Rapid Fluorescence Imaging of miR-21 in Cells Using Templated Staudinger Reaction

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Supplemental Information

Figure S1. MiR-21 imaging in BT474 cells (related to figure 5).

Figure S2. MiR-21 imaging in HeLa cells (related to figure 5).

Figure S3. MiR-21 imaging in JIMT1 cells (related to figure 5).

Figure S4. MiR-21 imaging in 293T cells (related to figure 5).

Figure S5. Quantification of fluorescence detection of miR-21 in different cell lines (related to figure 5).

Figure S6. Imaging of miR-21 level changes in HeLa cells upon transfection (top – cells transfected with control plasmid, bottom – cells transfected with miR21 plasmid) (related to figure 6).

Figure S7. Quantification of fluorescent miR-21 imaging in transfected HeLa cells (related to figure 6).

Figure S8. MiR-31 imaging in HeLa cells (related to figure 7).

Figure S9. MiR-31 imaging in MCF7 cells (related to figure 7).

Figure S5. Quantification of fluorescence detection of miR-31 in different cell lines (related to figure 7).

Supplemental Experimental Procedures

Characterization of used PNA probes
Figure S1. MiR-21 imaging in BT474 cells (related to figure 5). From left to right: merged image, fluorescence – Rho110 channel, DIC of cells treated with 1) first 100nM Lys(N3RhN3)_3 C*TG*AC*TA*C Arg then PBS alone (which were used as a negative control); 2) first Lys(N3RhN3)_3 C*TG*AC*TA*C Arg then 1mM tmTCEP (A cell permeable phosphine - these conditions were used as a positive control to define the maximum fluorescence); 3) first 100nM Lys(N3RhN3)_3 C*TG*AC*TA*C Arg then 200nM Arg AT*CG*AA*T dmTCEP (the perfect match probe - PM); 4) first 100nM Lys(N3RhN3)_3 C*TG*AC*TA*C Arg then 200nM Arg AT*GA*AA*T dmTCEP (the mismatched probe - MM).
Figure S2. MiR-21 imaging in HeLa cells (related to figure 5). From left to right: merged image, fluorescence – Rho110 channel, DIC of cells treated with 1) first 100nM Lys(N3RhN3) C*TG*AC*TA*C Arg then PBS alone (which were used as a negative control); 2) first Lys(N3RhN3) C*TG*AC*TA*C Arg then 1mM tmTCEP (A cell permeable phosphine - these conditions were used as a positive control to define the maximum fluorescence); 3) first 100nM Lys(N3RhN3) C*TG*AC*TA*C Arg then 200nM Arg AT*CG*AA*T dmTCEP (the perfect match probe - PM); 4) first 100nM Lys(N3RhN3) C*TG*AC*TA*C Arg then 200nM Arg AT*GA*AA*T dmTCEP (the mismatched probe - MM).
Figure S3. MiR-21 imaging in JIMT1 cells (related to figure 5). From left to right: merged image, fluorescence – Rhod110 channel, DIC of cells treated with 1) first 100nM Lys(N₃RhN₃) C*TG*AC*TA*C Arg then PBS alone (which were used as a negative control); 2) first Lys(N₃RhN₃) C*TG*AC*TA*C Arg then 1mM tmTCEP (A cell permeable phosphine - these conditions were used as a positive control to define the maximum fluorescence); 3) first 100nM Lys(N₃RhN₃) C*TG*AC*TA*C Arg then 200nM Arg AT*CG*AA*T dmTCEP (the perfect match probe – PM); 4) first 100nM Lys(N₃RhN₃) C*TG*AC*TA*C Arg then 200nM Arg AT*GA*AA*T dmTCEP (the mismatched probe - MM).
Figure S4. MiR-21 imaging in 293T cells (related to figure 5). From left to right: merged image, fluorescence – Rho110 channel, DIC of cells treated with 1) first 100nM Lys(N3RhN3) C*TG*AC*TA*C Arg then PBS alone (which were used as a negative control); 2) first Lys(N3RhN3) C*TG*AC*TA*C Arg then 1mM tmTCEP (A cell permeable phosphine - these conditions were used as a positive control to define the maximum fluorescence); 3) first 100nM Lys(N3RhN3) C*TG*AC*TA*C Arg then 200nM Arg AT*CG*AA*T dmTCEP (the perfect match probe - PM); 4) first 100nM Lys(N3RhN3) C*TG*AC*TA*C Arg then 200nM Arg AT*GA*AA*T dmTCEP (the mismatched probe - MM).
Figure S5. Quantification of fluorescence detection of miR-21 in different cell lines (related to figure 5). Quantification of fluorescence based on the average fluorescence at three distinct areas within the culture dish (error bars represent the standard deviations). The conversion with the perfect match probe (PM) and mismatch probe (MM) was calculated by taking the fluorescence intensity divided by the maximum intensity (maximum fluorescence - positive control - TCEP) corrected for the background fluorescence (no phosphine -negative control).
Figure S6. Imaging of miR-21 level changes in HeLa cells upon transfection (top – cells transfected with control plasmid, bottom – cells transfected with miR-21 plasmid) (related to figure 6). From left to right: merged image, fluorescence, DIC of cells treated with 1) first 100nM Lys(N$_3$RhN$_3$) C*TG*AC*TA*C Arg then PBS alone (which were used as a negative control); 2) first Lys(N$_3$RhN$_3$) C*TG*AC*TA*C Arg then 1mM tmTCEP (A cell permeable phosphine - these conditions were used as a positive control to define the maximum
fluorescence; 3) first 100nM Lys(N\textsubscript{3}RhN\textsubscript{3}) C\textsuperscript{T}G\textsuperscript{A}C\textsuperscript{T}A\textsuperscript{C} Arg then 200nM Arg AT\textsuperscript{C}G\textsuperscript{A}A\textsuperscript{T} dmTCEP (the perfect match probe - PM); 4) first 100nM Lys(N\textsubscript{3}RhN\textsubscript{3}) C\textsuperscript{T}G\textsuperscript{A}C\textsuperscript{T}A\textsuperscript{C} Arg then 200nM Arg AT\textsuperscript{G}A\textsuperscript{A}A\textsuperscript{T} dmTCEP (the mismatched probe - MM).

Figure S7. Quantification of fluorescent miR-21 imaging in transfected HeLa cells (related to figure 6). Quantification of fluorescence based on the average fluorescence at three distinct areas within the culture dish (error bars represent the standard deviations). The conversion with the perfect match probe (PM) and mismatch probe (MM) was calculated by taking the fluorescence intensity divided by the maximum intensity (maximum fluorescence - positive control - TCEP) corrected for the background fluorescence (no phosphine -negative control).
**Figure S8. MiR-31 imaging in HeLa cells (related to figure 7).** From left to right: merged image, Rho110 channel, DAPI channel, DIC of cells treated with 1) first 100nM Lys(N\textsubscript{3}RhN\textsubscript{3}) C*CG*TA*TC*G Arg then PBS alone (which were used as a negative control); 2) first Lys(N\textsubscript{3}RhN\textsubscript{3}) C*CG*TA*TC*G Arg then 1mM tmTCEP (A cell permeable phosphine - these conditions were used as a positive control to define the maximum fluorescence); 3) first 100nM Lys(N\textsubscript{3}RhN\textsubscript{3}) C*CG*TA*TC*G Arg then 200nM Arg CG*TT*CT*A dmTCEP (the perfect match probe - PM); 4) first 100nM Lys(N\textsubscript{3}RhN\textsubscript{3}) C*CG*TA*TC*G Arg then 200nM Arg CG*TT*AT*A dmTCEP (the mismatched probe - MM).

**Figure S9. MiR-31 imaging in MCF7 cells (related to figure 7).** From left to right: merged image, Rho110 channel, DAPI channel, DIC of cells treated with 1) first 100nM Lys(N\textsubscript{3}RhN\textsubscript{3}) C*CG*TA*TC*G Arg then PBS alone (which were used as a negative control); 2) first Lys(N\textsubscript{3}RhN\textsubscript{3}) C*CG*TA*TC*G Arg then 1mM tmTCEP (A cell permeable phosphine - these conditions were used as a positive control to define the maximum fluorescence); 3) first 100nM Lys(N\textsubscript{3}RhN\textsubscript{3}) C*CG*TA*TC*G Arg then 200nM Arg CG*TT*CT*A dmTCEP (the perfect match probe - PM); 4) first 100nM Lys(N\textsubscript{3}RhN\textsubscript{3}) C*CG*TA*TC*G Arg then 200nM Arg CG*TT*AT*A dmTCEP (the mismatched probe - MM).
Figure S10. Quantification of fluorescence detection of miR31 in different cell lines (related to figure 7). Quantification of fluorescence based on the average fluorescence at three distinct areas within the culture dish (error bars represent the standard deviations). The conversion with the perfect match probe (PM) and mismatch probe (MM) was calculated by taking the fluorescence intensity divided by the maximum intensity (maximum fluorescence - positive control - TCEP) corrected for the background fluorescence (no phosphine -negative control).

Supplemental Experimental Procedures

NMR spectra were recorded on Bruker Avance-400 instrument and calibrated by using residual undeuterated solvent as an internal reference. The following abbreviations were used to explain the multiplicities: s=singlet, d=doublet, m=multiplet. LC-MS were recorded by using an Agilent 1100 HPLC with a Surveyor MSQ spectrometer equipped with a Supelco C18 (5 cm x 2.1 mm, 5 μm particles) column (method A, linear elution gradient from 95% H2O 0.01% TFA to 100% MeCN 0.01% TFA in 8 minutes at a flow rate of 0.7 mLmin⁻¹) or by using a Thermo Scientific Accela HPLC with a Surveyor MSQ Plus spectrometer equipped with a Thermo C18 (5 cm x 2.1 mm, 1.9 μm particles) Hypersil gold column (method B, linear elution gradient for 95% H2O 0.01% TFA to 90% MeCN 0.01% TFA in 3.6 minutes at a flow rate of 1.0 mLmin⁻¹; method C, linear elution gradient for 95% H2O 0.01% TFA to 90% MeCN 0.01% TFA in 2.2 minutes at a flow rate of 1.0 mLmin⁻¹). The MALDI spectra were measured using Bruker Daltonics Autoflex TOF/TOF spectrometer reverse-phase chromatography using a Biotage Isolera ONE equipped with a Biotage SNAP Cartridge KP-C18-HS 12g (linear gradient from 100% H2O 0.01% TFA to 30% MeCN 0.01% TFA in 22.5 minutes with a flow rate of 10 mLmin⁻¹).
Azidorhodamine synthesis

![Azidorhodamine structure]

**Carboxyrhodamine 110 (mixture of isomers):** Can be obtained from commercial sources or prepared according to the following procedure: Trimellitic acid (5.00 g, 23.8 mmol) and 3-aminophenol (8.68g, 78.5 mmol) were combined in concentrated sulfuric acid (55mL) and stirred at 180°C for 4h. After cooling to ambient temperature reaction mixture was poured onto 60g of ice and then neutralized with solid Na₂CO₃ while stirred in ice bath. Methanol (260mL) was added to precipitate inorganic salts which were then filtered. Filtration cake was washed with additional 170mL of methanol and combined filtrates evaporated. The organic residues were purified by column chromatography (dry pack, 0-20% MeOH in acetone) to obtain 4.31g (48%) of 1:1 mixture of 5- and 6-carboxyrohodamines 110 as a red crystalline solid.

![Carboxyrhodamine structure]

**Diazidocarboxyrhodamine 110:** Mixture of isomers of 5/6-carboxyrhodamine 110 (1.90 g, 5.08 mmol) was dissolved in water (84 mL) and THF (34 mL) and resulting solution was cooled down to 0°C in ice bath. Then concentrated H₂SO₄ (5mL) was added dropwise followed by solution of NaNO₂ (0.86 g, 12.18 mmol, 2.4 equiv) in water (7 mL). After 1h of stirring at 0°C solution of NaN₃ (1.12g, 16.71 mmol, 3.3 equiv) in water (7 mL) was added dropwise. Cooling bath was then removed and reaction mixture was allowed to warm up to ambient temperature while stirred overnight. Brine (100 mL) was added to quench the reaction and product was recovered by extraction with CH₂Cl₂ (3 x 150mL). Combined organic layers were washed with water, dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residues were redissolved in CH₂Cl₂ and after drypacking purified by flash chromatography (5% MeOH in CH₂Cl₂) to obtain 140mg (0.33mmol, 6.5%) of diazidodervative. **¹H NMR (acetone) δ(ppm):** 8.56 (1H, s, 5-isomer), 8.41 (1H, d, J = 7.6 Hz, 5-isomer), 8.36 (1H, d, J = 7.6 Hz, 6-isomer), 8.15 (1H, d, J = 8.0 Hz, 6-isomer), 7.89 (1H, s, 5-isomer), 7.49 (1H, d, J = 8.0 Hz, 6-isomer), 7.02-6.98 (2H+2H, m, both isomers), 6.91-6.89 (2H+2H, m, both isomers); **¹³C NMR (CDCl₃) δ(ppm):** 167.4, 165.0, 156.2, 151.8, 143.1, 136.4, 133.1, 129.9, 126.9, 126.2, 124.5, 115.5, 115.3, 107.2, 94.2; **LCMS (ESI):** RT = 1.96 min, 2.03 min (2 isomers) m/z calcld for C₂₁H₁₁N₆O₅ [M+H]⁺: 427.08, found: 427.07 [M+H]⁺, 399.07 [M+H-N₂]⁺, 371.06 [M+H-2N₂]⁺; **HRMS (MALDI TOF) m/z** calcld for C₂₁H₁₁N₂O₅ [M+H-2N₂]⁺: 371.0668, found: 371.0678[M+H-2N₂]⁺.
**dmTCEP**

Tricarboxyethylphosphine hydrochloride (TCEP-HCl, 200 mg, 0.208 mmol.) was stirred with 200 mg of sulfonic acid resin (Amberlyst) in 3 mL of methanol at room temperature for 40 minutes. The resin was then removed by filtration and the filtrates, containing a mixture of mono-, di- and tri-methylester, were fractionated by flash chromatography (dry packing, 0, 5, 10, 15% of MeOH in DCM) to give dmTCEP as colorless oil (67mg, 34% yield).

**LCMS (ESI):** RT = 0.71 min m/z calcd for C_{11}H_{19}O_{6}P [M+H]^+: 279.10, found: 279.16 [M+H]^+

**PNA probes synthesis and labeling**

**General procedure for leading of the first residue with reduction of resin loading to 0.2 μmol/g.**

NovaPEG Rink amide resin (0.44 mmol/g, NovaBiochem) was swollen in CH₂Cl₂ for 20 min. The Fmoc protected amino acid (0.45 equiv, reduction of the resin loading to 0.2 mmol/g) was dissolved in anhydrous NMP and HOBT (2.5 equiv) followed by diisopropylcarbodiimide (DIC, 7.5 equiv) were added. The mixture was stirred for 15 min prior the addition to the resin and then shaken for 3 hours with the resin. The unreacted amino groups were capped (20 min) with a solution of acetic anhydride (5.3 equiv) and 2,6-lutidine (6.4 equiv) in DMF. Then, the resin was washed extensively with DMF and CH₂Cl₂, and dried.

**Diazo[carboxyrhodamine 110 coupling to lysine on resin.**

The resin loaded with Lys(Mtt)NHFmoc (200 mg, 40 μmol, 1.0 equiv), was swollen in CH₂Cl₂ for 20 min and washed with a solution 1:1 of hexafluoroisopropanol (HFIP) in dichloroethane (2 mL) 15 cycles of 30 sec washes followed by a last incubation of 5 min. The resin was then washed with DMF and CH₂Cl₂. Diazo[carboxyrhodamine 110 (36.5 mg, 85.5 mmol, 2.13 equiv), HATU (1.5 equiv), DIPEA (2.13 equiv) and 2,6-lutidine (3.2 equiv) were combined in NMP (2 mL) and after 5 minutes of incubation added to the resin. Resin was shaken for 2h at ambient temperature, washed with DMF and treated with Ac₂O (5.3 equiv), 2,6-lutidine (6.4 equiv) in NMP (2 mL) for capping. At the end, the resin was washed extensively with DMF and CH₂Cl₂, and dried.

**General procedure for PNA synthesis using Boc-protected nucleobases and Fmoc protected monomers.**

The PNA monomers were prepared as previously described (Pianowski, Z., K. Gorska, et al. (2009). "Imaging of mRNA in live cells using nucleic acid-templated reduction of azidorhodamine probes." J. Am. Chem. Soc. 131(18): 6492-6497). The resin (10 mg, 2 μmol, 1.0 equiv) was swollen in CH₂Cl₂ (300 μL) for 20 min and deprotected with 20% piperidine in DMF (2 and 4 min). The resin was then washed with DMF and CH₂Cl₂ and treated with a preactivated (10 min) solution of the corresponding PNA monomer or aminoacid (5.0 equiv), HATU (4.4 equiv), DIPEA (5.0 equiv) and 2,6-lutidine (7.5 equiv) during 20 min in NMP. This process was repeated once (double couplings). Each coupling was followed by a capping step with Ac₂O (5.3 equiv), 2,6-lutidine (6.4 equiv) in NMP (100 μL).

**General procedure for dmTCEP coupling.**

The resin (10 mg, 2 μmol, 1.0 equiv) was swollen in CH₂Cl₂ for 20 min and deprotected with 20% piperidine in DMF (200 μL) for 10 min; then it was washed with DMF and CH₂Cl₂ and treated with dmTCEP (6.0 equiv) in
DMF (100 μL) previously activated for one minute with DIC (24 equiv) and HOBt (12 equiv) for 30 min. The resin was then washed first with 5% AcOH in CH₂Cl₂ then with CH₂Cl₂ and DMF.

**General procedure for cleavage from resin and purification.**

The resin was treated with TFA (95% in H₂O, 500 μL for 2 μmol of resin) for 4 hours. The TFA solution was precipitated in Et₂O (10 times TFA volume) and centrifuged to recover the product as a pellet. The precipitate was redisssolved in H₂O (500 μL for crude cleaved from 10 mg of resin) and purified by reverse-phase chromatography using a Biotage Isolera ONE equipped with a Biotage SNAP Cartridge KP-C18-HS 12g (linear gradient from 100% H₂O 0.01% TFA to 30% MeCN 0.01% TFA in 22.5 minutes with a flow rate of 10 mLmin⁻¹).

**Characterisation of used PNA probes** (*denotes positions where GPNA monomer was incorporated)

**Probes targeting miR21:**

Azidorhodamine probe 14mer:
Lys(N₃RhN₃) GT*CTG*ACT*ACA*ACT* Arg MW:4956.41 MALDI-TOF m/z found: 4990.24 [M+H-2N₂⁺]

Azidorhodamine probe 8mer:
Lys(N₃RhN₃) C*TG*AC*TA*C Arg MW: 3233.43 MALDI-TOF m/z found: 3178.44[M+H-2N₂⁺]

dmTCEP probe PM
Arg AT*CG*AA*T dmTCEP MW: 2630.33 MALDI-TOF m/z found: 2632.03 [M+H⁺]

dmTCEP probe MM
Arg AT*GA*AA*T dmTCEP MW: 2654.34 MALDI-TOF m/z found: 2655.49 [M+H⁺]

**Probes targeting miR31:**

Azidorhodamine probe 8mer:
(N₃RhN₃) C*CG*TA*TC*G Arg MW:3249.43 MALDI-TOF m/z found: 3194.98[M+H-2N₂⁺]

dmTCEP probe PM
Arg CG*TT*CT*A dmTCEP MW: 2597.30 MALDI-TOF m/z found: 2598.58 [M+H⁺]

dmTCEP probe MM
Arg CG*TT*AT*A dmTCEP MW: 2622.32 MALDI-TOF m/z found: 2623.15 [M+H⁺]
DNA sequences used as templates:

PM miR21- template: TAGCTTATCAGACTGATGTTGA

MM N3 miR21- template: TAGCTTATCAGACGTATGTTGA

PM miR31- template: AGGCAAGATGCTGGCATAGCT

MM Ph miR31- template: AGGCAATATGCTGGCATAGCT

RANDOM - template: CTGTGCATGACAGCAGGCTGA