Supporting Information

Oligonucleotide Probe for Transcriptome *in Vivo* Analysis (TIVA) of Single Neurons with Minimal Background

Sean B. Yeldell,[†] Linlin Yang,[†] Jaehee Lee,[‡] James H. Eberwine,[‡] and Ivan J. Dmochowski*[,†]

[†]Department of Chemistry, University of Pennsylvania, 231 South 34th Street, Philadelphia, Pennsylvania 19104-6323, USA

[‡]Department of Pharmacology, University of Pennsylvania, 38 John Morgan Building, 3620 Hamilton Walk, Philadelphia, Pennsylvania 19104-6084, USA

To whom correspondence should be addressed: ivandmo@sas.upenn.edu

Contents

Figure S1. HPLC traces for TIVA oligo synthesis. (A) RP-HPLC purification of crude 22/12/8 $(GC)_2$ TIVA probe after solid-phase synthesis. (B) AX-HPLC purification of $(D\text{-}Arg)_9$-conjugated 22/12/8 $(GC)_2$ probe.

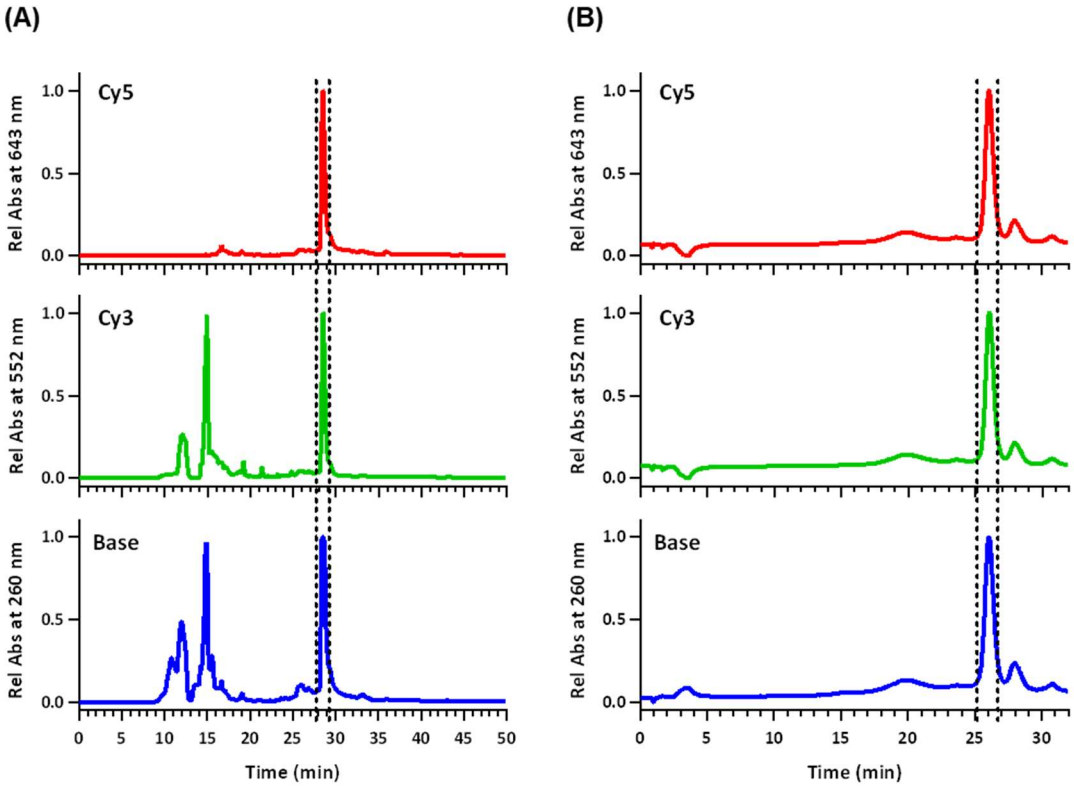


Figure S2. General characterization of 22/12/8 $(GC)_2$ +$(D\text{-}Arg)_9$ (A) ESI-MS (B) Melting temperature.

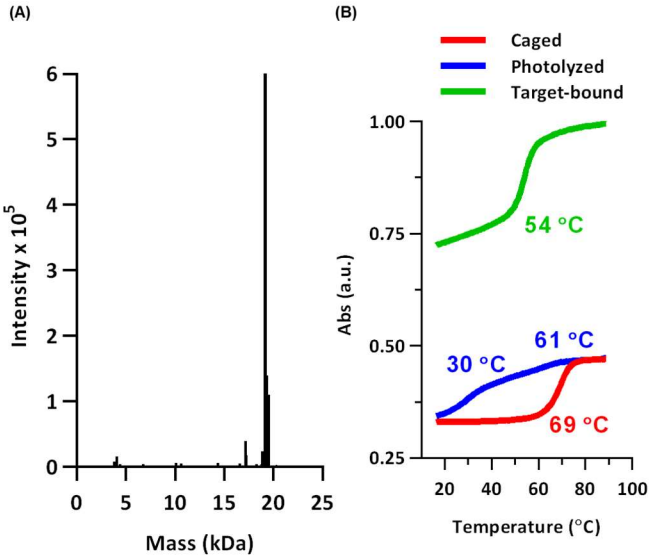


Figure S3. Synthesis and characterization of TIVA-NT and TIVA-CPP. (A) AX-HPLC purification and (B) ESI-MS of TIVA-CPP probe after $(D\text{-}Arg)_9$ conjugation; (C) RP-HPLC purification after DMT removal and (D) ESI-MS of TIVA-NT probe.

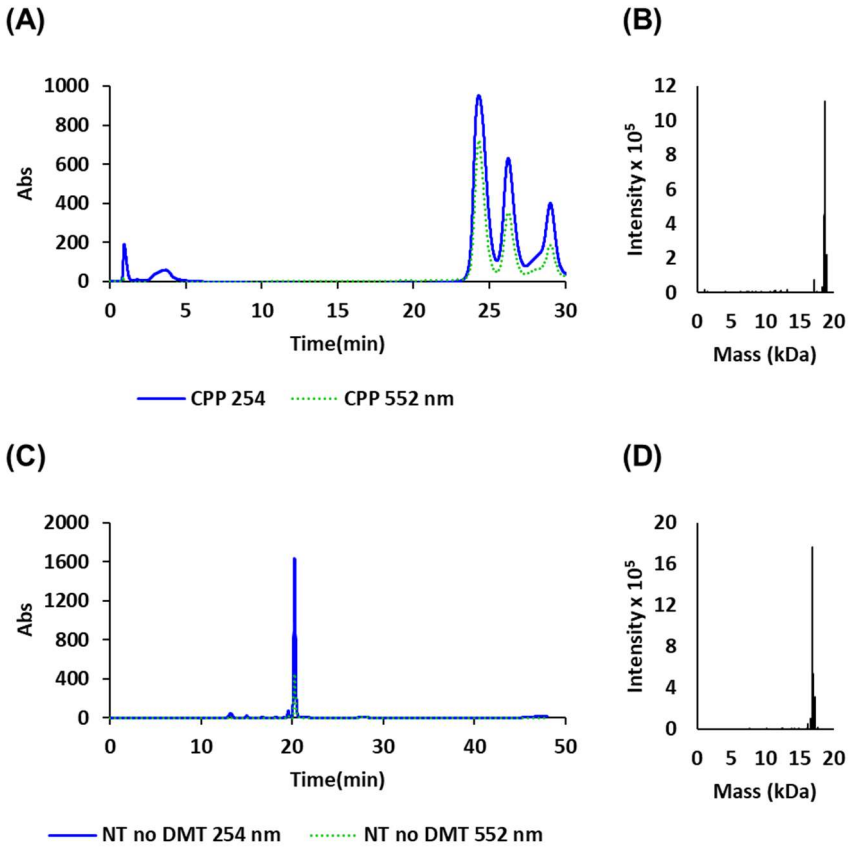


Figure S4. (A) DLS characterization and (B) Zeta potential of TIVA-CPP and TIVA-NT at 10 μM in PBS buffer (pH = 7).

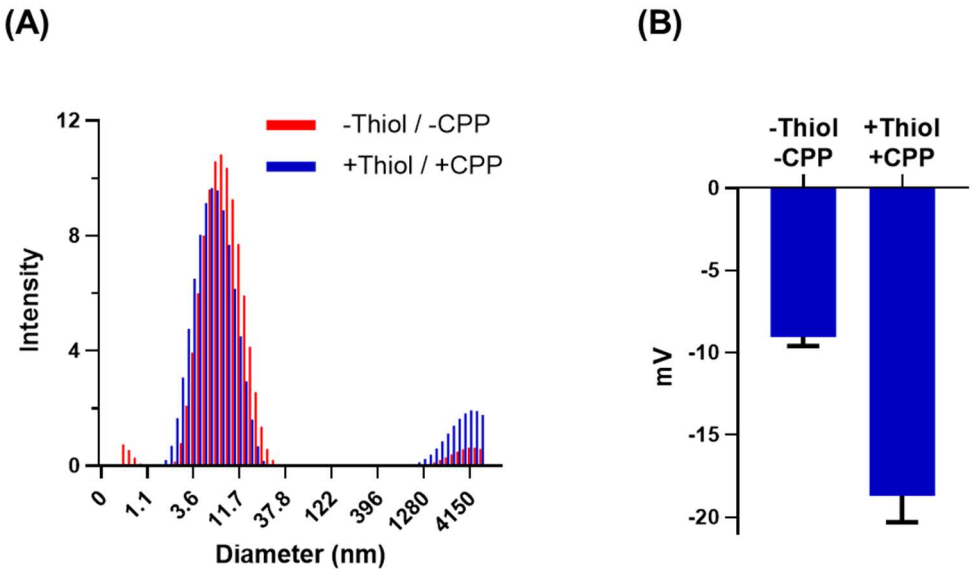


Figure S5. Uptake of TIVA-NT and TIVA-CPP monitored by Cy3 fluorescence measured by confocal laser scanning microscopy. (A) Time course fluorescence intensity (arbitrary units) for both TIVA-NT and TIVA-CPP. (B) Uptake of TIVA-CPP with treatment of amiloride.

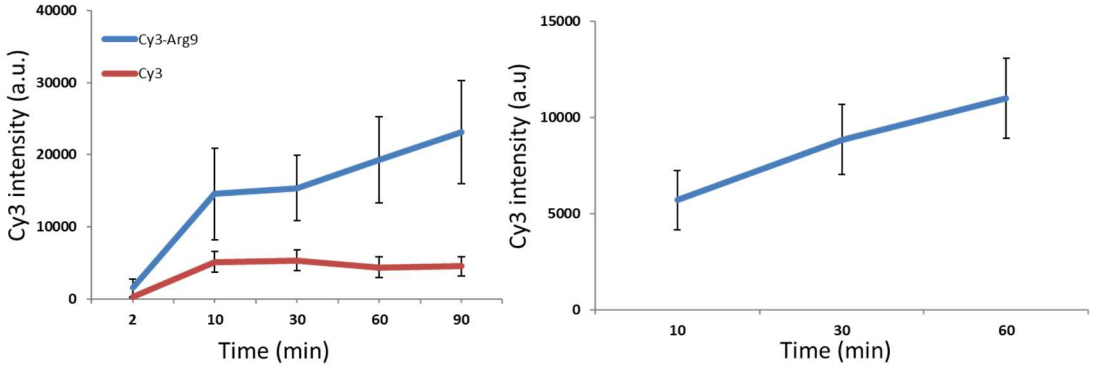
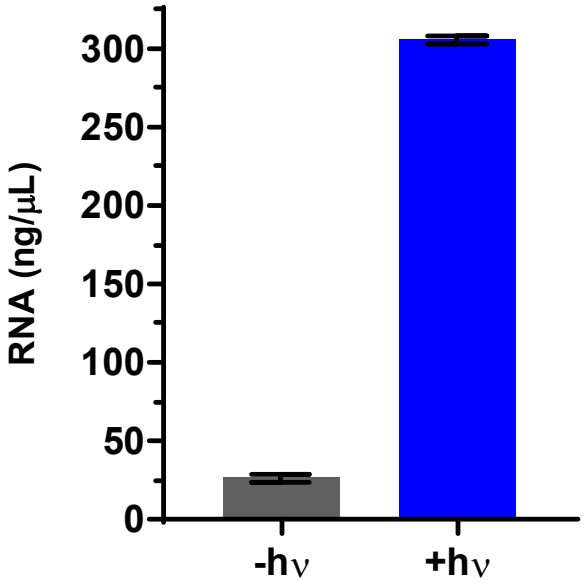


Figure S6. Concentration of mRNA from human fibroblast lysate after pull-down with unactivated ("-hv") or photoactivated 22/12/8 $(GC)_2$ +$(D\text{-}Arg)_9$ TIVA probe ("+hv"). Intensity measured on 3+ pull-down replicates using a Qubit 2.0 fluorometer.



METHODS

TIVA Probe Synthesis

Synthesis of 22/12/8 $(GC)_2$ +$(D\text{-}Arg)_9$ TIVA probe proceeded similarly to the published method for 22/9/9 (GC) TIVA with a few modifications. All phosphoramidites, including the TEG spacer (10-1909-XX) introduced in the 22/12/8 $(GC)_2$ TIVA probe, were supplied by Glen Research (Sterling, VA). In brief, the oligo was synthesized by solid-phase phosphoramidite chemistry on an ABI 394 synthesizer utilizing a BiotinTEG CPG (20-2955-XX) and a 5' terminal DMT-capped thiol-modifier. The DMT-cap was left on during NH_4OH cleavage, the NH_4OH was removed by vacufuge, and the crude mixture was purified by reverse-phase (RP) HPLC (Agilent 1100S) on a C18 column at 1.0 mL/min, 40 °C. A gradient of increasing acetonitrile (B) in 0.1 M TEAA (A) on a C18 column was employed for purification (B starting at 30%, reaching 40% at 30 min, 90% at 35 min, 90% at 38 min, 30% at 46 min). The product eluted at roughly 28 min, based upon monitoring absorbance of base (254 nm), Cy3 (552 nm), and Cy5 (643 nm) (Figure S1). After RP-HPLC purification the product was concentrated, the 5' disulfide was reduced, and the released DMT-alkyl-thiol moiety was separated in a NAP-10 Sephadex column. The probe's liberated 5' thiol was then reacted with 1 μM Cys(Npys)-$(D\text{-}Arg)_9$ peptide in formamide buffer as previously described. Anion exchange (AX) HPLC (Agilent 1100S, 1.0 mL/min, RT) with a Resource Q column (GE Healthcare) was utilized to purify $(D\text{-}Arg)_9$-conjugated probe. The gradient used 100% buffer A (20 mM Tris-HCl, pH 6.8, 50% formamide) and 0% buffer B (20 mM Tris-HCl, pH 6.8, 50% formamide, 400 mM $NaClO_4$) transitioning to 0% buffer A and 100% buffer B over 30 min. 22/12/8 $(GC)_2$ TIVA probe eluted at 26 min. The recovered probe was buffer-exchanged to 1x STE buffer pH 8.0 (Fisher Scientific) with 4+ washes in a 10k MWCO spin column, aliquoted into 3.0 nmol portions in amber microtubes, wrapped in foil, and stored at -20 °C until use. The final yield for the CPP-conjugated product was 20 - 60 nmol, for a total yield ranging from 2 to 6%, based on A_{260} [ε_{260} = 463,000 L/(mole·cm estimated via idtdna.com/calc/analyzer)] and starting scale of 1.0 μmol.

TIVA-CPP was synthesized by the same method except Cy5 was not included in the sequence. Synthesis of TIVA-NT skipped the step of $(D\text{-}Arg)_9$ peptide conjugation. Terminal DMT group was removed by 10% acetic acid for 2 h at rt. Acetic acid was removed by vacufuge after the reaction and the final product was desalted with NAP-5 column.

Procedures for synthesis and characterization of 22/9/9 (GC) and 18/7/7 TIVA probes were previously reported.(*1, 2*)

Mass Determination

Oligonucleotide masses were determined by Novatia, LLC using a LCMS system with electrospray injection (Oligo HTCS). Analysis of 22/12/8 $(GC)_2$ +$(D\text{-}Arg)_9$ verified an expected principal mass of 19,168.8 Da with an observed mass of 19,168.2 Da (Figure S2). Observed masses for TIVA-NT and TIVA-CPP were 16,914.5 Da and 18,633.8 Da (Figure S3), which matched expected masses of 16,914.1 Da and 18,635.2 Da.

T_m and FRET Sample Preparation

Four 60 μL (FRET) or 250 μL (T_m) TIVA probe samples were prepared at 1.0 μM in 1x STE buffer: two samples mixed with one equivalent of 30mer poly-A 2'-OMe RNA as a model poly-A tail ("+polyA") and two samples without poly-A RNA ("-polyA"). One -polyA and one +polyA sample were then photoactivated ("+hv"), while the remaining two samples were left unactivated ("-hv"). Photoactivation was performed using a TL-355R Ultraviolet Transilluminator (Spectroline, Westbury, NY), irradiating the samples at 365 nm, 9

mW/cm^2 for 8 min, followed by brief mixing, and then irradiating for an additional 8 min. Finally, the samples were incubated at 37 °C for 5 min and then cooled to rt.

Melting Point Determination

The thermal stability of TIVA probe samples was assessed using a Beckman Coulter DU800 UV-Vis spectrophotometer and Peltier temperature controller. Samples were heated at 1.0 °C/min from 15 °C to 90 °C, held at 90 °C for 10 min, cooled at 1.0 °C/min from 90 °C back to 15 °C, and A_{260} was measured each minute. T_m values were assigned to each phase using first-derivative analysis.

FRET Measurement

Cy3-Cy5 FRET emission was measured on a Cary Eclipse fluorimeter (Varian) with Cary Temperature Controller (Agilent) set to 20 °C. Cy3 was excited at 552 nm and the emission spectrum was collected from 555 nm to 705 nm. FRET efficiency was approximated using the formula FRET= I_a / (I_a + (I_d*γ)), where I_a is the emission intensity of the FRET acceptor at 665 nm, I_d is the emission intensity of the FRET donor at 565 nm, and γ is a correction factor of 2.0 for the two fluorophores' differing quantum yields. We chose to utilize this approximation method over more complex photobleaching or single-fluorophore probe methods as it can also be used to estimate the FRET signal from TIVA probe in real-time microscopy applications.

Streptavidin Bead Procedure

Dynabeads (ThermoFisher Sci 65001) were first removed from 4 °C storage and warmed on benchtop for 30 min to rt. Dynabeads were vortexed for 1 min and an aliquot of X µL beads (X = 150 µL per sample for GFP-poly(A) pull-down, later reduced to 50 µL per sample for cell lysate pull-down) was removed to a LoBind 1.5 mL microtube (Eppendorf 022431021) and washed three times on a magnetic stand using 1x BW buffer [prepared as 2x BW stock: 10 mM Tris (pH 7.5), 1 mM EDTA, 2 M NaCl]. Beads were fully resuspended and then allowed to settle for at least 60 s on the magnetic stand for each wash. As a precaution against contaminating RNA, the beads were incubated with X µL 0.1 M NaOH, 0.05 M NaCl for 2 min, then set on the magnetic stand for 1 min, and the wash was removed and then replaced for another 2 min treatment. The beads were then similarly incubated twice for 2 min with X µL 0.1 M NaCl, and then resuspended in the original volume of BW buffer. On the day of the pull-down experiment, to block the beads against nonspecific hydrophobic interactions an additional X µL of 10 ng/µL purified BSA and 0.1*X µL 1 M spermidine were added, and the bead mixture was rotated for 1 h at rt. After blocking, the beads were washed three times with 1x BW buffer, and then resuspended in the original volume (X µL) of 1x BW buffer.

GFP-poly(A) RNA Expression, Pull-down, and Detection

To obtain GFP-poly(A) transcript, pRS314-T7-GFP plasmid (Addgene ID 33130) was initially expressed in *E. coli* as described previously (Ping et al., 2017), and was subsequently purified by Maxiprep (Thermo Fisher Scientific) and digested with XhoI restriction enzyme for 1 h. Linearized pRS314-T7-GFP RNA was purified by QIAquick affinity columns (Qiagen), and then T7-mediated in vitro transcription of the GFP gene was performed with a MEGAscript T7 Transcription Kit (Invitrogen) per manufacturer's protocol. GFP transcripts were polyadenylated with a Poly(A) Tailing Kit (Invitrogen AM1350) per manufacturer's instructions (Figure 6). After polyadenylation, GFP-poly(A) RNA expected 1.2-1.4 kb size was verified by capillary gel electrophoresis (Figure 6).

For pull-down, 1,500 ng of GFP-poly(A) RNA with 1 µL of RNaseIn in 1x STE buffer (5 µL total) were heat denatured at 70 °C for 5 min. 10 molar eq. of 22/12/8 $(GC)_2$ +$(D\text{-}Arg)_9$ TIVA probe (1-2 µL in

STE) were added and the +light TIVA+RNA samples were placed under a transilluminator for 7 min, mixed briefly, and then irradiated for an additional 7 min to ensure complete photolysis. Both +light and -light tubes were incubated at 37 °C for 5 min, and then placed on ice. To each ~7 µL TIVA probe+RNA sample, 0.6 µL 1 M spermidine and 150 µL of blocked beads were added, and the mixtures were rotated for 40-60 min at 18 °C. After binding, the tubes were placed on a magnetic stand for 3 min, and the supernatant was subsequently discarded. Bead pellets were washed three times with 200 µL of ice-cold 1x BW buffer to remove unbound material. During these washes, the bead pellets were gently rinsed while bound to the magnetic stand but the pellets were not fully re-solubilized, allowing only 30 s to lightly settle after each wash. Finally, the beads were resuspended in 120 uL of nuclease-free water with 1 µL of RnaseIn, and captured mRNA was released by incubating at 70 °C for 5 min. Heated tubes were immediately transferred to the magnetic stand and allowed to settle for 2 min, and 118 µL of the eluent was collected to avoid transferring any beads left at the bottom of the tube. To further eliminate any residual beads, the 118 µL eluent tubes were placed back onto the magnetic stand, and after 2 min an aliquot (116 µL) was transferred to fresh Lobind tubes. 5 µL of 100 mM NaCl / 75 mM Tris buffer was added, and the solutions were concentrated to 10 µL. 1 µL of this solution was loaded onto a Bioanalyzer Nanochip, and the electrophoresis was performed according to manufacturer's instructions.

Fibroblast Culture, Lysis Preparation, Pull-down, and Detection

CCD-1112Sk human neonatal foreskin-derived fibroblasts (ATCC, Manassas, VA) were cultured in IMDM with 10% FBS and penicillin/streptomycin in a 37 °C incubator with 5% CO_2. For pull-down experiments fibroblasts were seeded into wells of a 24-well plate and grown to confluency overnight. On the day of the pull-down experiment the wells were gently washed twice with 400 µL of PBS buffer, and then incubated with 250 µL of 0.5% trypsin at 37 °C for 5 min. 750 µL IMDM media with 10% FBS was added to quench the trypsinization, and the cell mixtures were transferred to individual sterile Lobind 1.5 mL tubes. Cell mixtures were centrifuged at 15,000 g for 5 min and washed with 200 µL PBS, then 5 µL RNaseIn were added followed by 50 µL of ice cold 5x First-Strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$). To aid in lysis, tubes were snap-frozen in liquid nitrogen and then thawed, and this freeze-thaw cycle was performed twice. Fibroblast lysate tubes were set aside on ice.

Aliquots of 200 pmol of 22/12/8 $(GC)_2$ +$(D\text{-}Arg)_9$ TIVA probe in 50 µL PBS were incubated at 37 °C, while +light samples were irradiated for 5 min under a 410 nm LED. TIVA aliquots were combined with lysate aliquots, and 50 µL blocked beads and 100 µL of 1x BW buffer were added to each tube, and the mixtures were rotated at 18 °C for 40-60 min. Tubes were then washed three times with ice-cold 1x BW buffer as described earlier, before being resuspended in 25 µL of nuclease-free water for elution at 70 °C for 5 min. The eluent was returned to the magnetic stand and 23 µL captured in fresh Lobind tubes. The concentration of RNA in these tubes was later assessed on a Qubit 2.0 fluorescence detector according to manufacturer instructions.

DLS and Zeta Potential

Hydrodynamic diameter of TIVA complexes was determined by dynamic light scattering (DLS) and zeta potential by Laser Doppler Velocimetry (LDV) at 25 °C using a Zetasizer Nano ZS apparatus (Malvern Panalytical). TIVA probes lacking Cy5, with or without CPP (TIVA-CPP, TIVA-NT) were prepared in PBS buffer diluted to 10 µM. We determined that Cy5 interfered with these measurements, due to significant absorbance of the 633 nm light used for sample interrogation, but Cy3 did not. Samples were assessed in disposable low-volume cuvettes protected from ambient light. Each sample was measured 3 times and data presented in 'relative intensity'. Zeta potential was also measured by the same instrument. Samples were prepared in water at 2 µM in Folded Capillary Zeta Cell (Malvern Panalytical) protected from ambient light. Each sample was measured 3 times.

Stability Assay

TIVA 22/12/8 and TIVA 18/7/7 probes were diluted to 1.0 μM in PBS and incubated at 37 °C for 24 h with or without 10% FBS. Cy3 emission at 565 nm and Cy5 emission at 665 nm were recorded every 10 min by the Infinite® M1000 plate reader (Tecan). FRET efficiencies were calculated as indicated above. FRET efficiency at each time point was normalized to the starting point.

TIVA Probe Loading into Cells and Activation

The cortical brain slices (250 μm) were prepared from 30~50 day old freshly isolated C57BL/6 mouse brain using a vibratome (Leica VT 2000). The brain slices were incubated in artificial cerebrospinal fluid (aCSF, in mM: NaCl 122, $CaCl_2$ 2, $NaHCO_3$ 28, KCl 3.5, glucose 5.5, HEPES 10, $MgCl_2$ 1, pH 7.4 with 5% CO_2/ 95% O_2 gas) for 30 min followed by loading of the 22/12/8 $(GC)_2$ +$(D\text{-}Arg)_9$ TIVA probe into cells by incubation with the 3 μM TIVA-probe in aCSF for 90 min at rt. After loading, excitation and uncaging was performed while imaging the FRET signal by 514 nm excitation and simultaneous capturing of images in the Cy3 (538-599nm) and Cy5 (637-704nm) emission ranges. The photolysis was performed in visually identified cells in the cortex while live tissue/cell imaging on the confocal microscope (Zeiss 710meta) using a laser power at 80% and 100.85 μs dwell time per pixel. After uncaging, the targeted region including adjacent cells was isolated using a glass pipet and the TIVA/RNA hybrids isolated and the RNA processed for sequencing.

TIVA Probe Uptake

The cellular uptake was tested with mouse brain slices in aCSF prepared as mentioned. 3 μM TIVA-NT or TIVA-CPP was loaded to brain tissue samples. Uptake was shown by Cy3 intensity and imaged under a confocal microscope (same setting). Images were taken at 2, 10, 30, 60, 90 min. For amiloride study, cells were pre-incubated in aCSF with 10 mM amiloride for 30 min, and TIVA-CPP was loaded in the above-mentioned way. Confocal images were taken at 10, 30, 60 min.

TIVA aRNA Amplification, Library Construction and Sequencing

As the TIVA/RNA complex is tagged with a biotin moiety, the complex can be isolated using streptavidin to bind to the biotin in the complex. To accomplish this, Dynabeads MyOne Streptavidin C1 (Thermo Fisher Scientific, Catalog #65001) were washed according to the manufacturer's instructions, and resuspended in Binding and Washing (B&W) Buffer (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1M NaCl). The Dynabeads were blocked by rotating desired volume of the blocking mixture (0.2 μg/μL washed Dynabeads, 4 ng/μL BSA, 4 mM spermidine) at rt for 1 h. Blocked Dynabeads was then washed with B&W buffer for three times, and resuspended in B&W buffer to the final concentration of 10 mg/mL. The collected target cell was mixed with 60 μL B&W buffer, 0.3 μL 1 M spermidine, 1 μL blocked Dynabeads and rotated for 30 min at 12 °C with vortexing at 5 min intervals. The tube was then placed onto a magnetic stand for 3 min and the pellet was washed 3 times with 12 °C B&W buffer. After air-drying for 3 min, Dynabeads were resuspended in 6 μL water and incubated at 70 °C for 5 min. The suspension was placed on magnetic stand for 2 min and 5 μL of deannealed solution containing the cellular RNA was used as template for two rounds of aRNA amplification (1). A 1:4 million dilution of ERCC RNA Spike-In (Life Technologies) was added to each sample to control for technical variation between samples. Briefly, for the first round of amplification, a synthesized oligo(dT)-T7 primer that contained a poly-T and phage T7 RNA polymerase promoter

sequence was hybridized to the mRNA in the sample and used to create double-stranded cDNA. The T7 promoter included in the oligo was used to initiate T7 polymerase–mediated linear amplification of the RNA. For second round of amplification, aRNA was converted into cDNA initiated by random hexanucleotide primers for first strand and oligo(dT)-T7 primer for second strand. The amplified aRNA was purified and then assessed using Bioanalyzer RNA Pico kit (Agilent, Catalog #5067-1513). This material was then made into a sequencing library using Illumina TruSeq Stranded mRNA library Prep kit (Catalog #20020594) according to the manufacturer's instructions and submitted for NextSeq550 sequencing. These protocols were adapted from Lovatt et al.(*2, 3*)

References

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