Nucleic-acid templated chemical reaction in a live vertebrate

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Table S1: miRNA target sequences and PNA sequences for templated reactions (non capitalized letters in PNA sequence denote a modified PNA with a serine side-chain at the γ position[1], chemical structures can be found later in this supplemental information)

Name	Target RNA Sequence	Rhodamine PNA conjugate (PM)	Ruthenium PNA sequence	Mismatched rhodamine PNA conjugate (MM)
miR9	5'-UCU UUG GUU AUC UAG CUG UAU G– 3'	PEG-aGa AaC cA-Linker(N3)-Rh	Ru-Lys-Ag AtC gAc AtA-NHAc	PEG-aAa AgC cA-Linker(N3)-Rh
miR196	5'-UAG GUA GUU UCA UGU UGU UGG G – 3'	PEG-aTc CaT cA-Linker(N3)-Rh	Ru-Lys-TaC aAc AaC cC-NHAc	PEG-aAc CgT cA-Linker(N3)-Rh
miR206	5'-UGG AAU GUA AGG AAG UGU GUG G– 3'	PEG-cTt AcA tT-Linker(N3)-Rh	Ru-Lys-CcT tCa CaC aC-NHAc	PEG-cCt AtA tT-Linker(N3)-Rh

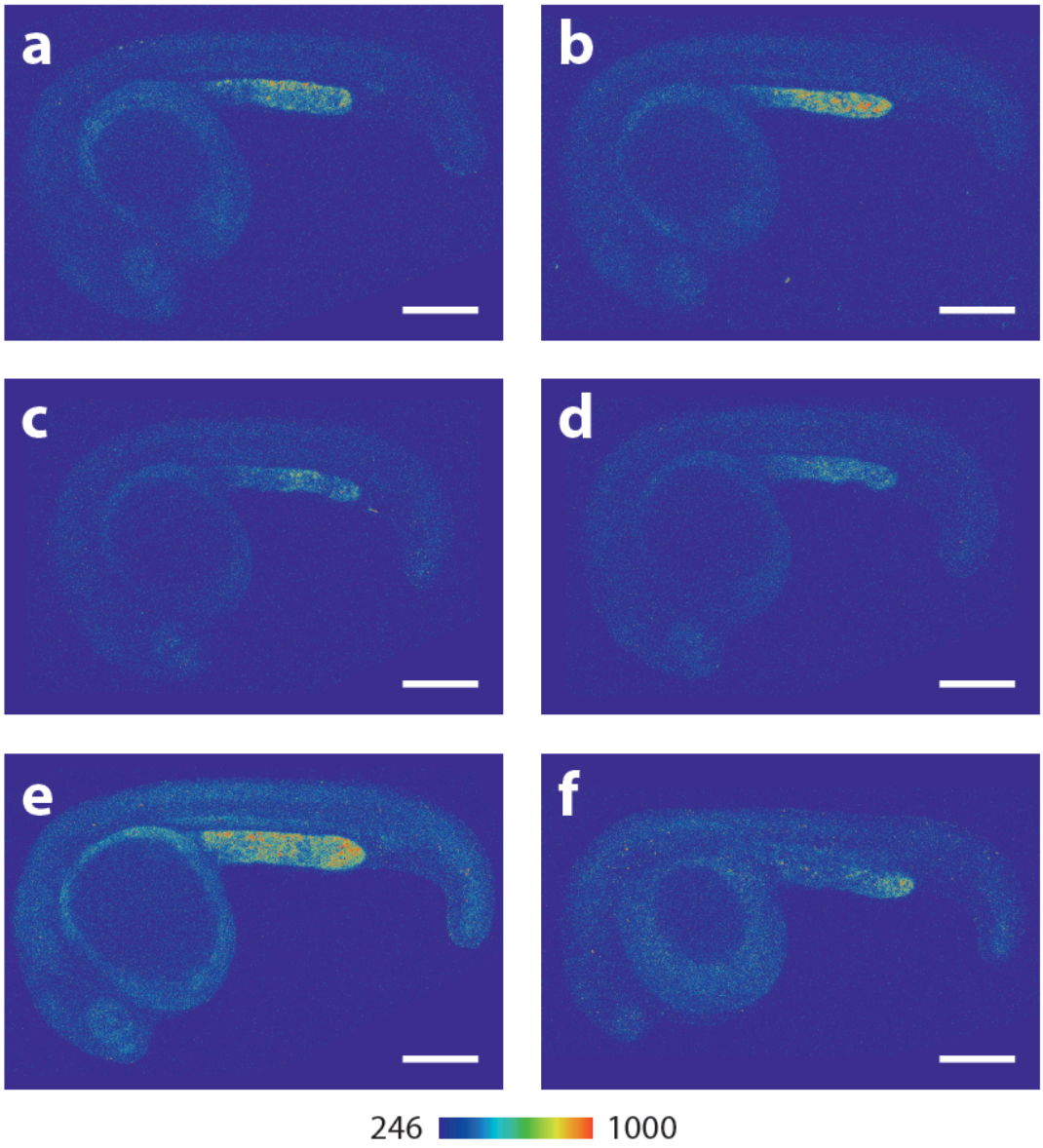


Figure S1. **Fluorescence imaging of wt uninjected zebrafish embryos at 24 hpf (rhodamine channel).** Maximum intensity projections of image stacks showing rhodamine fluorescence in individual embryos. Images were assembled from tile scans using ImageJ. Color lookup-tables are identical for each PM-MM pair. Scale bar = 250 µm. Lookup table (LUT) of fluorescence intensity (bottom).

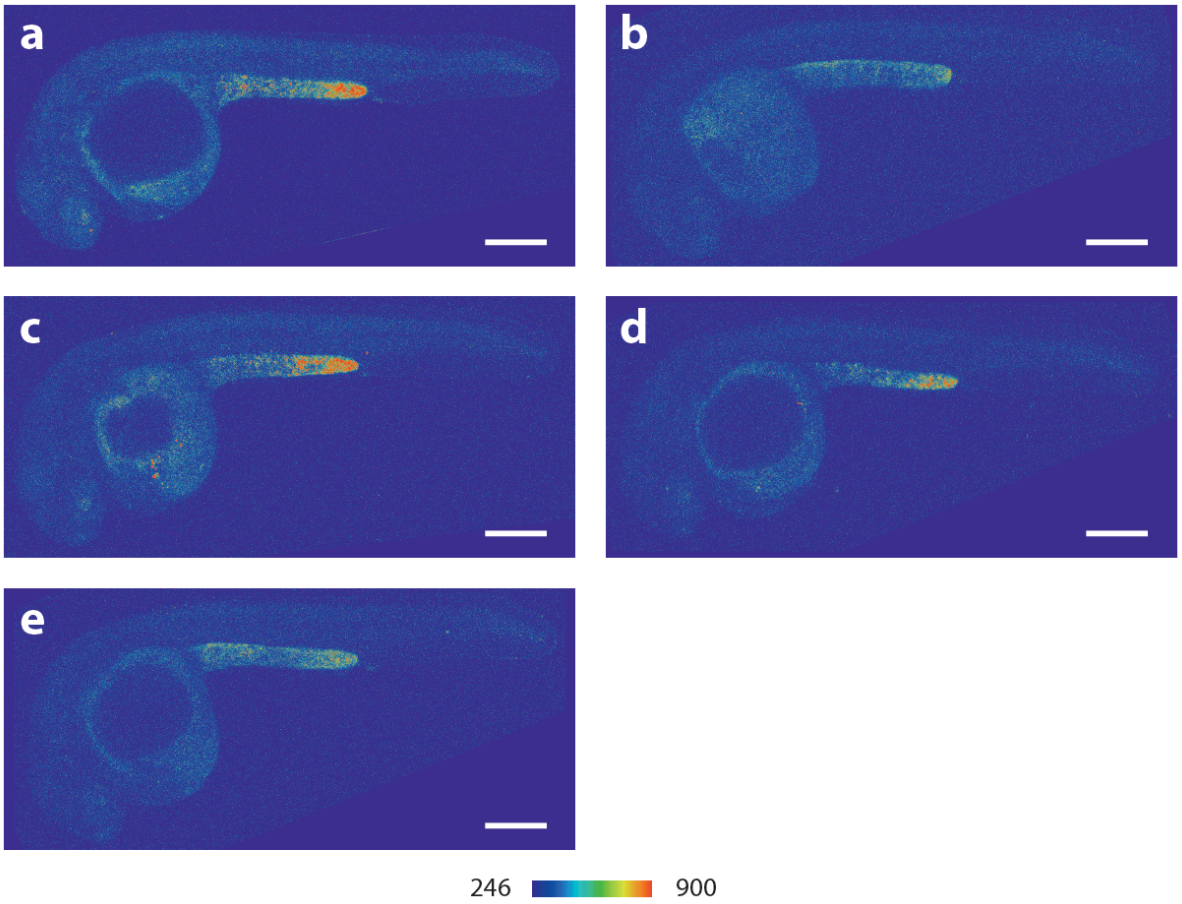


Figure S2. **Fluorescence imaging of wt uninjected zebrafish embryos at 36 hpf (rhodamine channel).** Maximum intensity projections of image stacks showing rhodamine fluorescence in individual embryos. Images were assembled from tile scans using ImageJ. Color lookup-tables are identical for each PM-MM pair. Scale bar = 250 µm. Lookup table (LUT) of fluorescence intensity (bottom).

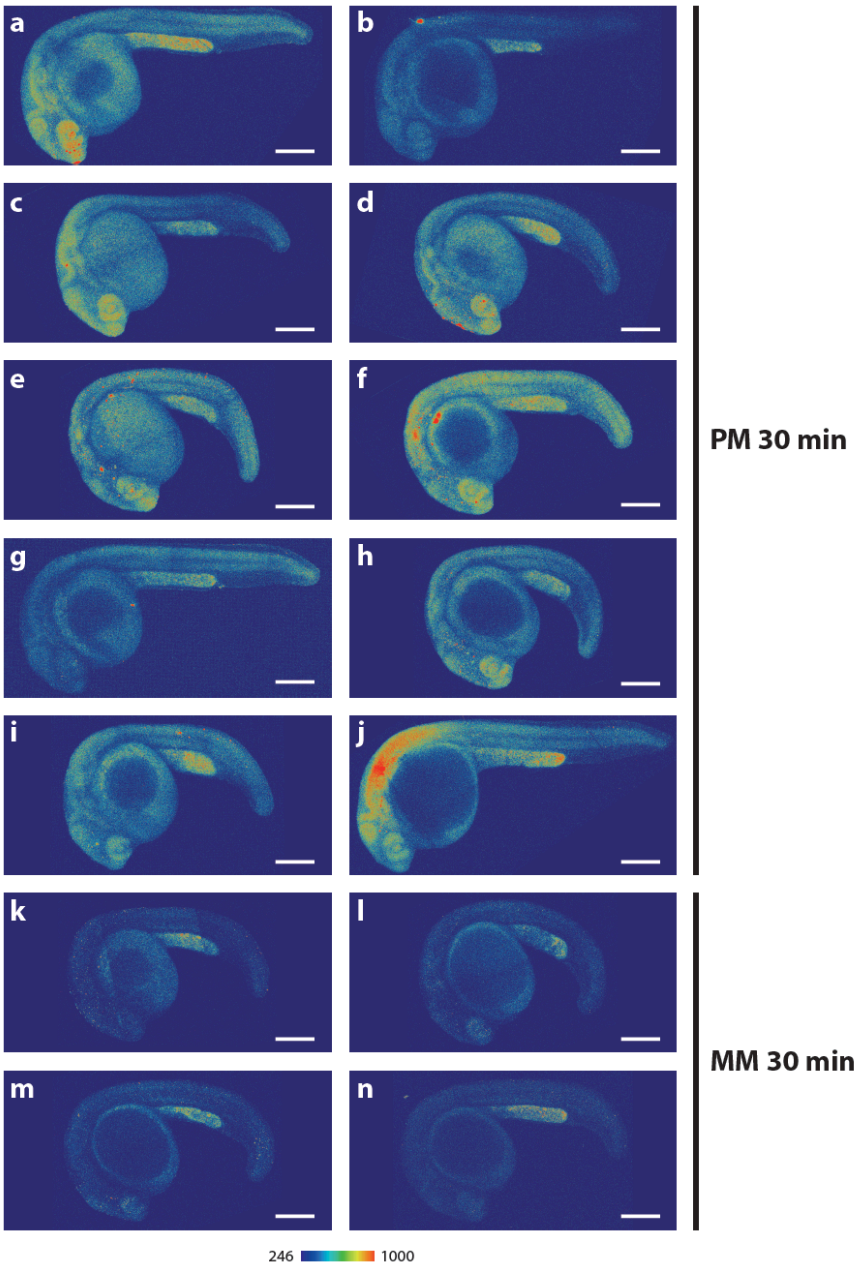


Figure S3. **Fluorescence imaging of zebrafish embryos injected with miR-9 probes at 24 hpf.** Wild type zebrafish embryos were injected with 0.5 nL mixture of appropriate Rho-PNA and Ru-PNA probes (ratio 4:1). Maximum intensity projections of image stacks showing rhodamine fluorescence in individual live zebrafish embryos injected with either matched probes (PM: **a**-**j**) or mismatched probes (MM: **k**-**n**) after 30 minutes of irradiation with 455 nm light. Images were assembled from tile scans using ImageJ. Color lookup-tables are identical for each PM-MM pair. Scale bar = 250 μm. Lookup table (LUT) of fluorescence intensity (bottom).

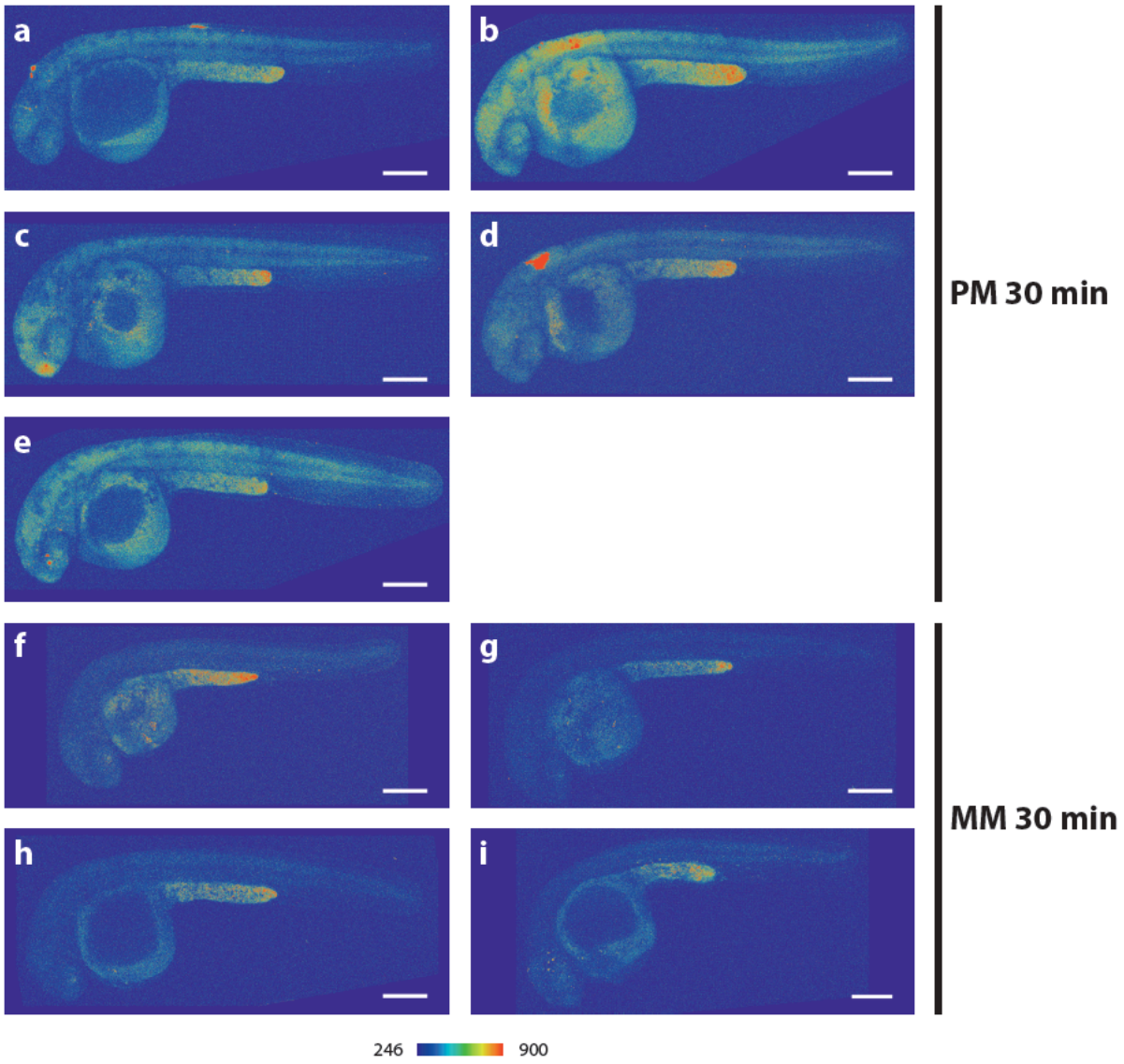


Figure S4. **Fluorescence imaging of zebrafish embryos injected with miR-9 probes at 36 hpf.** Wild type zebrafish embryos were injected with 0.5 nL mixture of appropriate Rho-PNA and Ru-PNA probes (ratio 4:1). Maximum intensity projections of image stacks showing rhodamine fluorescence in individual live zebrafish embryos injected with either matched probes (PM: **a-e**) or mismatched probes (MM: **f-i**) after 30 minutes of irradiation with 455 nm light. Images were assembled from tile scans using ImageJ. Color lookup-tables are identical for each PM-MM pair. Scale bar = 250 µm. Lookup table (LUT) of fluorescence intensity (bottom).

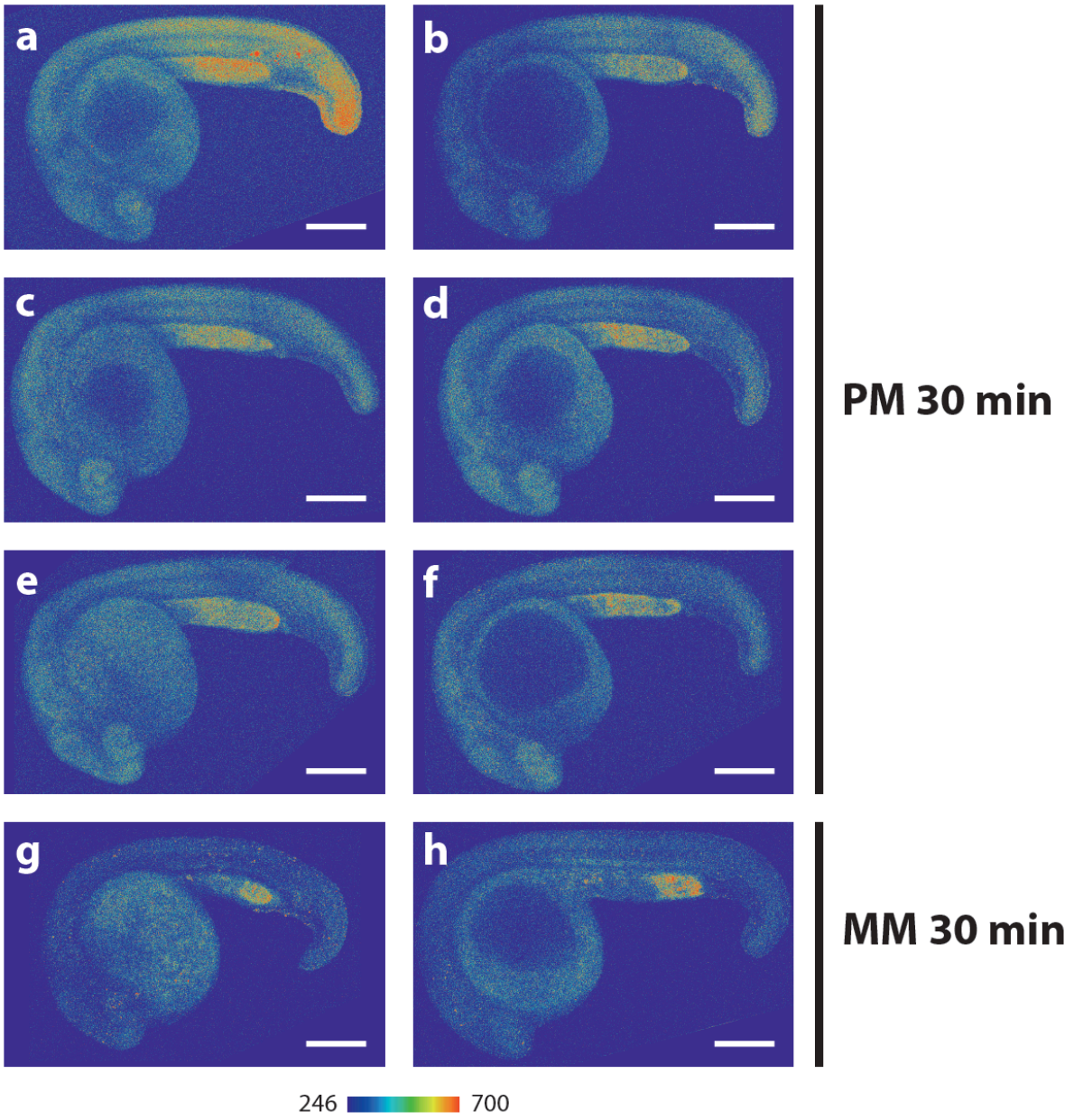


Figure S5. **Fluorescence imaging of zebrafish embryos injected with miR-196 probes at 24 hpf.** Wild type zebrafish embryos were injected with 0.5 nL mixture of appropriate Rho-PNA and Ru-PNA probes (ratio 4:1). Maximum intensity projections of image stacks showing rhodamine fluorescence in individual live zebrafish embryos injected with either matched probes (PM: **a-f**) or mismatched probes (MM: **g-h**) after 30 minutes of irradiation with 455 nm light. Images were assembled from tile scans using ImageJ. Color lookup-tables are identical for each PM-MM pair. Scale bar = 250 µm. Lookup table (LUT) of fluorescence intensity (bottom).

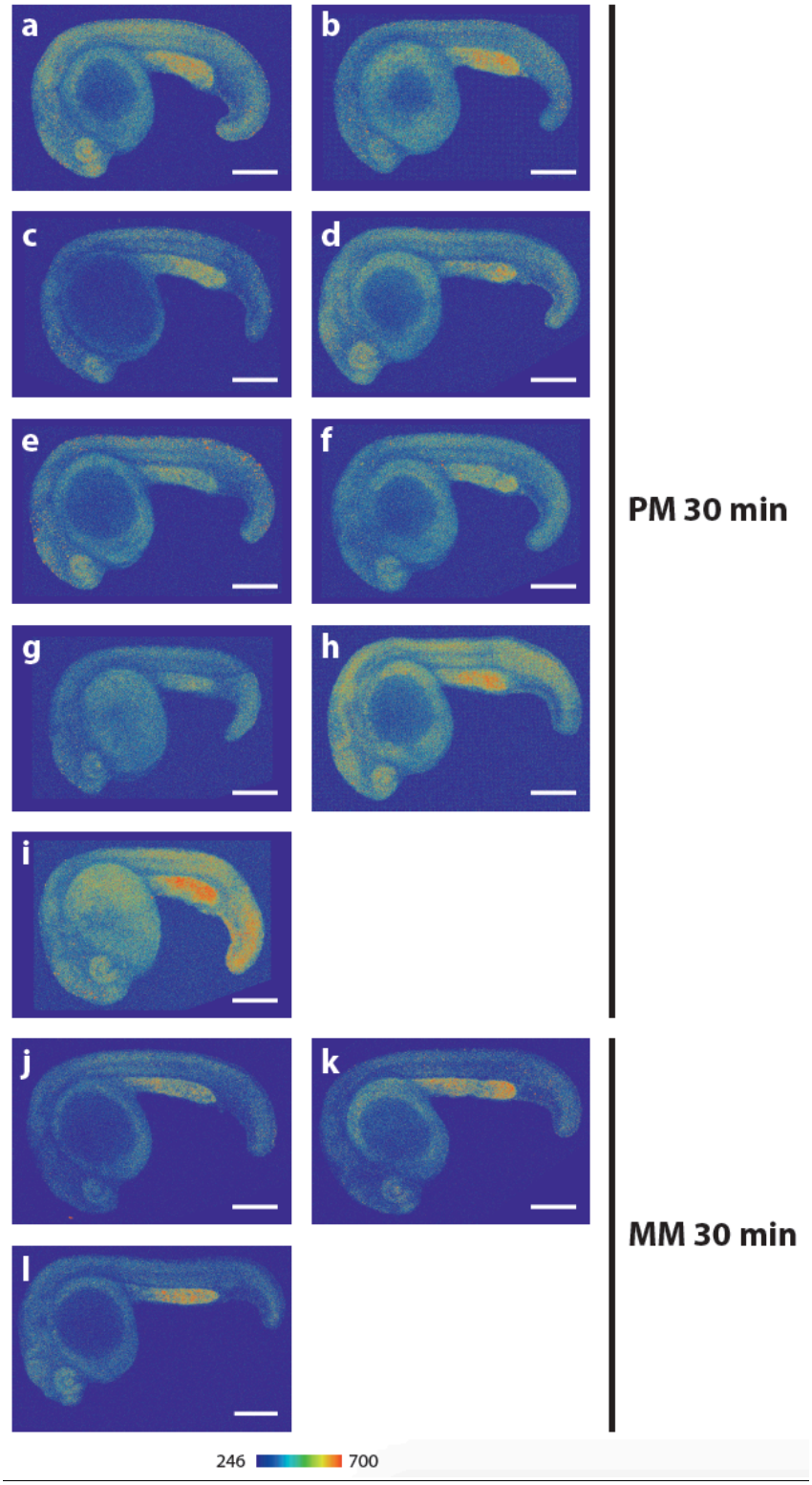


Figure S6. **Fluorescence imaging of zebrafish embryos injected with miR-206 probes at 24 hpf.** Wild type zebrafish embryos were injected with 0.5 nL mixture of appropriate Rho-PNA and Ru-PNA probes (ratio 4:1). Maximum intensity projections of image stacks showing rhodamine fluorescence in live zebrafish embryos injected with either matched probes (PM: **a-i**) or mismatched probes (MM: **j-l**) after 30 minutes of irradiation with 455 nm light. Images were assembled from tile scans using ImageJ. Color lookup-tables are identical for each PM-MM pair. Scale bar = 250 µm. Lookup table (LUT) of fluorescence intensity (bottom).

Staging and maintenance of zebrafish embryos

Wild-type (AB) zebrafish strains were maintained as previously described.[2] Embryos were staged as previously described.[3] We verified by re-sequencing ourselves that sequences used to design the different probes[4] correspond to the genomic sequence of the wt strain (AB) used in our laboratory.

***In vivo* templated chemistry in zebrafish embryos**

Rho-PNA and Ru-PNA probes were premixed in a 4:1 ratio (unless otherwise specified: [Rho-PNA]=500 μM in DMF, [Ru-PNA]=125 μM in H_2O). 0.5 nL of this mixture was injected into the cell of 1-cell stage wild-type zebrafish embryos. Embryos were raised in the dark at 28°C. At the desired stage, embryos were placed in a small petri dish (without lid) and irradiated with 455 nm LED (1W) light (Thorlabs Inc, Newton, NJ, USA) for a period of 30 minutes. Embryos were screened for fluorescence using a fluorescent stereomicroscope after which they were prepared for confocal imaging.

Confocal imaging

Live embryos were dechorionated if necessary and were transferred to a droplet of 1% low melting point agarose (Sigma Aldrich, Buchs, Switzerland) in 0.3X Danieau medium (17.40 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4 \bullet 7H_2O$, 0.18 mM $Ca(NO_3)_2$, 1.50 mM HEPES, pH 7.6), placed on a glass bottom dish (MatTek, Ashland, MA, USA). Live embryos were anesthetized by adding 0.020% tricaine (ethyl 3-aminobenzoate methanesulfonate, Sigma Aldrich, Buchs, Switzerland) to the agarose. Three-dimensional confocal images were acquired using a Zeiss LSM710 upright confocal microscope with a 20X/1.0 water dipping objective (Carl Zeiss, Germany) using the 488 nm laser for the Rhodamine probe. Z-spacing between planes was typically 5 μm.

A Leica SP5 inverted confocal microscope was used to directly image the Ruthenium probe. Ruthenium was excited using a white laser at 470 nm, while using time gating (time gate start: 5.00 ns, time gate end: 12.00 ns) for the detection of the fluorescence after passing through an 600-640 nm emission filter.[5]

Image analysis and quantification

Confocal images were analyzed and quantified using the FIJI software.[6] Tile stitching was done using the BioFormats-plugin.[7] To analyze the fluorescent signal, background fluorescence levels were estimated from noise outside the embryo and the corresponding value was removed from each plane. Image stacks were then projected onto a two-dimensional image using a maximum intensity projection. Fluorescent intensity in different parts of the embryo was measured by calculating the average intensity in selected regions with an area of 10-40 cells in the maximum projection.

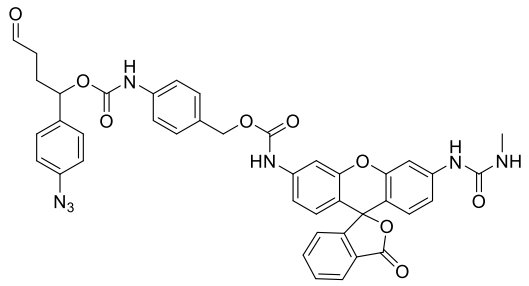
Possible off-target hits analysis

In order to find possible off-target hits, we generated a list of all possible combinations of single mismatches and gap sizes (from zero to four) for each miRNA, both for the PM and MM pair. The resulting list (about half a million sequences for each pair) was then compared to the NCBI RefSeq Danio Rerio mRNA database (Early February 2016) by running the NCBI command line tool 'blastn' locally.[8] No off-target hits were found for any of the probe pairs for gap sizes between 0 and 4 nt while not allowing for any mismatches in the probes themselves. Only for miR-206 MM we found one off-target hit when we allowed one of the probes to have a mismatch nucleotide. For the other probe pairs we found off-target hits only when we allowed both probes to have a mismatch nucleotide.

General techniques for PNA synthesis

Anhydrous solvents were obtained by passing them through a commercially available alumina column (Innovative technology, MA). Solid phase syntheses were performed with NovaPEG Rink amide resin obtained from EMD Millipore. PNA synthesis was performed according to previously reported protocols[1,9] using an automated peptide synthesizer (Intavis MultiPep instrument in 500 µL fritted tubes.). LC-MS were recorded by using a Thermo Scientific Accela HPLC equipped with a Thermo C18 (5 cm x 2.1 mm, 1.9 µm particles) Hypersil gold column coupled with A: Surveyor MSQ Plus spectrometer or B: LCQ Fleet mass spectrometer (both ESI, Thermo Scientific). Method: linear elution gradient for 95% H_2O 0.01% TFA to 90% MeCN 0.01% TFA in 4 minutes at a flow rate of 1.0 mL/min. The MALDI spectra were measured using Bruker Daltonics Autoflex TOF/TOF spectrometer. Final compounds were purified by reverse-phase chromatography using a Biotage Isolera ONE equipped with a Biotage SNAP Cartridge KP-C18-HS (linear gradient from 100% H_2O 0.01% TFA to 100% MeCN 0.01% TFA with a flow rate of 5 mL/min) or using an Agilent 1100 series HPLC equipped with DAD and with a Agilent ZORBAX Eclipse XDB-C18 (4.6 x 250 mm, 5µm) column (linear gradient from 100% H_2O 0.1% TFA to 1000% MeCN 0.1% TFA with a flow rate of 1 mL/min).

Synthesis of the fluorogenic rhodamine – immolative linker conjugate



The compound was synthesized as described previously.[10]

PNA SYNTHESIS

Loading of the first residue

NovaPEG Rink amide resin (0.44 mmol/g, Novabiochem) was swollen in dichloromethane for 30 minutes. Fmoc-Lys(Boc)-OH or Fmoc-AEEA-OH (0.45 equiv, in order to reduce the loading of the resin to 0.2 mmol/g) was dissolved in anhydrous NMP (1 mL/100mg resin), then HOBt (5 equiv) and DIC (15 equiv) were added to the mixture. The mixture was stirred for 15 minutes. The resin was then washed with DMF prior to addition of the activated carboxylic acid and shaken overnight. The unreacted amino groups were capped then the resin was washed with DMF and DCM and dried.

Automated PNA synthesis

PNAs were synthesized in 500 mL fritted tubes by using an Intavis MultiPep instrument in a fully automated fashion. The resin (5 mg, 1 µmol per column) was swollen in DCM (300 µL) for 20 min and deprotected using 20% piperidine in DMF (2x5 min). The resin was then washed with DMF and DCM and treated with a preactivated (5 min) solution of the corresponding Mtt-protected PNA monomer (5 equiv), HATU (4 equiv), DIPEA (5 equiv), and 2,6-lutidine(7.5 equiv) for 20 min in NMP. This process was repeated once (double couplings). Each coupling was followed by a capping step with Ac2O (5.3 equiv) and 2,6-lutidine (6.4 equiv) in DMF (150 µL per column).

Mtt deprotection

The resin was treated with a solution of HOBt in a 1:1 mixture of hexafluoroisopropanol and 1,2-dichloroethane (150 µL per column) for 4 min, then washed with DCM. The process is repeated four times.

Deprotection and coupling of the PNA monomer is iterated until the desired sequence is obtained

Attachment of hydroxylamine spacer

After the deprotection of Mtt, the resin was treated with activated 2-Boc(aminooxy) acetic acid for 30 min. The process is repeated twice. The activation of 2-Boc(aminooxy)acetic acid (10 µmol, 10 equiv) in 100 µl NMP was performed by addition of HATU (9 µmol, 9 equiv), 2,6-lutidine (12 µmol, 12 equiv), and DIPEA (8 µmol, 8 equiv). Then the resin was washed with DMF and DCM.

Preparation of final ruthenium derivative

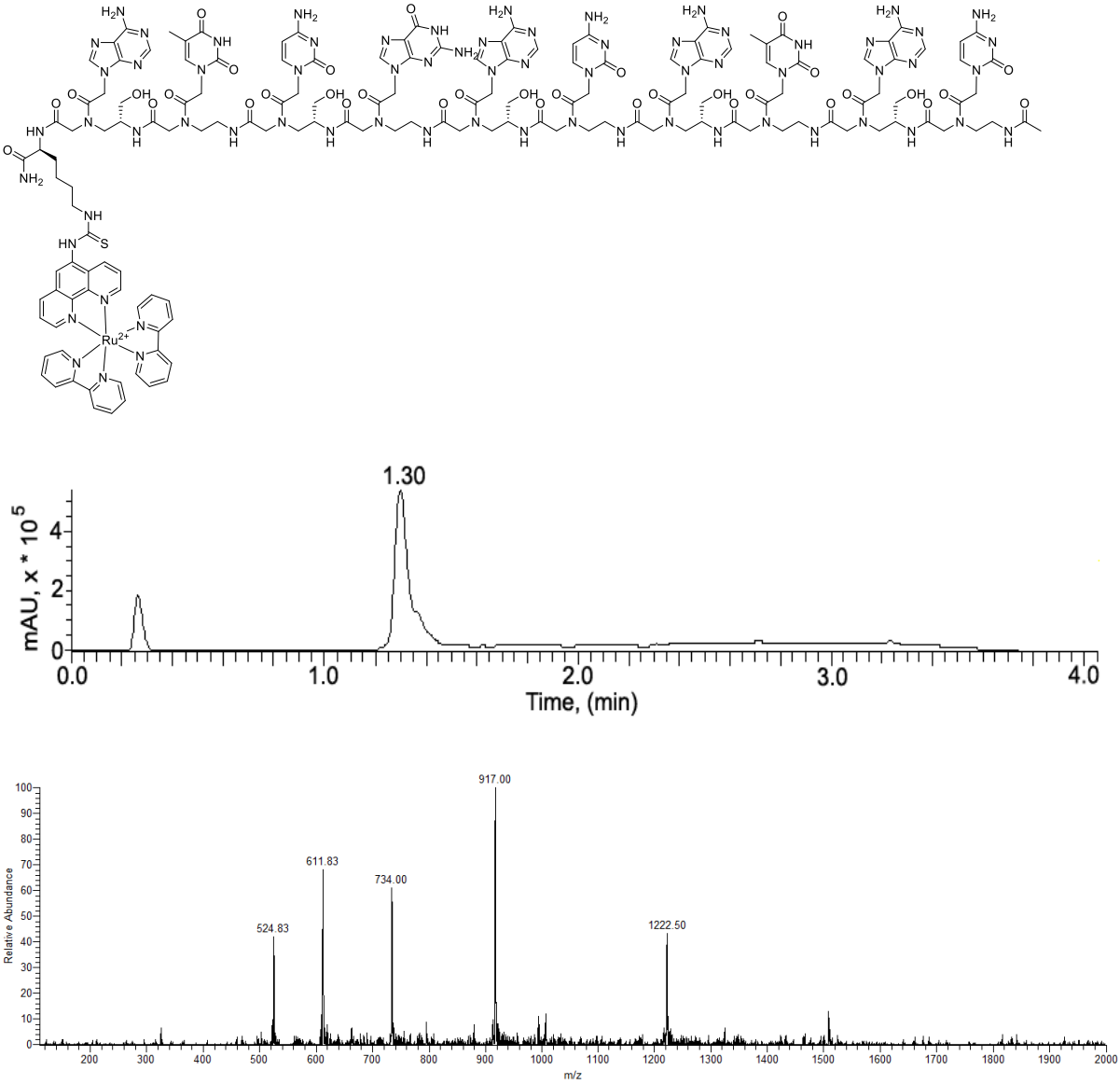
The N-terminus of the PNAs was capped. The resin was suspended in TFA (200 µL for 5mg resin) for 1 h. The solution was then precipitated in diethylether (10 times the volume of TFA) and centrifuged to recover the product as a pellet.
The compound isolated from resin (0.50 µmol, 1 equiv) was dissolved in DMF (70 µL) and commercially available bis(2,2'-bipyridine)-(5-isothiocyanatophenanthroline)-ruthenium bis(hexafluorophosphate) (0.7 mg, 0.75 µmol, 0.75 equiv) in DMF (10 µl) and DIPEA (2.5µl, 15µmol, 30 equiv) were added. The reaction mixture was shaken overnight at RT and then diluted with DMF up to 200 µL and purified by HPLC.

Preparation of final rhodamine derivative

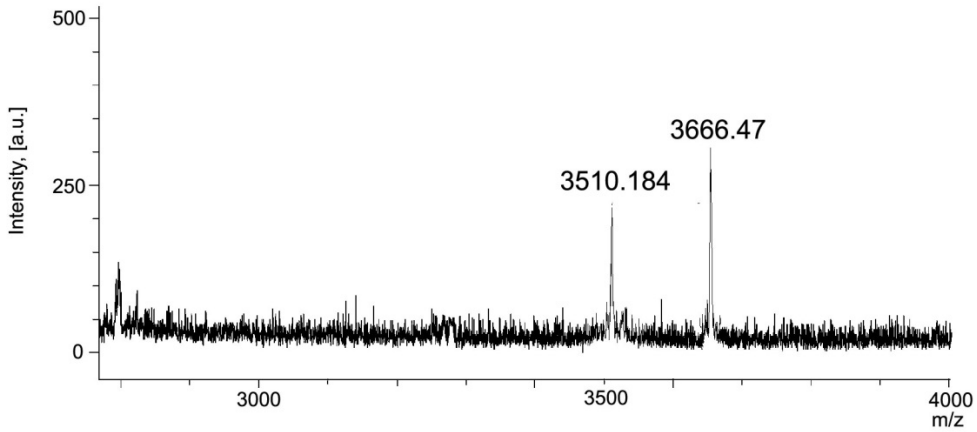
The resin was suspended in TFA (200 µL for 5mg resin) and NH_2OH 50% solution in water (10 µL) for 1 h. The solution was then precipitated in diethylether (10 times the volume of TFA) and centrifuged to recover the product as a pellet.
The compound isolated from resin (0.50 µmol, 1 equiv) was dissolved in a water/acetonitrile 1:1 mixture (50 µL). The rhodamine compound (0.8 mg, 1 µmol, 2 equiv) was dissolved in acetonitrile (50 µL). The two solutions were mixed and shaken overnight. The reaction mixture was diluted with water up to 200 µL and purified by HPLC.

Structure and characterization of PNA probes

miR-9:
Ru-PNA conjugate: Calculated MW ($C_{153}H_{182}N_{68}O_{35}RuS$): 3665.33 Da. LC-MS (ESI) RT= 1.30 min *m/z* found:. 1222.50 $[M+3H]^{3+}$, 917.00 $[M+4H]^{4+}$, 734.00 $[M+5H]^{5+}$, 611.83 $[M+6H]^{6+}$, 524.83 $[M+7H]^{7+}$; MALDI-TOF *m/z* found: 3666.470 $[M+H]^{+}$, 3510.184 $[M+H\text{-bpy}]^{+}$.

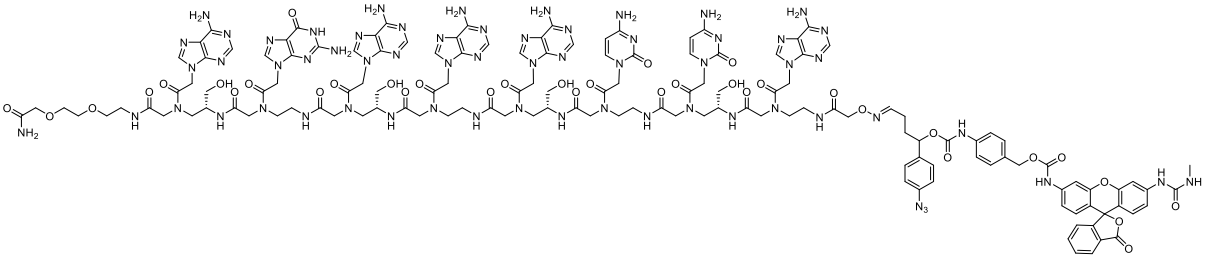


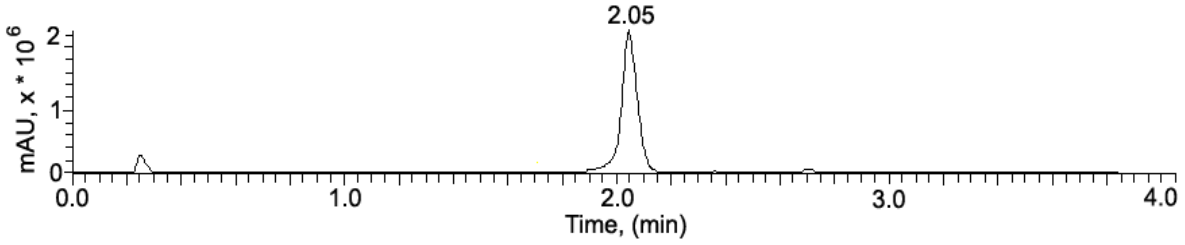
LC/MS analysis of miR-9 Ru-PNA

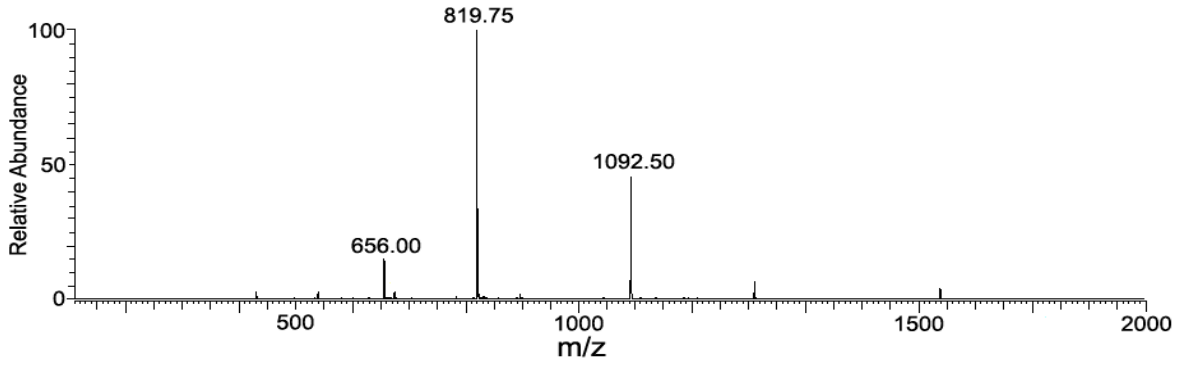


MALDI-TOF MS of miR-9 Ru-PNA.

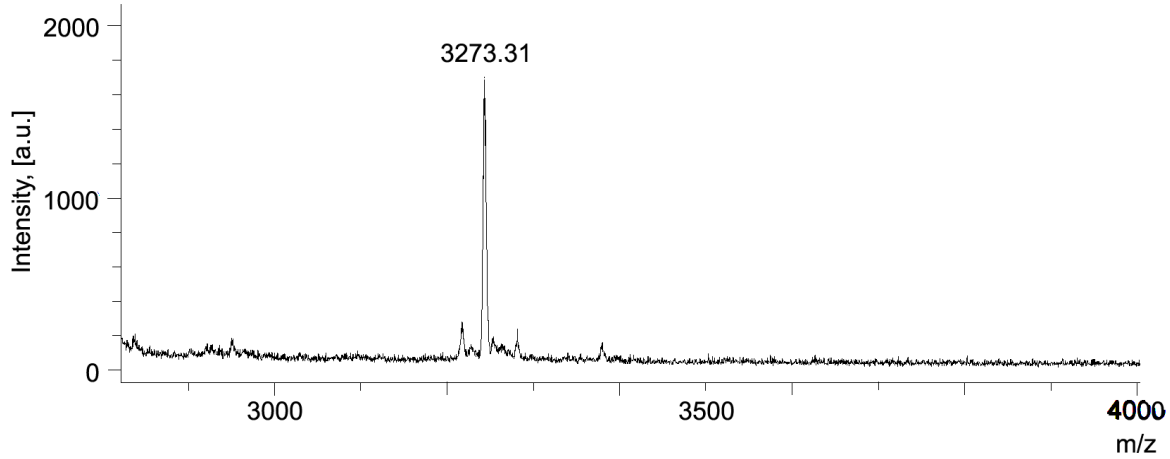
PM Rhodamine conjugate: Calculated MW ($C_{127}H_{147}N_{49}O_{38}$): 3273.26 Da. LC-MS (ESI) RT= 2.05 min *m/z* found:.1092.50 $[M+3H]^{3+}$, 819.75 $[M+4H]^{4+}$, 656.00 $[M+5H]^{5+}$; MALDI-TOF *m/z* found: 3273.31 $[M+H]^{+}$





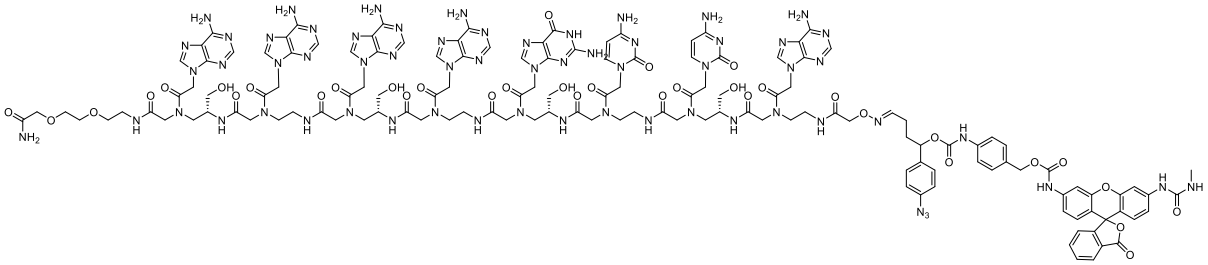


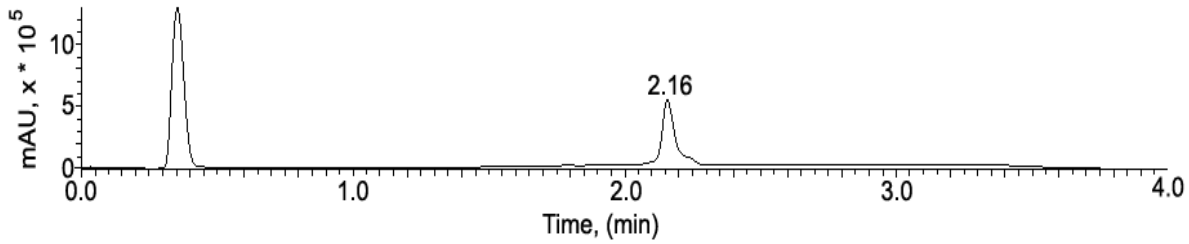
LC/MS analysis of miR-9 PM Rho-PNA.

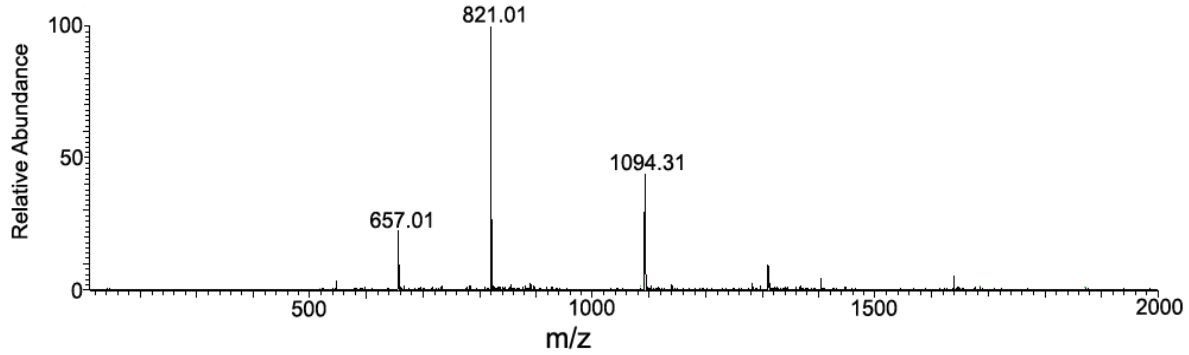


MALDI-TOF spectrum of of miR-9 PM Rho-PNA.

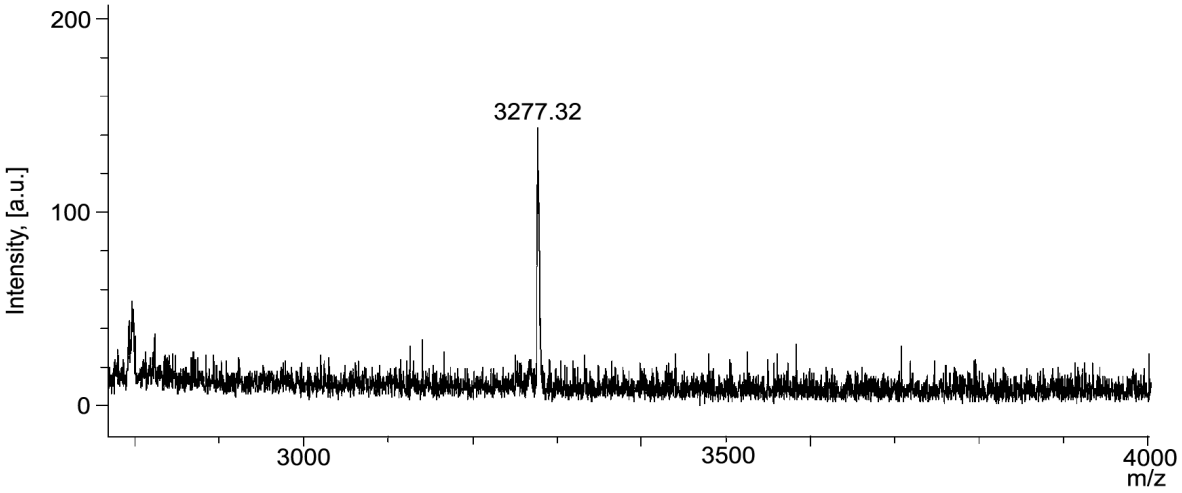
MM Rhodamine conjugate: Calculated MW ($C_{139}H_{160}N_{62}O_{36}$): 3280.20 Da. LC-MS (ESI) RT= 2.16 min *m/z* found:.1094.31 $[M+3H]^{3+}$, 821.01 $[M+4H]^{4+}$,657.01 $[M+5H]^{5+}$; MALDI-TOF *m/z* found: 3277.32 $[M+H]^{+}$







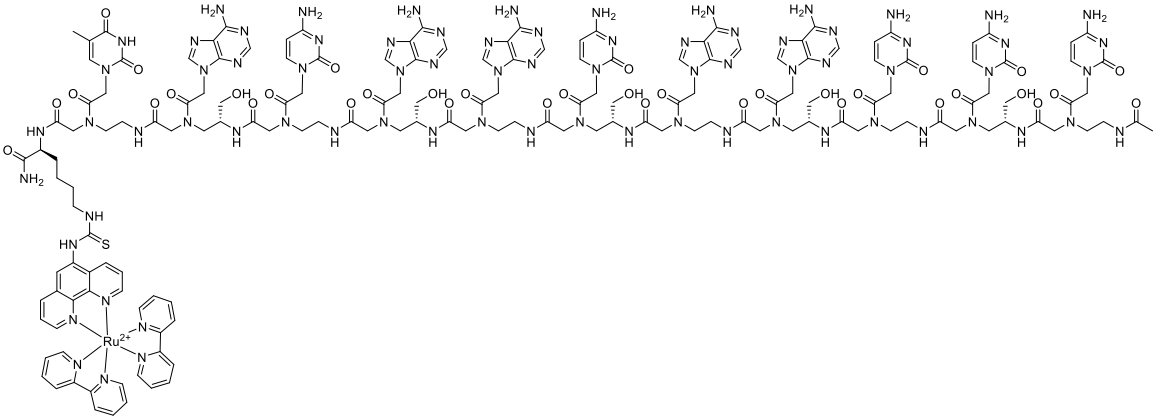
LC/MS analysis of miR-9 MM Rho-PNA.

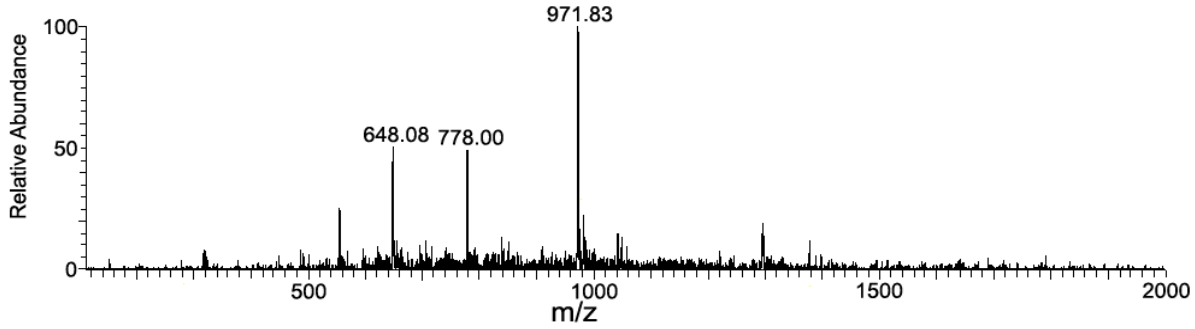


MALDI-TOF MS of miR-9 MM Rho-PNA.

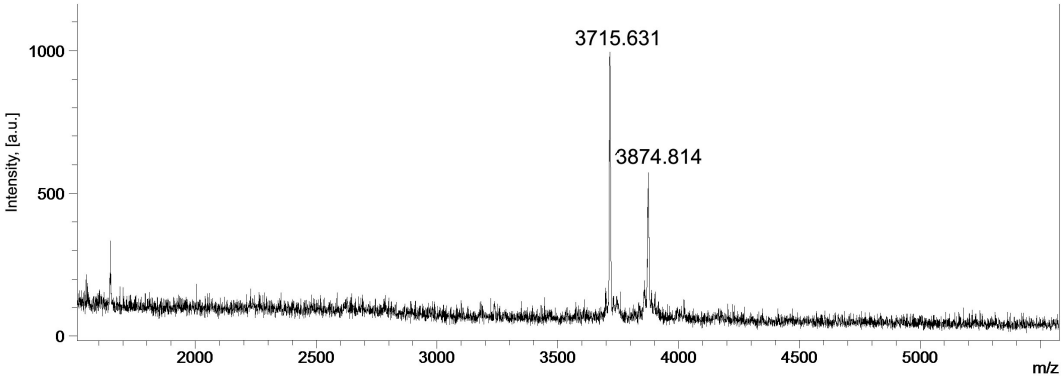
miR-196:

Ru-PNA conjugate: Calculated MW ($C_{162}H_{195}N_{75}O_{36}RuS$): 3877.11 Da. LC-MS (ESI) RT= 1.26 min *m/z* found: 971.83 $[M+4H]^{4+}$, 778.00 $[M+5H]^{5+}$, 648.08 $[M+6H]^{6+}$; MALDI-TOF *m/z* found: 3874.814 $[M+H]^{+}$, 3715.631 $[M+H\text{-bpy}]^{+}$.



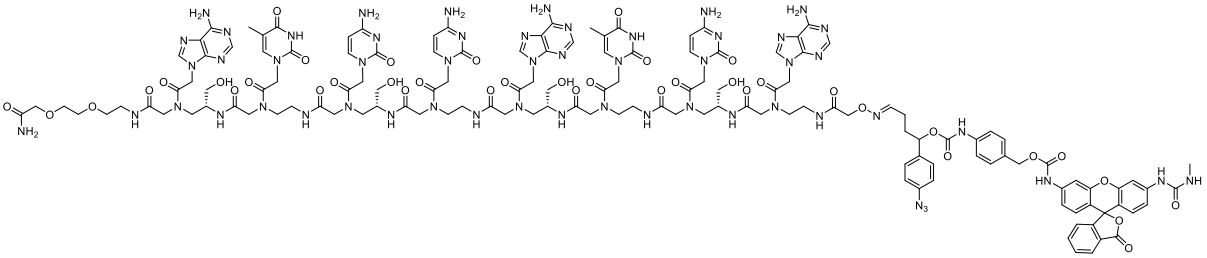


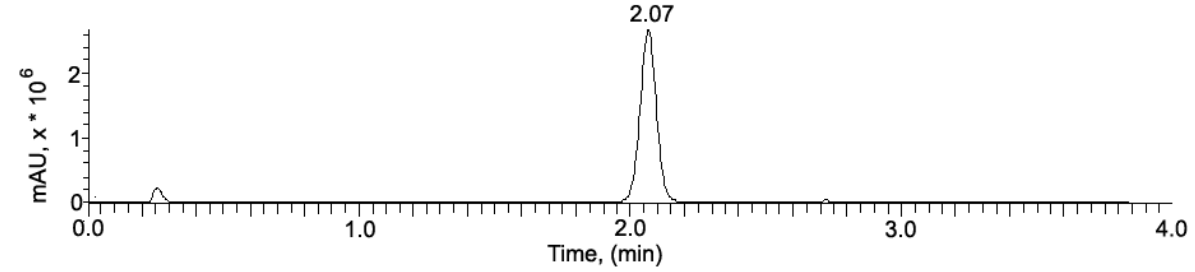
LC/MS analysis of miR-196 Ru-PNA.

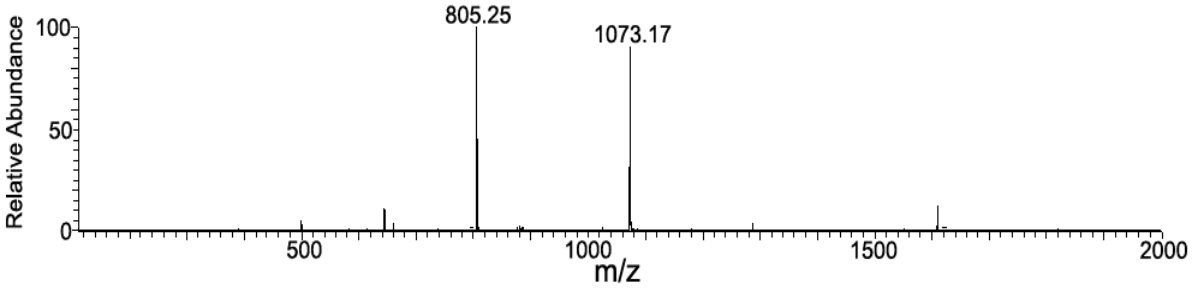


MALDI-TOF MS of miR-196 Ru-PNA.

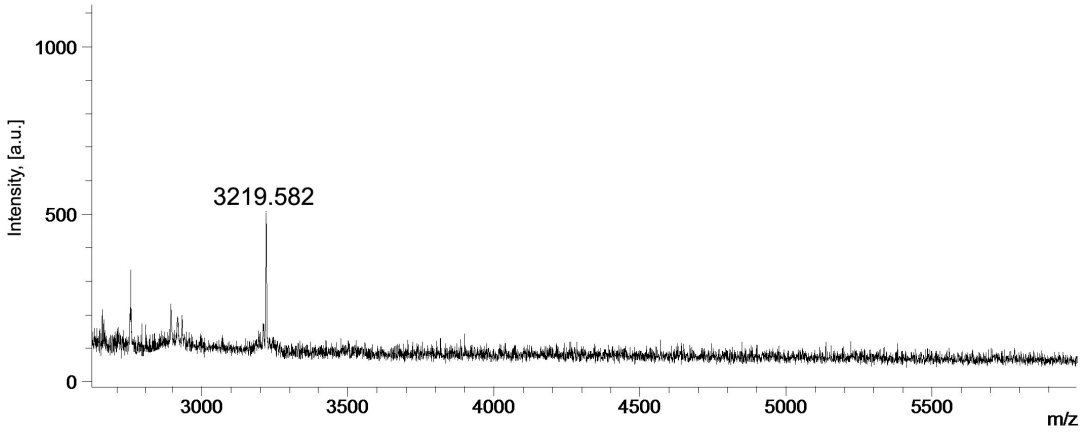
PM Rhodamine conjugate: Calculated MW ($C_{138}H_{162}N_{54}O_{40}$): 3217.04 Da. LC-MS (ESI) RT= 2.07 min *m/z* found: 1073.17 $[M+3H]^{3+}$, 805.25 $[M+4H]^{4+}$, MALDI-TOF *m/z* found: 3219.582 $[M+H]^{+}$.





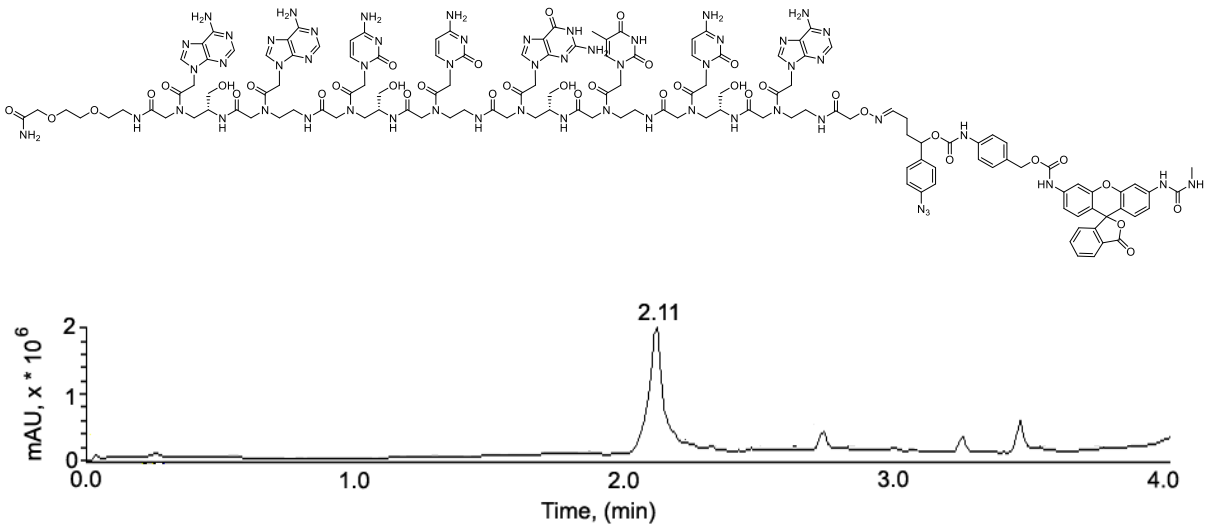


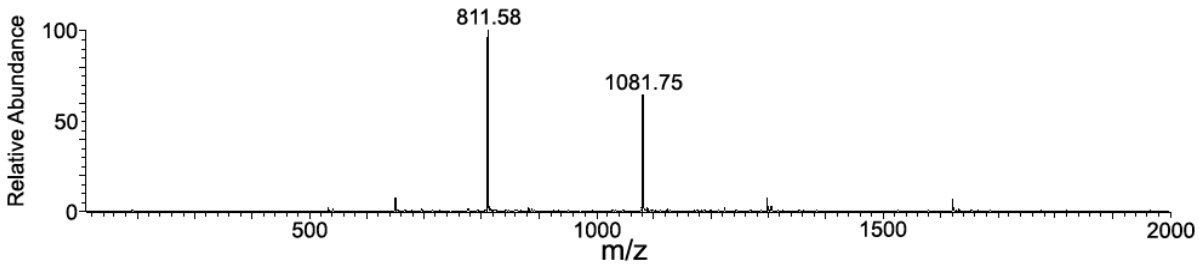
LC/MS analysis of miR-196 PM Rho-PNA.



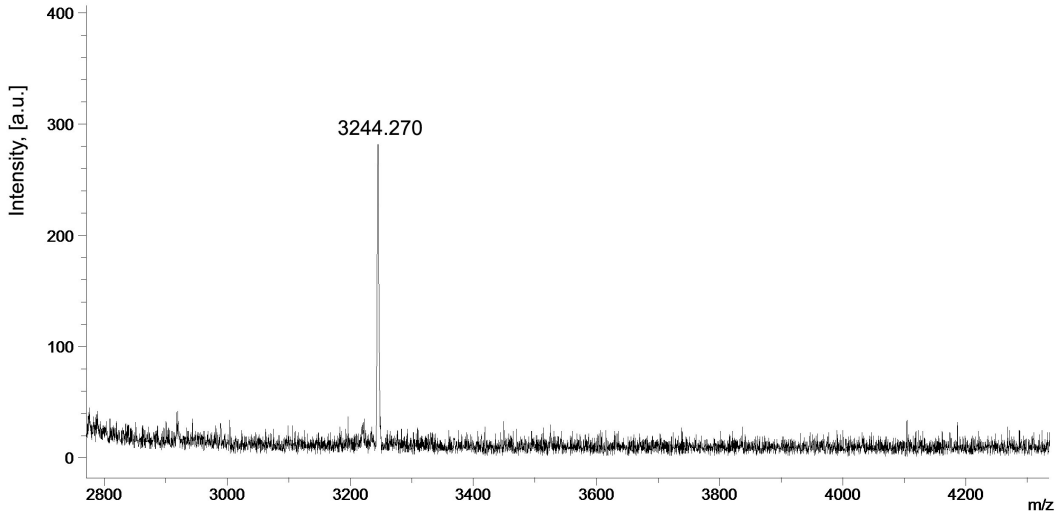
MALDI-TOF MS of miR-196 PM Rho-PNA.

MM Rhodamine conjugate: Calculated MW ($C_{138}H_{161}N_{57}O_{39}$): 3242.05 Da. LC-MS (ESI) RT= 2.11 min *m/z* found: 1081.75 $[M+3H]^{3+}$, 811.58 $[M+4H]^{4+}$, MALDI-TOF *m/z* found: 3244.270 $[M+H]^{+}$





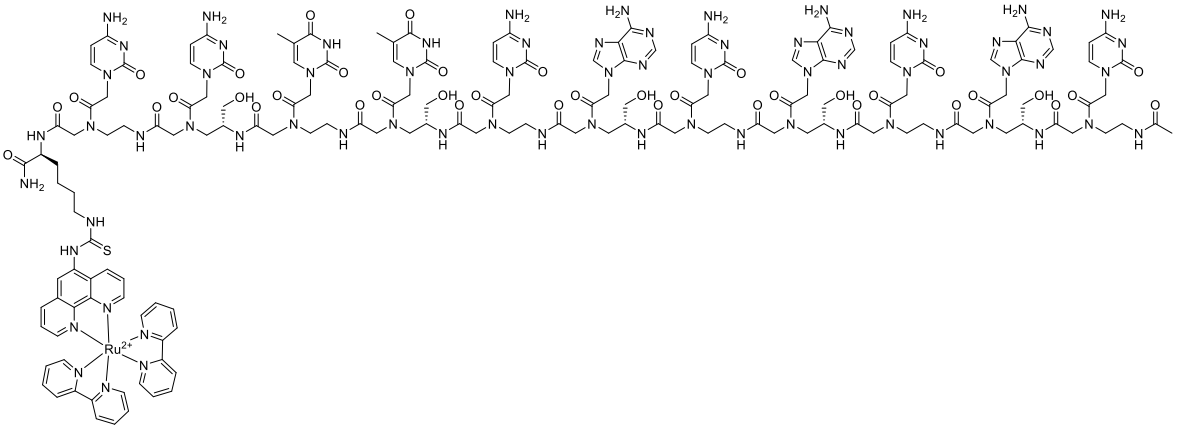
LC/MS analysis of miR-196 MM Rho-PNA.

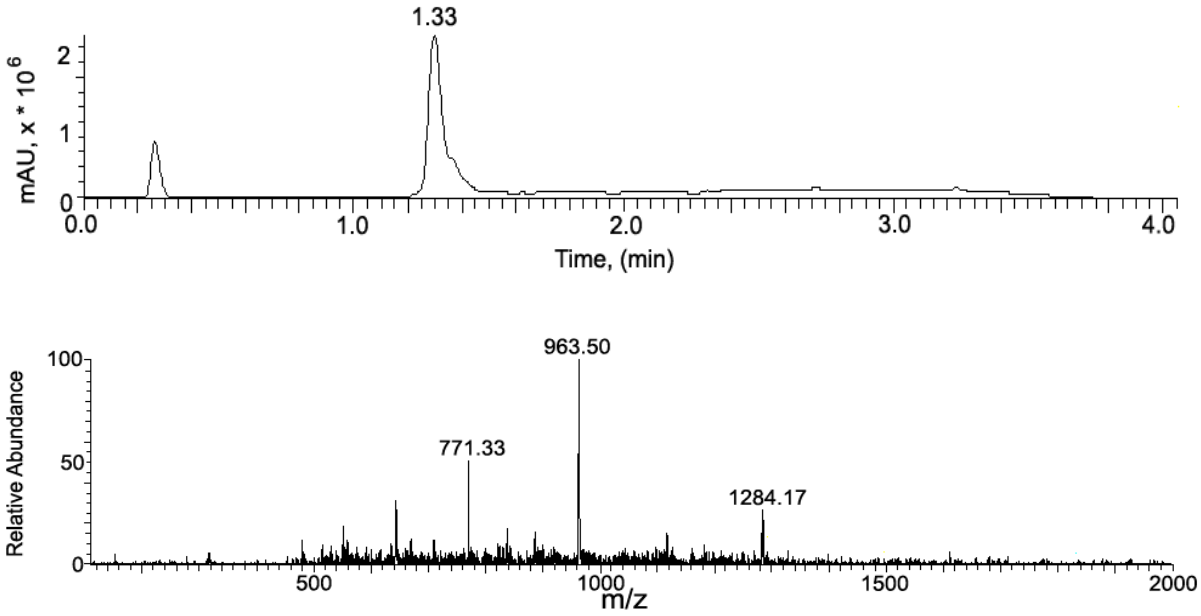


MALDI-TOF MS of miR-196 MM Rho-PNA.

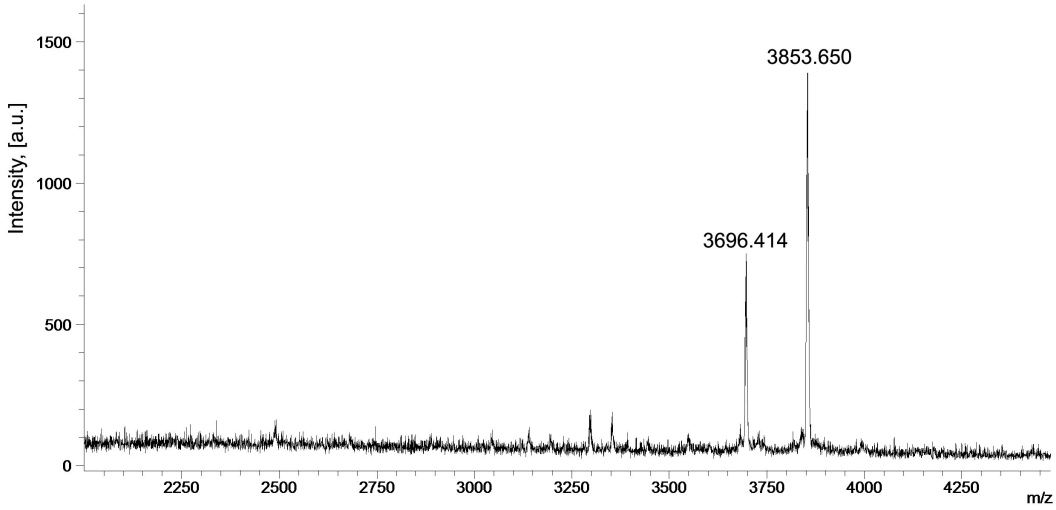
miR-206:

Ru-PNA conjugate: Calculated MW ($C_{162}H_{197}N_{69}O_{40}RuS$): 3852.30 Da. LC-MS (ESI) RT= 1.33 min *m/z* found: 1284.17 $[M+3H]^{3+}$, 963.50 $[M+4H]^{4+}$, 771.33 $[M+5H]^{5+}$, MALDI-TOF *m/z* found: 3853.650 $[M+H]^{+}$, 3696.414 $[M+H\text{-bpy}]^{+}$,



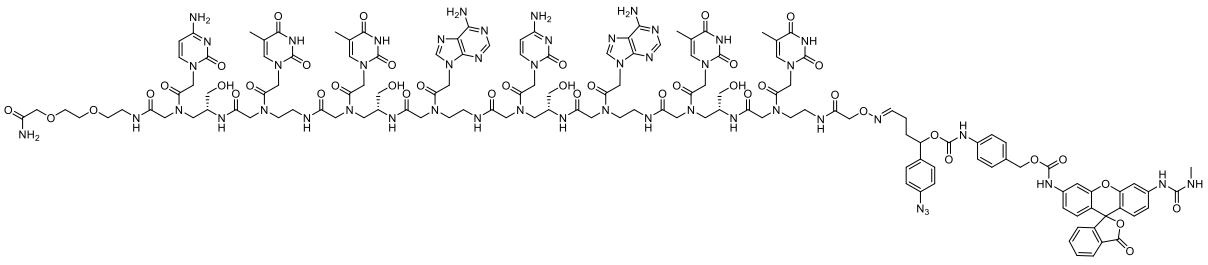


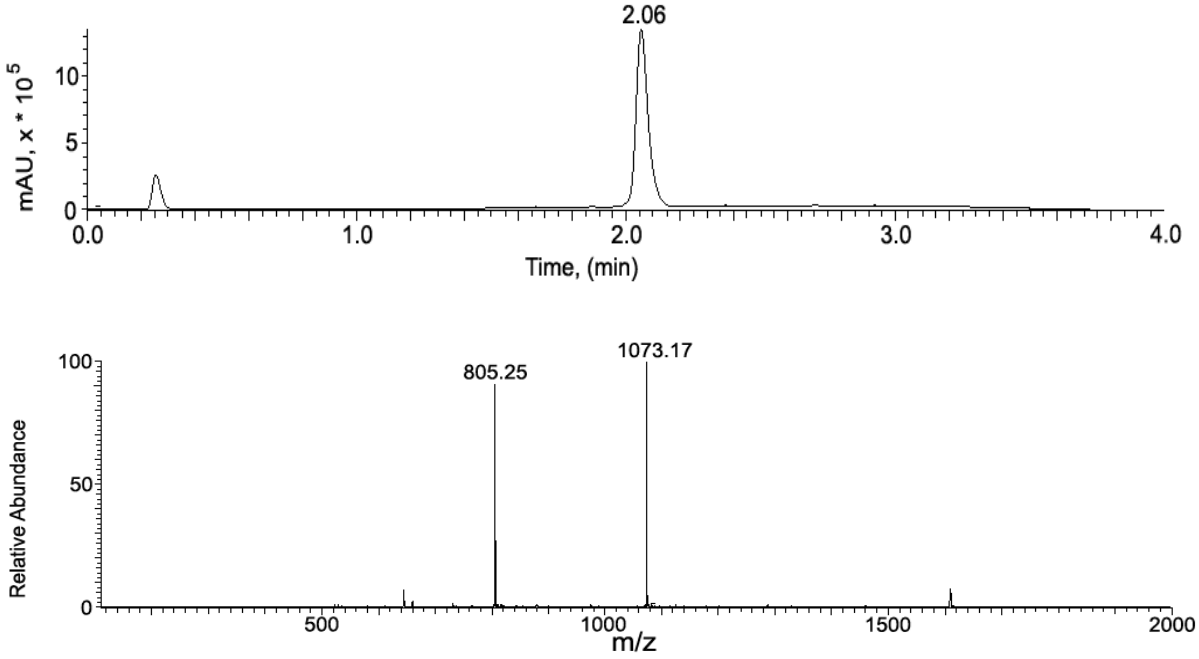
LC/MS analysis of miR-206 Ru-PNA.



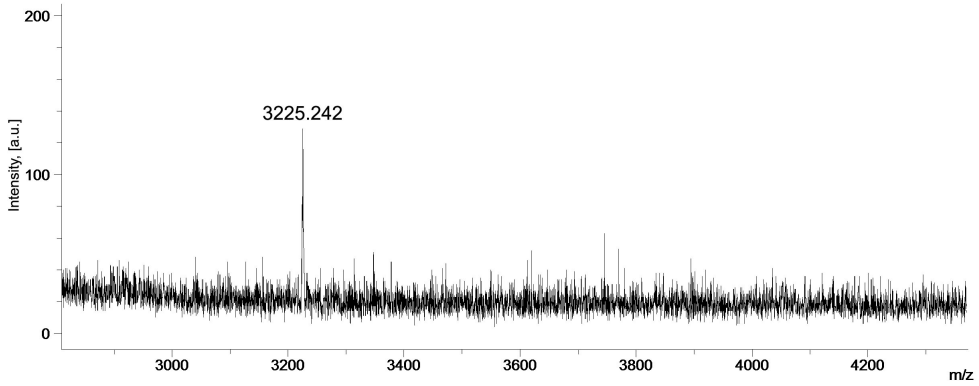
MALDI-TOF MS of miR-206 Ru-PNA.

PM Rhodamine conjugate: Calculated MW ($C_{139}H_{164}N_{50}O_{43}$): 3223.15 Da. LC-MS (ESI) RT= 2.06 min *m/z* found: 1073.17 $[M+3H]^{3+}$, 805.25 $[M+4H]^{4+}$, MALDI-TOF *m/z* found: 3225.242 $[M+H]^{+}$



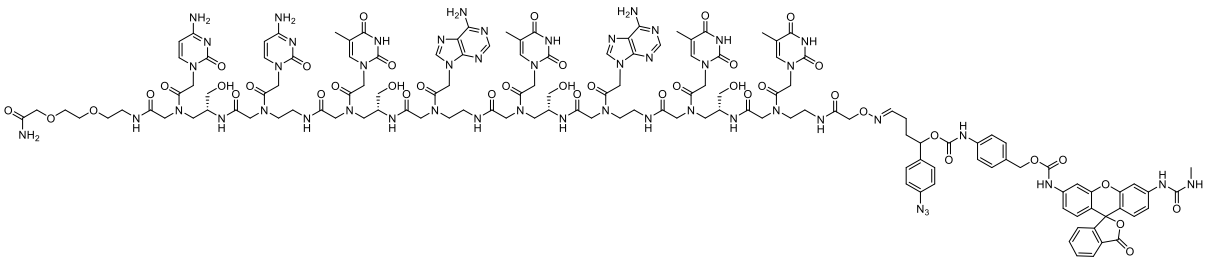


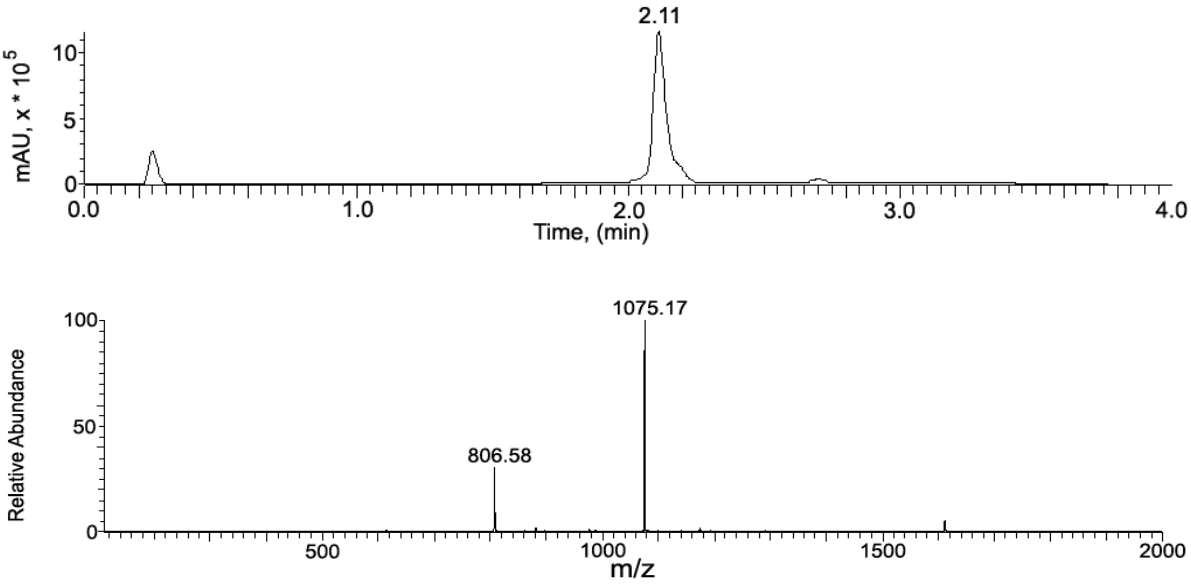
LC/MS analysis of miR-206 PM Rho-PNA.



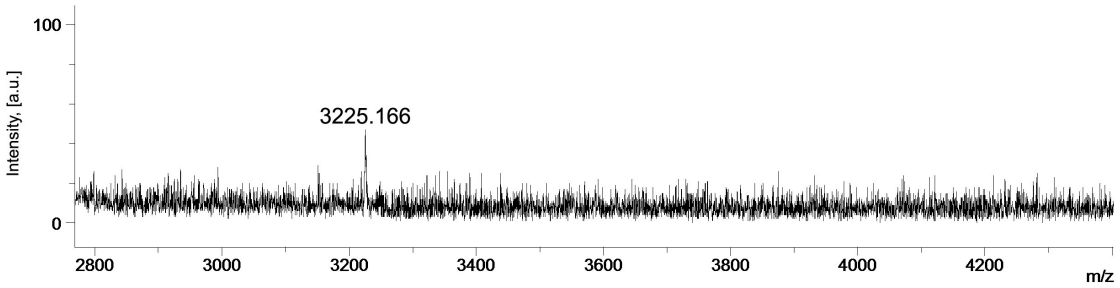
MALDI-TOF MS of miR-206 PM Rho-PNA.

MM Rhodamine conjugate: Calculated MW ($C_{139}H_{164}N_{50}O_{43}$): 3223.15 Da. LC-MS (ESI) RT= 2.11 min *m/z* found: 1075.17 $[M+3H]^{3+}$, 806.58 $[M+4H]^{4+}$, MALDI-TOF *m/z* found: 3225.166 $[M+H]^{+}$





LC/MS analysis of miR-206 MM Rho-PNA.



MALDI-TOF MS of miR-206 MM Rho-PNA.

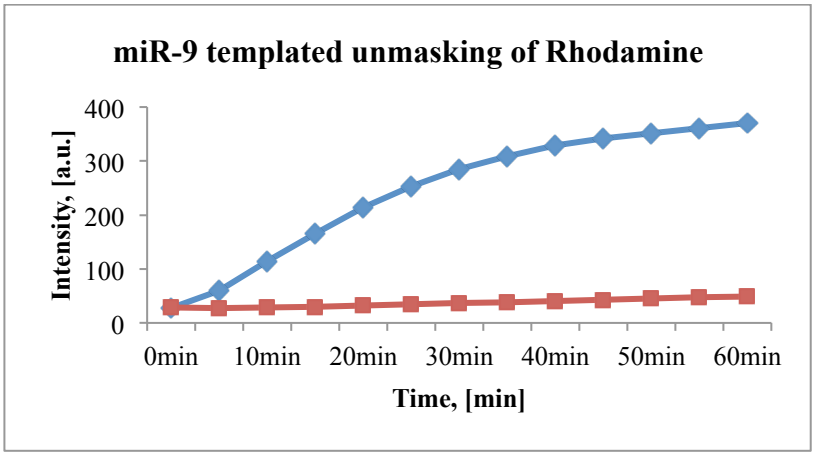
Templated reactions with synthetic template

The photoreactions were carried out in a 96 well plate (Nunc) in 0.5 M Tris buffer, 10 mM sodium ascorbate pH 7.4 at room temperature. Stock solutions of final derivatives (1 mM in dimethylformamide for rhodamine derivatives and 1 mM in deionized water for ruthenium derivatives) were diluted separately within reaction buffer and then added to wells (final reaction volume 100 μL). Signals were measured at 490/530nm excitation/emission wavelength in a 96-well plate using SpectraMax M5 from Molecular Devices. Reader control and data reduction Software was SoftMax Pro 6.3. Each experiment was performed in duplicates or triplicates. After measurement of the starting point fluorescence (ex: 490 nm, em: 530 nm) the plate was irradiated with a 455 nm LED (1 W, 30 cm distance, Thorlabs Inc). After different time intervals, fluorescence readouts were performed as before. The experiments were protected from external light.

i) Templated unmasking of Rhodamine with miR-9, miR-196a, and miR-206

MM/PM-Rho-PNA (nM)	Ru-PNA (nM)	miR9,196a, 206 (nM)
250	50	50

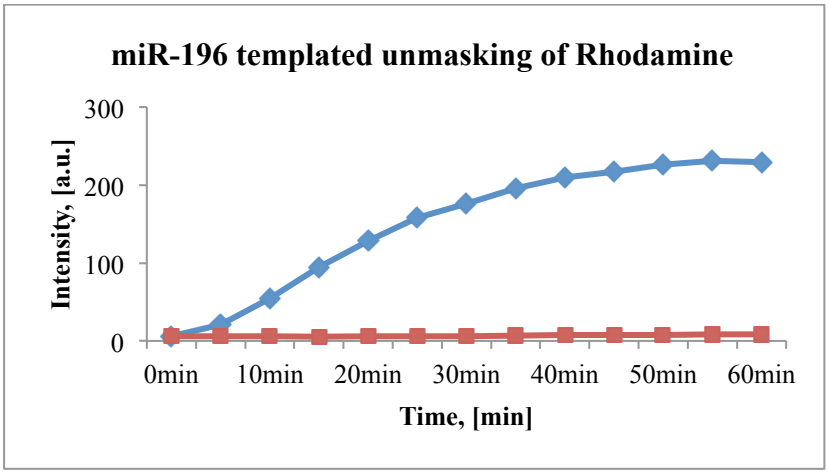
Target model DNA template: 5'-TCT TTG GTT ATC TAG CTG TAT G– 3'
Perfectly matched Rh-PNA: PEG-aGa AaC cA-Linker(N3)-Rh
Mismatched Rh-PNA: PEG-aAa AgC cA-Linker(N3)-Rh
Ru-PNA: Ru-Lys-aTc GaC aTa C-NHAc



Blue: perfectly matched probe reaction
Red: mismatched probe reaction

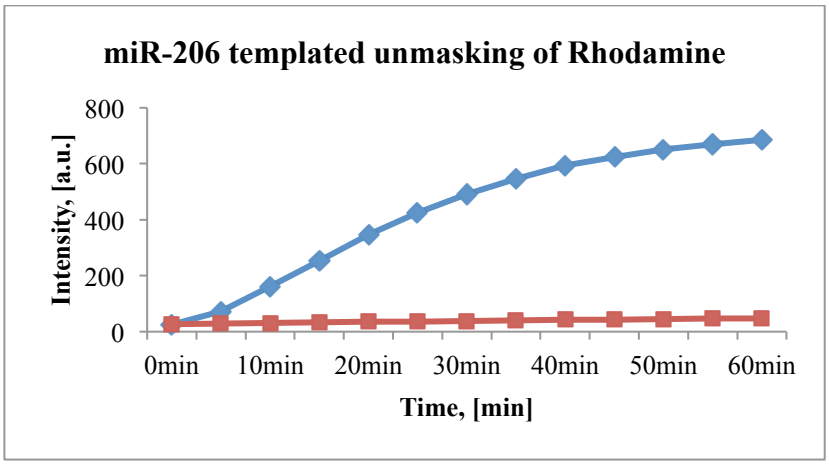
miRNA196:

Target model DNA template: 5'-TAG GTA GTT TCA TGT TGT TGG G – 3'
Perfectly matched Rh-PNA: PEG-aTc CaT cA-Linker(N3)-Rh
Mismatched Rh-PNA: PEG-aAc CgT cA-Linker(N3)-Rh
Ru-PNA: Ru-Lys-TaC aAc AaC cC-NHAc



Blue: perfectly matched probe reaction
Red: mismatched probe reaction

miRNA206:
Target model DNA template: 5'-TGG AAT GTA AGG AAG TGT GTG G– 3'
Perfectly **matched Rh-PNA**: PEG-cTt AcA tT-Linker(N3)-Rh
Mismatched Rh-PNA: PEG-cCt AtA tT-Linker(N3)-Rh
Ru-PNA: Ru-Lys-CcT tCa CaC aC-NHAc



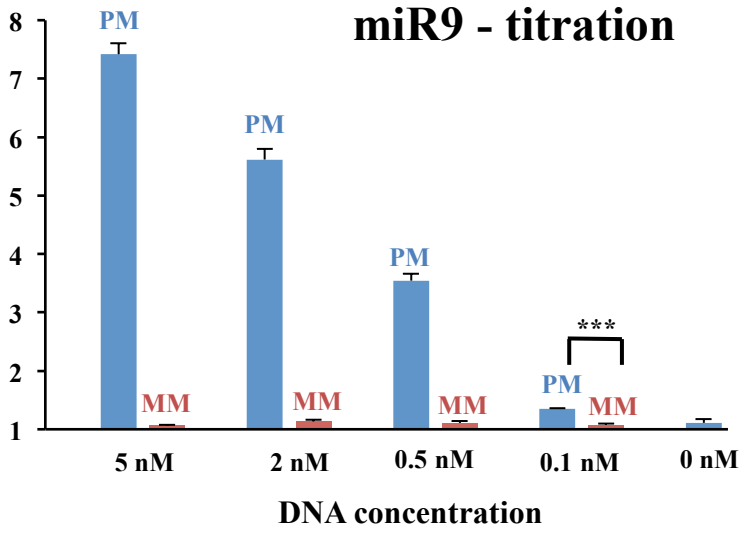
Blue: perfectly matched probe reaction
Red: mismatched probe reaction

ii) Templated unmasking of Rhodamine with miR-9 at constant ratios, different overall concentrations

	PM-Rho-PNA (nM)	Ru-PNA (nM)	miR9 or miR206 (nM)
250 nM	250	50	50
100 nM	100	20	20
25 nM	25	10	10
10 nM	10	2	2

Samples were irradiated for 30 min at room temperature and fluorescence was measured as described above (n = 3).

iii) Templated unmasking of Rhodamine with miR-9 at different DNA concentrations.



Blue: perfectly matched probe reaction (miR9)
Red: mismatched probe reaction (miR206)

Target model DNA template: 5'-TCT TTG GTT ATC TAG CTG TAT G– 3': 0-5 nM
Perfectly matched Rh-PNA: PEG-aGa AaC cA-Linker(N3)-Rh: 25 nM
Ru-PNA: Ru-Lys-aTc GaC aTa C-NHAc; 5 nM.
Mismatched Rh-PNA: PEG-aAa AgC cA-Linker(N3)-Rh
Samples were irradiated for 30 min at room temperature, and further incubated for 30 min and fluorescence was measured as described above (n = 4).

(1) Sadhu, K. K.; Winssinger, N. Detection of miRNA in Live Cells by Using Templated RuII-Catalyzed Unmasking of a Fluorophore, *Chem. Eur. J.* **2013**, *19*, 8182-8189.

(2) Westerfield, M. In *The zebrafish book : a guide for the laboratory use of zebrafish (Brachydanio rerio)*; Westerfield, M. Ed.; University of Oregon Press: Eugene, 1993.

(3) Kimmel, C. B.; Ballard, W. W.; Kimmel, S. R.; Ullmann, B.; Schilling, T. F. Stages of Embryonic-Development of the Zebrafish, *Dev. Dynam.* **1995**, *203*, 253-310.

(4) Wienholds, E.; Kloosterman, W. P.; Miska, E.; Alvarez-Saavedra, E.; Berezikov, E.; de Bruijn, E.; Horvitz, H. R.; Kauppinen, S.; Plasterk, R. H. A. MicroRNA expression in zebrafish embryonic development, *Science* **2005**, *309*, 310-311.

(5) Zhong, W.; Urayama, P.; Mycek, M. A. Imaging fluorescence lifetime modulation of a ruthenium-based dye in living cells: the potential for oxygen sensing, *J. Phys. D Appl. Phys.* **2003**, *36*, 1689-1695.

(6) Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; Tinevez, J. Y.; White, D. J.; Hartenstein, V.; Eliceiri, K.; Tomancak, P.; Cardona, A. Fiji: an open-source platform for biological-image analysis, *Nat. Methods* **2012**, *9*, 676-682.

(7) Linkert, M.; Rueden, C. T.; Allan, C.; Burel, J. M.; Moore, W.; Patterson, A.; Loranger, B.; Moore, J.; Neves, C.; MacDonald, D.; Tarkowska, A.; Sticco, C.; Hill, E.; Rossner, M.; Eliceiri, K. W.; Swedlow, J. R. Metadata matters: access to image data in the real world, *J. Cell Biol.* **2010**, *189*, 777-782.

(8) Camacho, C.; Coulouris, G.; Avagyan, V.; Ma, N.; Papadopoulos, J.; Bealer, K.; Madden, T. L. BLAST plus : architecture and applications, *Bmc Bioinformatics* **2009**, *10*, 421.

(9) Chouikhi, D.; Ciobanu, M.; Zambaldo, C.; Duplan, V.; Barluenga, S.; Winssinger, N. Expanding the Scope of PNA-Encoded Synthesis (PES): Mtt-Protected PNA Fully Orthogonal to Fmoc Chemistry and a Broad Array of Robust Diversity-Generating Reactions, *Chem. Eur. J.* **2012**, *18*, 12698-12704.

(10) Gorska, K.; Manicardi, A.; Barluenga, S.; Winssinger, N. DNA-templated release of functional molecules with an azide-reduction-triggered immolative linker, *Chem. Commun.* **2011**, *47*, 4364-4366.