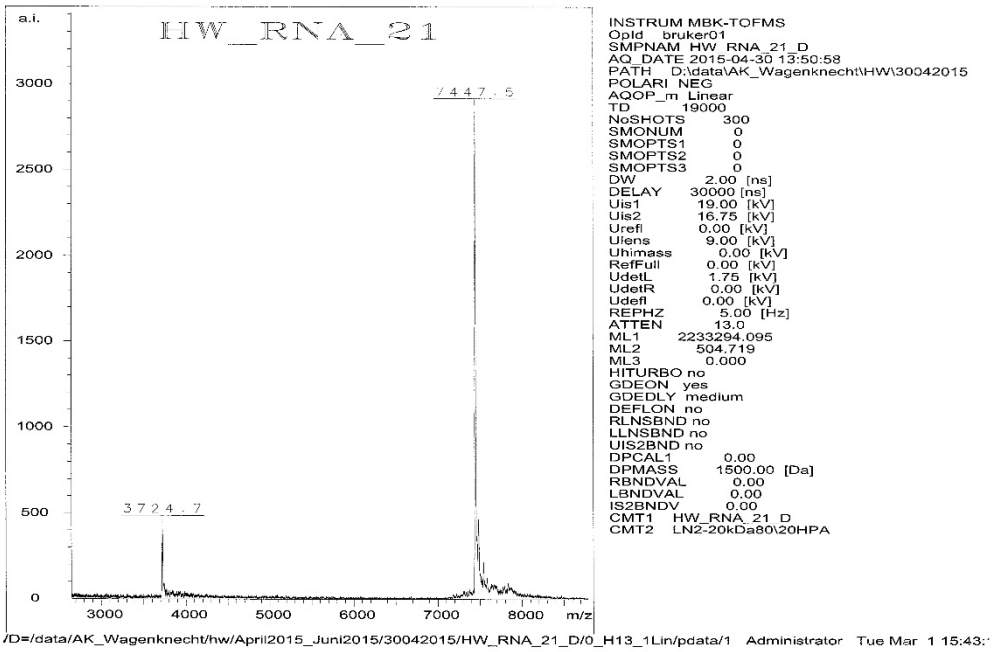


**Figure S10.** MALDI-TOF MS analysis of purified **RNA4**; calculated: 7441.2 Da, found: 7447.5 Da.

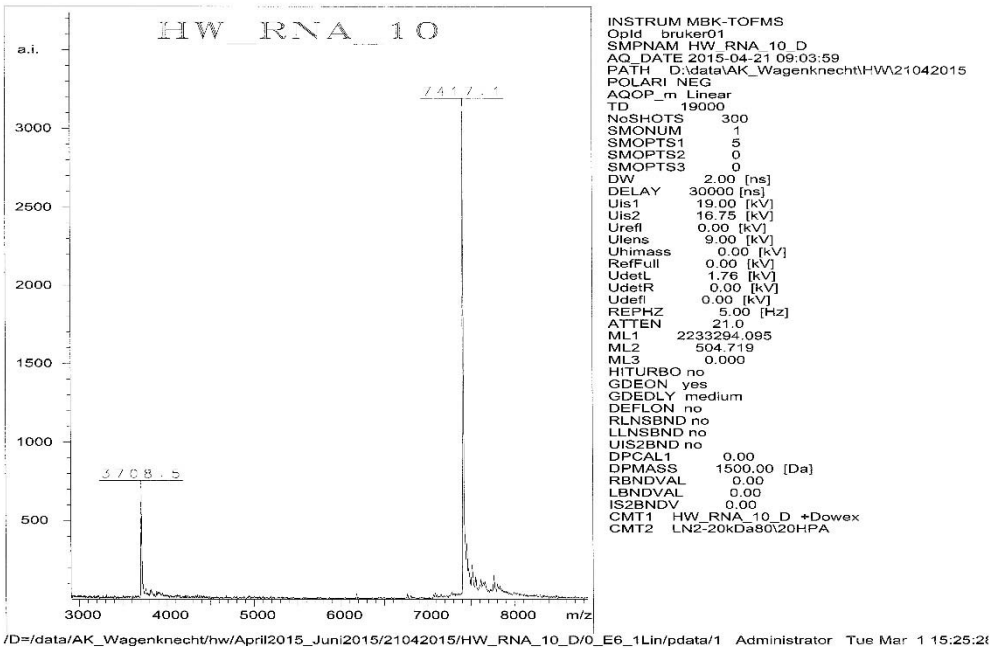


**Figure S11.** MALDI-TOF MS analysis of purified **RNA5**; calculated: 7410.2 Da, found: 7417.1 Da.

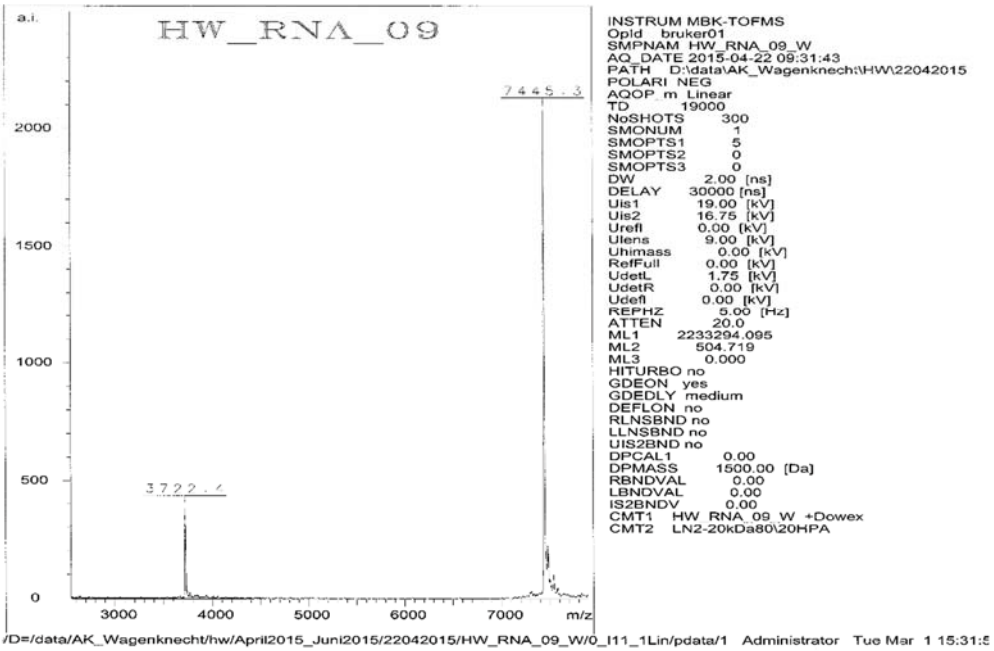


**Figure S12.** MALDI-TOF MS analysis of purified **RNA6**; calculated: 7441.2 Da, found: 7445.3 Da.

**3. Optical spectroscopy**

All spectra were recorded at 20 °C and are corrected for Raman emission from the buffer solution. Duplexes were formed by heating to 90 °C (10 min) followed by slow cooling to room temperature.

Spectroscopic measurements were recorded in $NaP_i$-buffer solution (10 mM, pH = 7) with 250 mM NaCl in quartz glass cuvettes (10 mm). The UV-vis absorbance spectra of the different probes were recorded on a Cary 100 spectrophotometer (Varian, Palo Alto, CA). Fluorescence emission spectra of the same samples were measured on a Fluorolog-3 spectrofluorometer (HORIBA JobinYvon, Edison, NJ, USA), with excitation of dye **1** at 488 nm, dye **2** at 530 nm and, in presence of both dyes, dye **1** at 400 nm. Quantum yields were determined with Quantaurus QY C11347 of Hamamatsu. For the measurements, samples were kept in quartz cuvettes (Hellma GmbH & Co KG, Müllheim, Germany) with a path length of 3 mm.

Fluorescence lifetime measurements: The RNA samples were prepared at a concentration of 2.5 µM in a home-built glass cylinder passivated with bovine serum albumin. The fluorescence lifetimes were recorded on a confocal microscope (Microtime 200, PicoQuant, Berlin) coupled to time-correlated single photon counting (TCSPC) data acquisition system. Dye 1 and 2 were excited with pulsed lasers at 488 nm (LDH-P-C-485, Picoquant) and 532 nm (LDH-P-FA-530, Picoquant), respectively, at 26.7 MHz. The lasers were focused into the sample by a water immersion objective (UPLSAPO 60XW, 60×/1.2 W, Olympus, Hamburg, Germany). The fluorescence emission was collected via the same objective, focused onto a pinhole of 150 µm and passed through a bandpass filter. For constructs labeled with just one dye, a 545/70 nm (wavelength / center width, AHF, Tübingen, Germany) bandpass was used to detect dye 1 emission and a 645/75 nm band pass (wavelength / center width, AHF, Tübingen, Germany) for dye 2 emission. Bandpass filters 515/30 and 740/40 (wavelength / center width, AHF, Tübingen, Germany) were used for dye1 and dye2, respectively, for measuring the doubly-modified double strand in order to avoid cross-talk. Photons were counted with a time resolution of 8 ps by a single photon avalanche photodiode (PD1CTC Micro Photon Devices, Bolzano, Italy). The instrumental response function (IRF) was measured with the Rose Bengal dye (Sigma–Aldrich, St. Louis, MO), which has a fluorescence lifetime of 77 ps[2]. The IRF had a full width at half maximum of 0.7 ns at 488 nm excitation and 0.5 ns at 532 nm excitation.

The fluorescence decay curves were calculated with the SymPhoTime software (PicoQuant). The lifetime decays of the singly-modified single and double strands were fitted with a two-component exponential function, using a home written Matlab script,

$$y = A_1\, e^{(-t/\tau_1)} + A_2\, e^{(-t/\tau_2)} + y_0 \qquad (2)$$

with amplitudes $A_1$ and $A_2$, decay times $\tau_1$ and $\tau_2$ and the number of background photons, $y_0$ .

The lifetime decays of the doubly-modified double strands were described by a three-component exponential function. In this model, the fast component describes the interacting dye 1 which contributes to FRET and the two slow components correspond to non-interacting (unquenched) dye 1. Thus, $\tau_2$ and $\tau_3$ were fixed respectively to the bi-exponential fluorescent decays of dye 1 in the double strand and the ratios of the amplitude were also fixed.

The fraction of each species i, $f_i$, was calculated as

$$f_i = \frac{A_i}{A_1 + A_2 + A_3}, \tag{3}$$

with amplitude $A_i$ and fluorescence lifetime, $\tau_i$, of component *i*, with *i* = 1, 2, 3 and $f_1 + f_2 + f_3 = 1$.

The amplitude weighted average lifetime, $\tau_{av}$, was calculated as

$$\tau_{av} = f_1 \cdot \tau_1 + f_2 \cdot \tau_2 + f_3 \cdot \tau_3. \tag{4}$$

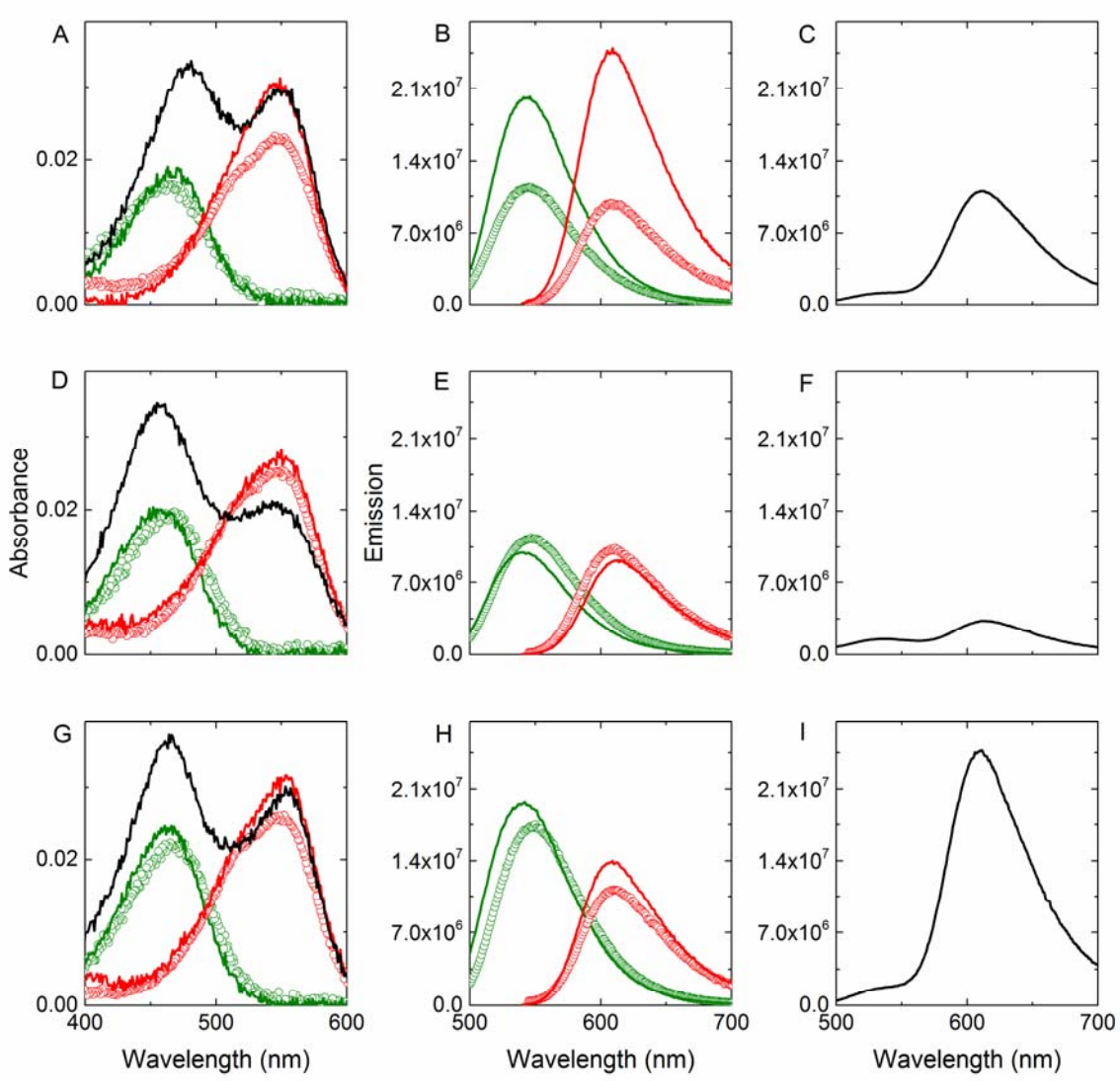


**Figure S13.** Optical spectra of dyes **1** and **2** within the different RNA strands. Left column: absorption spectra, center column: fluorescence emission spectra upon excitation at 488 nm (green) and 530 nm (red), right column: fluorescence emission spectra upon excitation at 400 nm. (A, B, C) RNA1-2, (D, E, F) RNA3-4 and (G, H, I) RNA5-6. Color code: green, dye 1; red, dye 2; black, dye1 and dye2. Solid lines, RNA double strands. Symbols, RNA single strands.

**Table S3.** Parameters to describe the fluorescence decay curves of dye **1** in the different RNA constructs.

| RNA | $A_1$ | $\tau_1$ (ns) [a] | $f_1$ (%) | $A_2$ | $\tau_2$ (ns) [a] | $f_2$ (%) | $A_3$ | $\tau_3$ (ns) [a] | $f_3$ (%) | $\tau_{av}$ (ns) |
|---|---|---|---|---|---|---|---|---|---|---|
| **ssRNA1** | 3240 ± 30 | 1.3 | 46 | 3400 ± 40 | 3.8 | 54 | | | | 2.6 ± 0.1 |
| **dsRNA1** | 1500 ± 100 | 1.8 | 27 | 4040 ± 270 | 4.2 | 73 | | | | 3.6 ± 0.2 |
| **dsRNA1-2**[b] | 57260 ± 1990 | 0.1 | 84 | 8330 ± 70 | 1.0 | 12 | 2450 ± 40 | 3.1 | 4 | 0.3 ± 0.1 |
| **ssRNA3** | 4120 ± 30 | 1.1 | 51 | 3970 ± 50 | 3.4 | 49 | | | | 2.2 ± 0.1 |
| **dsRNA3** | 1910 ± 30 | 1.0 | 30 | 4490 ± 30 | 3.7 | 70 | | | | 2.9 ± 0.1 |
| **dsRNA3-4**[b] | 29050 ± 1640 | 0.1 | 79 | 5250 ± 111 | 1.2 | 14 | 2660 ± 50 | 3.2 | 7 | 0.5 + 0.1 |
| **ssRNA5** | 3330 ± 40 | 1.3 | 45 | 4040 ± 50 | 3.6 | 55 | | | | 2.6 ± 0.1 |
| **dsRNA5** | 1480 ± 20 | 1.2 | 27 | 4020 ± 30 | 4.1 | 73 | | | | 3.3 ± 0.1 |
| **dsRNA5-6**[b] | 25860 ± 1450 | 0.1 | 76 | 4717 ± 40 | 1.1 | 14 | 3423 ± 50 | 3.3 | 10 | 0.6 ± 0.1 |

[a] Estimated systematic error: ± 0.1 ns.

[b] Free fit with a three-component exponential function.

**Table S4.** Parameters to describe the fluorescence decay curves of dye **2** in the different RNA constructs.

| RNA | $A_1$ | $\tau_1$ [a] (ns) | $A_2$ | $\tau_2$ [a] (ns) | $\tau_{av}$ (ns) |
|---|---|---|---|---|---|
| **ssRNA2** | 2900 ± 30 | 1.1 | 4140 ± 30 | 3.8 | 2.7 ± 0.1 |
| **dsRNA2** | 1850 ± 50 | 1.6 | 3850 ± 70 | 4.1 | 3.3 ± 0.1 |
| **dsRNA1-2** | 1780 ± 20 | 0.9 | 1870 ± 20 | 3.6 | 2.3 ± 0.1 |
| **ssRNA4** | 3460 ± 30 | 1 | 4420 ± 30 | 3.6 | 2.5 ± 0.1 |
| **dsRNA4** | 5150 ± 40 | 0.7 | 4080 ± 20 | 3.9 | 2.1 ± 0.1 |
| **dsRNA3-4** | 2410 ± 40 | 0.4 | 1180 ± 10 | 3.1 | 1.3 ± 0.1 |
| **ssRNA6** | 3460 ± 30 | 0.9 | 4300 ± 30 | 3.7 | 2.5 ± 0.1 |
| **dsRNA6** | 5980 ± 50 | 0.5 | 3510 ± 10 | 4.5 | 2.0 ± 0.1 |
| **dsRNA5-6** | 2510 ± 20 | 0.8 | 2300 ± 20 | 3.8 | 2.2 ± 0.1 |

[a] Estimated systematic error: ± 0.1 ns.

**4. Molecular dynamics simulation**

The atomic structure of the double-stranded RNA species of sequence (5'-GCUAU(-Acceptor-Dye)ACGACCCUGAAGUUCAUA-3' 3'-GCCGAU(-Donor-Dye)AUGCUGGGACUUCAAG-5') was prepared in the canonical A-RNA conformation with the Nucleic Acid Builder. The RNA was placed in a periodic rhombic dodecahedron box. The size of the box was chosen to ensure a distance of at least 1.3 nm between the RNA molecule and any box face. The box was filled with ca. 24,650 water molecules, and 40 water molecules were replaced by sodium ions to compensate for the negative charge of RNA; the box contained 75,505 atoms in total. The RNA was described with the Amber ff99bsc0+parmχOL3 parameter set, translated into the Gromacs file format with the Ambconv utility. The fluorescent dyes were represented with the generalized Amber force field (GAFF), and the corresponding atomic charges were obtained with the RESP procedure performed on the HF/6-31G* level using molecular structures optimized on the B3LYP/6-31G* level; the quantum chemical calculations were performed with Gaussian09. The water molecules and the sodium ions were modeled with TIP3P and the parameters by Joung and Cheatham, respectively. All of the hydrogen atoms in RNA were considered as virtual sites, and all of the bond lengths were constrained to their respective equilibrium values. This treatment made it possible to integrate the Newton equations of motion with an extended time step of 4 fs. Unrestrained MD simulations were performed both for the ribo-configured and arabino-configured fluorescent dyes, employing the following protocol:

• 1000 steps of steepest descent energy minimization.

• Assignment of random initial velocities from the Maxwell–Boltzmann distribution at 10 K.
• $5 \cdot 10^6$ steps (20 ns) of constant-volume heating to 300 K using the Nosé–Hoover thermostat with a coupling time of 2 ps. Two separate thermostat coupling groups were considered, containing RNA with the dyes, and water together with ions.

• $5 \cdot 10^6$ steps (20 ns) of constant-pressure simulation at 300 K and 1 bar. Two separate Nosé–Hoover thermostats with a coupling time of 2 ps were considered. The Parrinello–Rahman barostat was applied with a coupling time of 2 ps.

• Finally, for the production simulation, $1 \cdot 10^9$ steps (4000 ns) of constant-pressure simulation were performed at 300 K and 1 bar using a single Nosé–Hoover thermostat (coupling time of 2 ps) and the Parrinello–Rahman barostat (coupling time of 2 ps).

The electrostatics was treated with the particle–mesh Ewald algorithm with a direct-space cut-off of 1.0 nm and the group cut-off scheme. The Lennard-Jones interactions were cut off at 1.0 nm. The equations of motion were integrated by means of the leap-frog algorithm with a time step of 4 fs. All of the hydrogen atoms were considered as virtual sites, for which no equations of motion were solved, rather their new positions were obtained with a lever rule and the forces obtained on these atoms were distributed on neighboring atoms. All of the bond lengths were constrained with p-LINCS. All of the simulations were carried out with the Gromacs 5.0 program package.

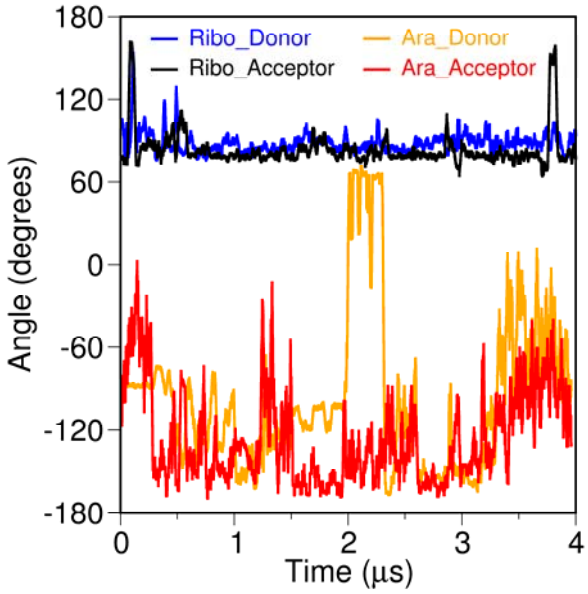


**Figure S14.** Torsional angle C1'-C2'-O2'-CA, which describes the rotation of the fluorescent dyes conjugated to the ribose / arabinose ring of RNA, in the course of the MD simulations of ribo-RNA and arabino-RNA.

**5. Imaging of HeLa cells**

**Cell culture and transfection.** Human cervix carcinoma cells (HeLa cells) were cultured under sterile conditions at 37 °C and 5 % $CO_2$ in Dulbecco's modified Eagle's medium (DMEM, high glucose, Invitrogen) with addition of 60 µg/ml penicillin, 100 µg/ml streptomycin and 10 % fetal calf serum (FCS, Sigma-Aldrich). Regular subculturing at 70- 80 % confluency was conducted.
For transfection experiments, 2 x $10^4$ HeLa cells (1 x $10^4$ for the 48 h experiments) were transferred into each well of an 8 well µ-slide (µ Slide 8 well ibiTreat, IBIDI). After 24 h, adherent cells were transfected with ScreenFect®siRNA transfection reagent (ScreenFect GmbH) with a final concentration of 5 pmol of the respective siRNA construct. Therefore, according to the manufactures protocol, a ScreenFect®siRNA reagent dilution with 2 µl ScreenFect®siRNA and 38 µl of the corresponding Dilution Buffer (ScreenFect GmbH) was prepared. For each approach, 10 µl were combined with a total amount of 5 pmol siRNA (2,5 µM stock solution in 10 mM $Na_2HPO_4$ buffer and 250 mM NaCl, pH 7) diluted in 8 µl Dilution Buffer, except for the control group, which did not contain siRNA. After incubation for 30 min at room temperature, 180 µl fresh culture medium was added to each approach and applied to the prepared adherent cells. The transfection process was stopped after 15 min, 4,5 h, 24 h or 48 h, respectively, by washing the cells 3 times with fresh culture medium.

**Live cell imaging by confocal microscopy.** After transfection, all siRNA constructs could be detected inside the cells via live cell confocal fluorescence microscopy using a Leica DMI8-CS inverse microscope with a HCPL APO CS2 40x/1.10 WATER objective. Image acquisition was conducted at a lateral resolution of 1024 × 1024 pixels and 8 bit depth using LAS-AF 1.1.0.12420 software. Dye **1** was excited using the 458 nm line of an argon ion laser and simultaneous detection of the fluorescence emission at 490–515 nm (dye **1**) and 670–795 nm (dye **2**), complemented with a brightfield image.

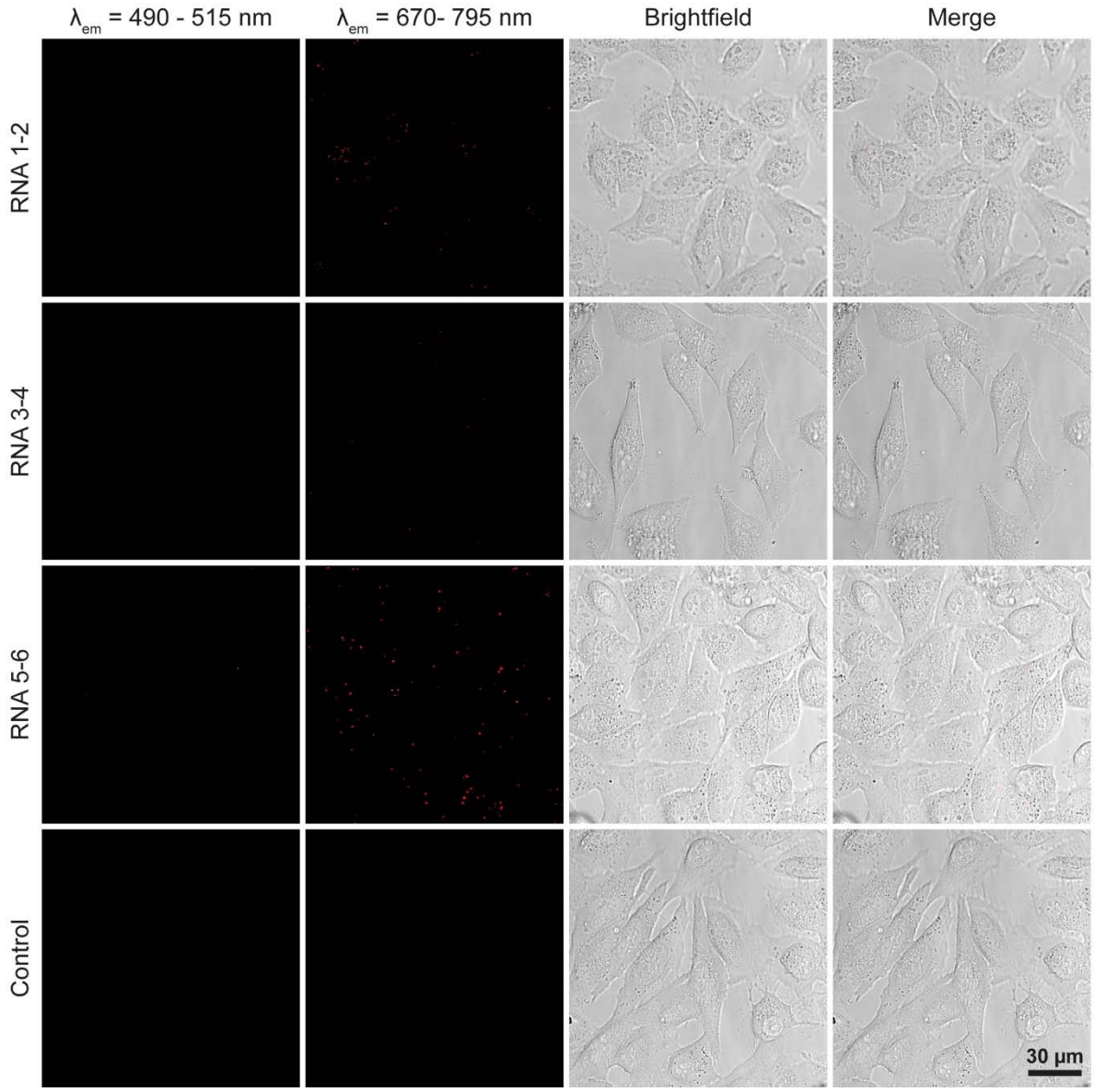


**Figure S15.** Live cell imaging by confocal microscopy: HeLa cells after 20 min transfection with 5 pmol siRNA (**RNA1-2**, **RNA3-4**, **RNA5-6**, no RNA in the control group), visualized by using a Leica DMI8-CS inverse microscope with a HCPL APO CS2 40x/1.10 WATER objective. Dye **1** (energy donor) was excited at $\lambda_{ex}$ = 458 nm, emission was captured at $\lambda_{em}$ = 490 - 515 nm (dye **1**, column 2) and $\lambda_{em}$ = 670 - 795 nm (dye **2** as energy acceptor, column 3). The control group showed no fluorescence with these settings. Scale bar: 30 µm.

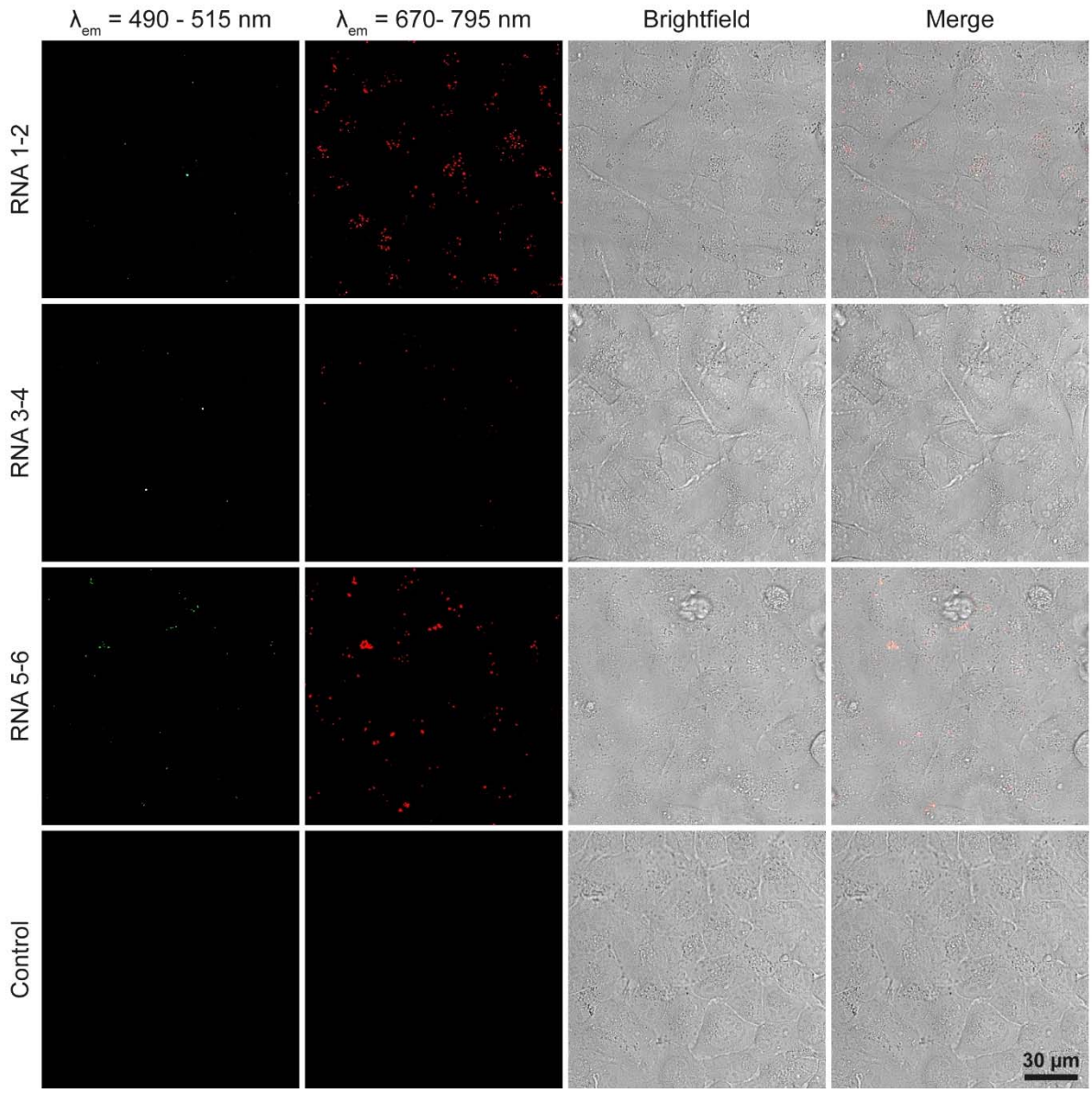


**Figure S16.** Live cell imaging by confocal microscopy: HeLa cells after 24 h transfection with 5 pmol siRNA (**RNA1-2**, **RNA3-4**, **RNA5-6**, no RNA in the control group), visualized by using a Leica DMI8-CS inverse microscope with a HCPL APO CS2 40x/1.10 WATER objective. Dye **1** (energy donor) was excited at $\lambda_{ex}$ = 458 nm, emission was captured at $\lambda_{em}$ = 490 - 515 nm (dye **1**, column 2) and $\lambda_{em}$ = 670 - 795 nm (dye **2** as energy acceptor, column 3). The control group showed no fluorescence with these settings. Scale bar: 30 µm.

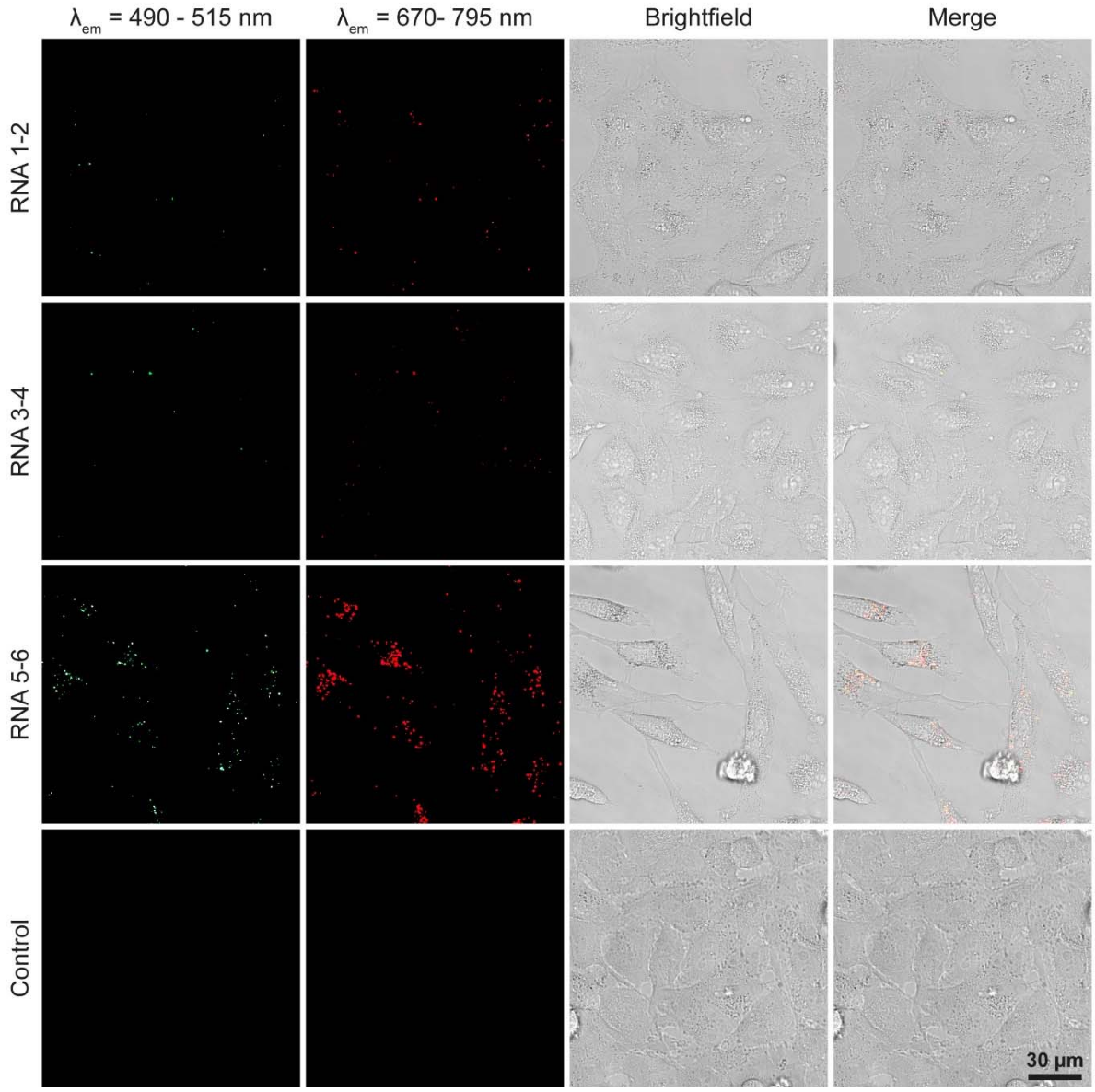


**Figure S17.** Live cell imaging by confocal microscopy: HeLa cells after 48 h transfection with 5 pmol siRNA (**RNA1-2**, **RNA3-4**, **RNA5-6**, no RNA in the control group), visualized by using a Leica DMI8-CS inverse microscope with a HCPL APO CS2 40x/1.10 WATER objective. Dye **1** (energy donor) was excited at $\lambda_{ex}$ = 458 nm, emission was captured at $\lambda_{em}$ = 490 - 515 nm (dye **1**, column 2) and $\lambda_{em}$ = 670 - 795 nm (dye **2** as energy acceptor, column 3). The control group showed no fluorescence with these settings. Scale bar: 30 µm.

## 6. References

[1] a) J. Steinmeyer, F. Rönicke, U. Schepers, H.-A. Wagenknecht, *ChemistryOpen* **2017**, *6*, 514-518; b) H.-K. Walter, P. R. Bohländer, H.-A. Wagenknecht, *ChemistryOpen* **2015**, *4*, 92-96.

[2] M. Szabelski, R. Luchowski, Z. Gryczynski, P. Kapusta, U. Ortmann, I. Gryczynski, *Chem. Phys. Lett.* **2009**, *471*, 153-159.