Electronic Supplementary Material (ESI) for RSC Chemical Biology.

Supplementary Information

Evaluating Riboglow-FLIM probes for RNA sensing

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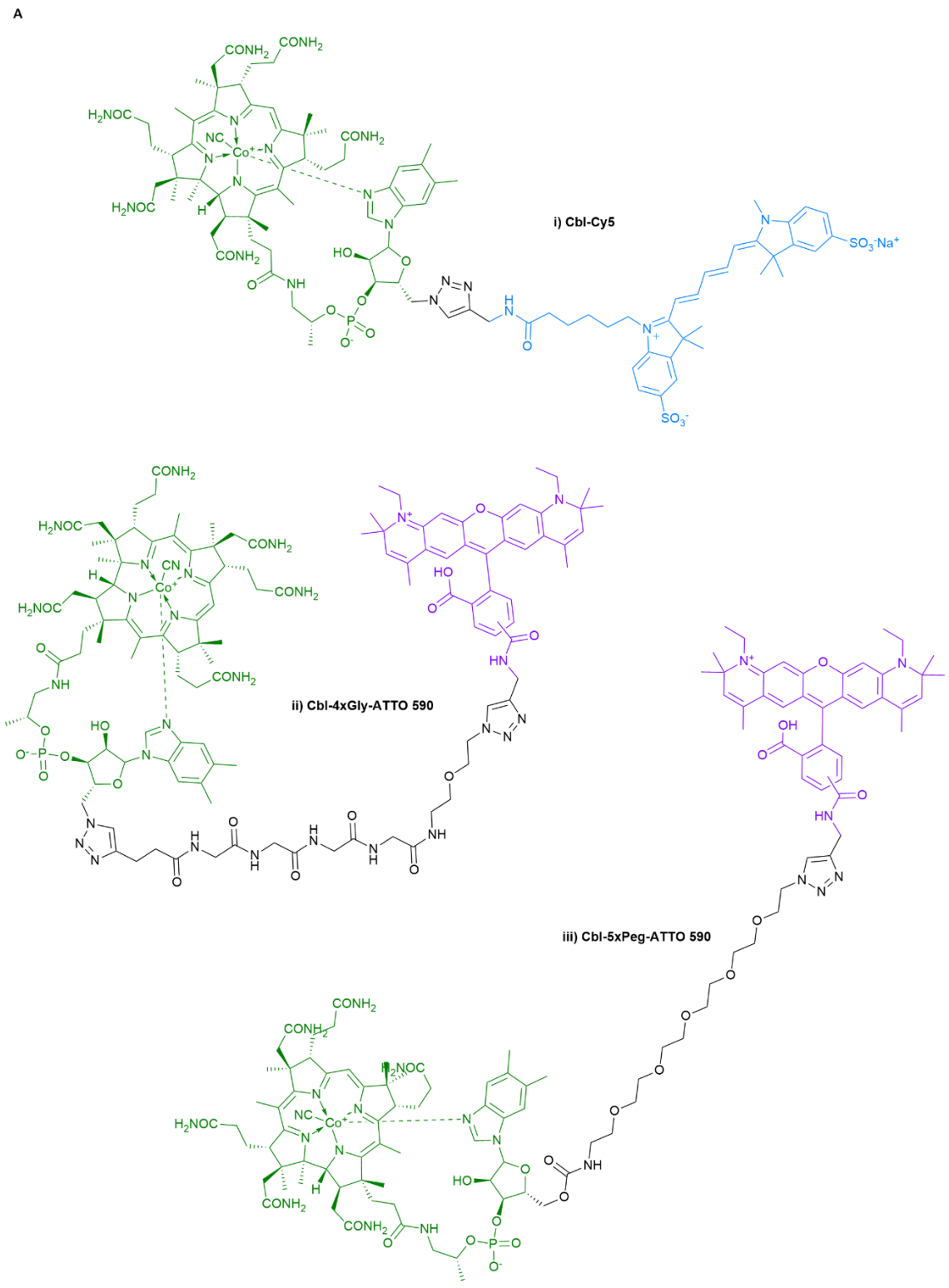
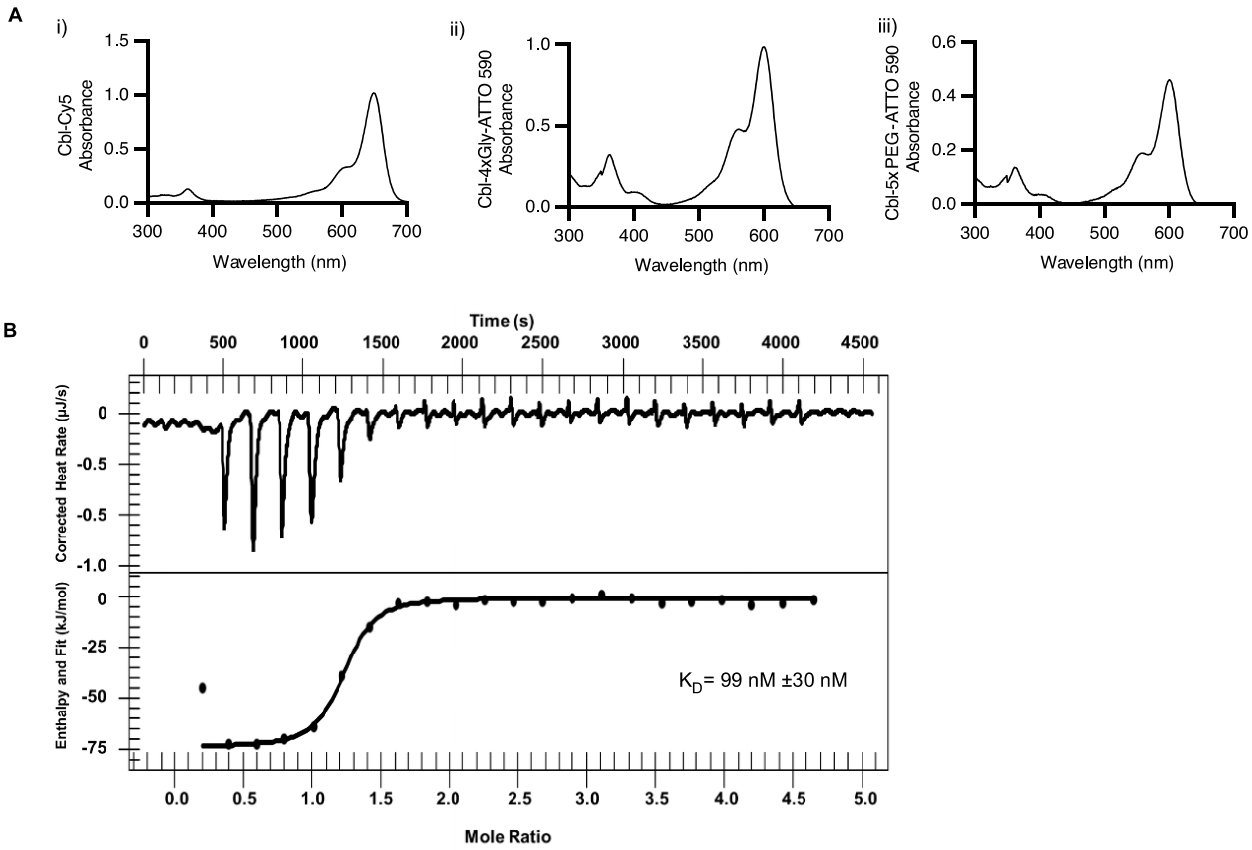
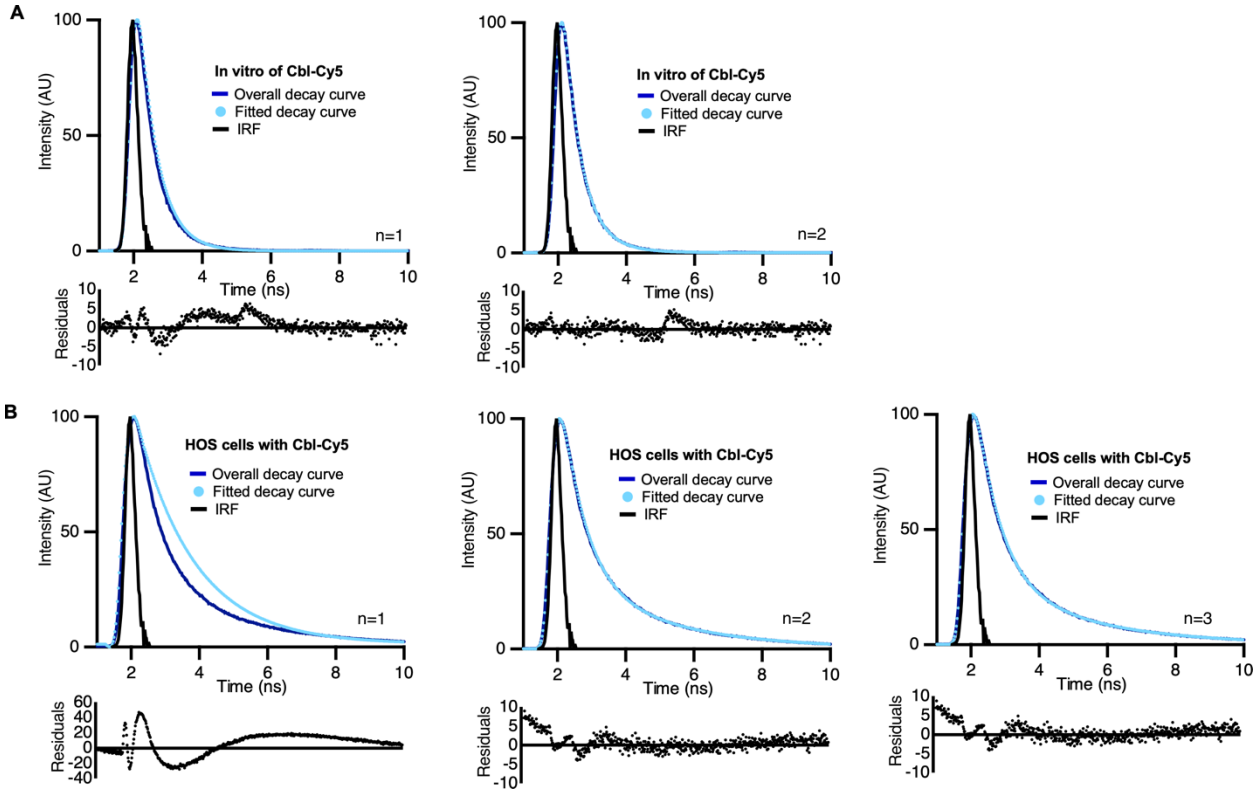


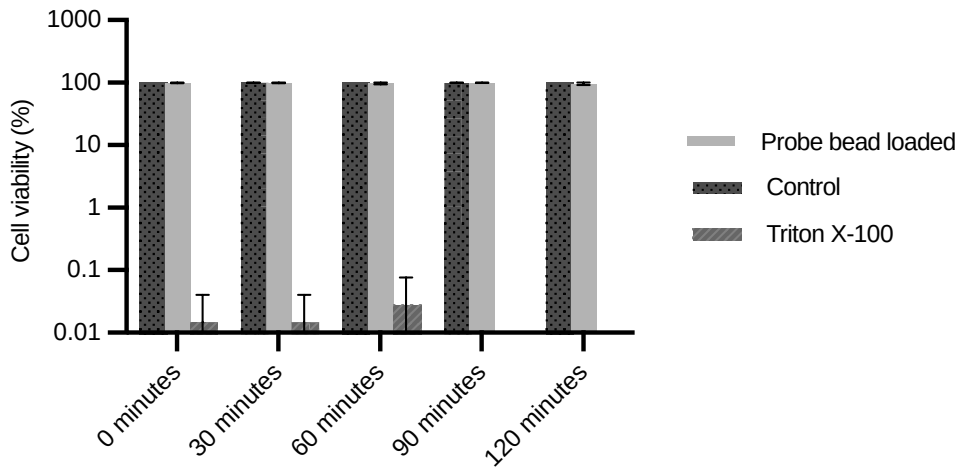
Supplementary Figure 1. Chemical structures of Riboglow probes. i) Cbl-Cy5, ii) Cbl-4xGly-ATTO 590, and iii) Cbl-5xPeg-ATTO 590. The Cbl-Cy5 probe fluorophore, Cy5 (blue), is not attached with a linker segment but is instead directly bound to cobalamin (green), whereas ATTO 590 (purple) is attached via shown linkers (black).[1]



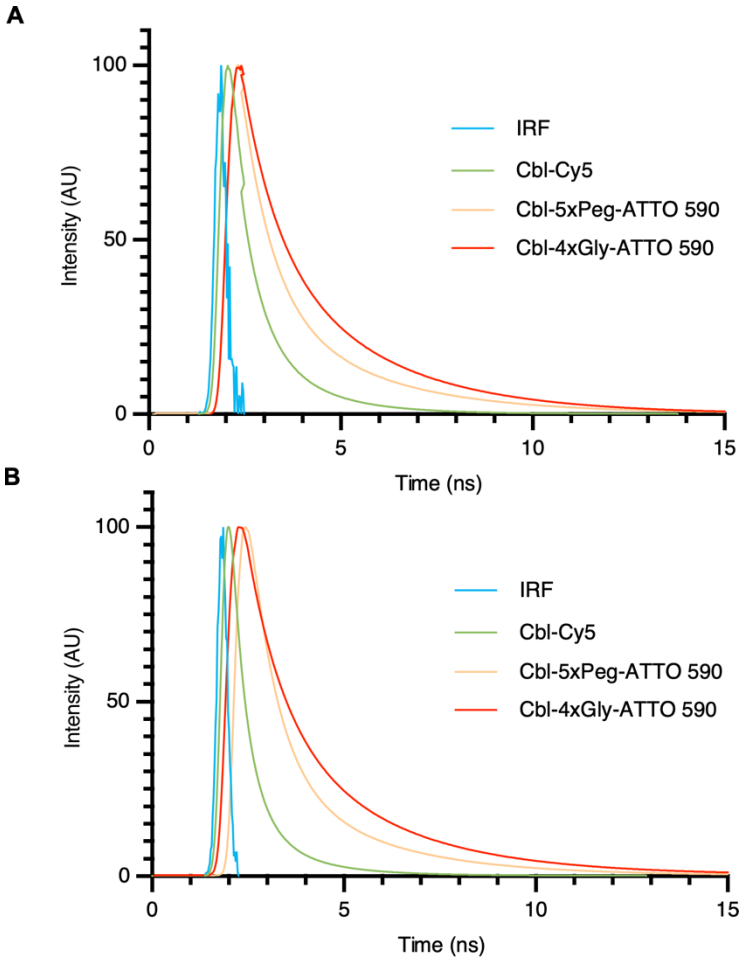
Supplementary Figure 2. In vitro characterization of fluorescence and binding. (A) UV-Vis absorbance spectra of i) Cbl-Cy5, ii) Cbl-4xGly-ATTO 590, and iii) Cbl-5xPeg-ATTO 590. (B) Isothermal titration calorimetry (ITC) thermogram of the purified RNA A-tag binding to Cbl (K_D = 99 nM $\pm$ 30 nM) fit to a 1:1 independent binding model.



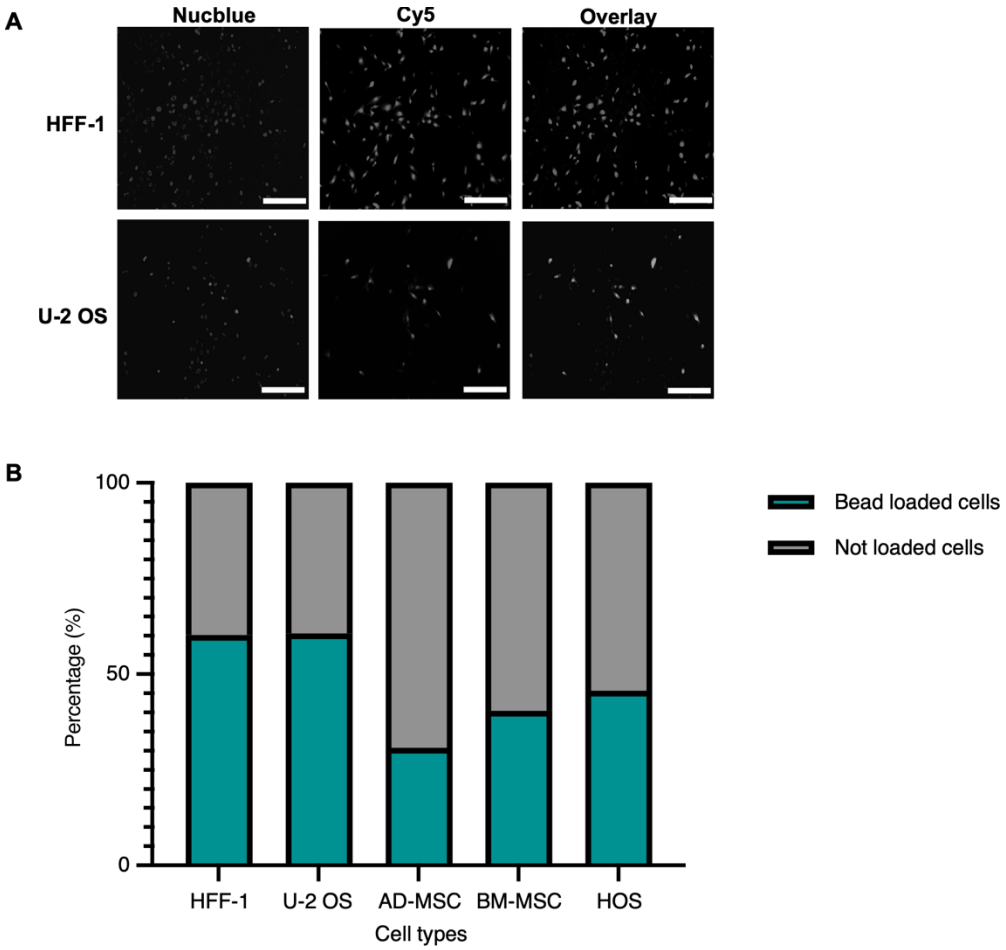
Supplementary Figure 3. Multiexponential reconvolution fitting of FLIM data for Cbl-Cy5 probe in vitro vs. in live cells. (A) Lifetime decay curves for Cbl-Cy5 in vitro, demonstrating good fits with both single (n=1) and double (n=2) exponential models. The dark blue line represents the overall decay curve, while the light blue circles depict the fitted decay curve. The residual map is shown below for each decay curve. (B) Lifetime decay curves for Cbl-Cy5 in a cellular environment (HOS cells), showcasing robust fits with double (n=2) or triple (n=3) exponential models, with minimal variations in fitting quality observed between n=2 and n=3.



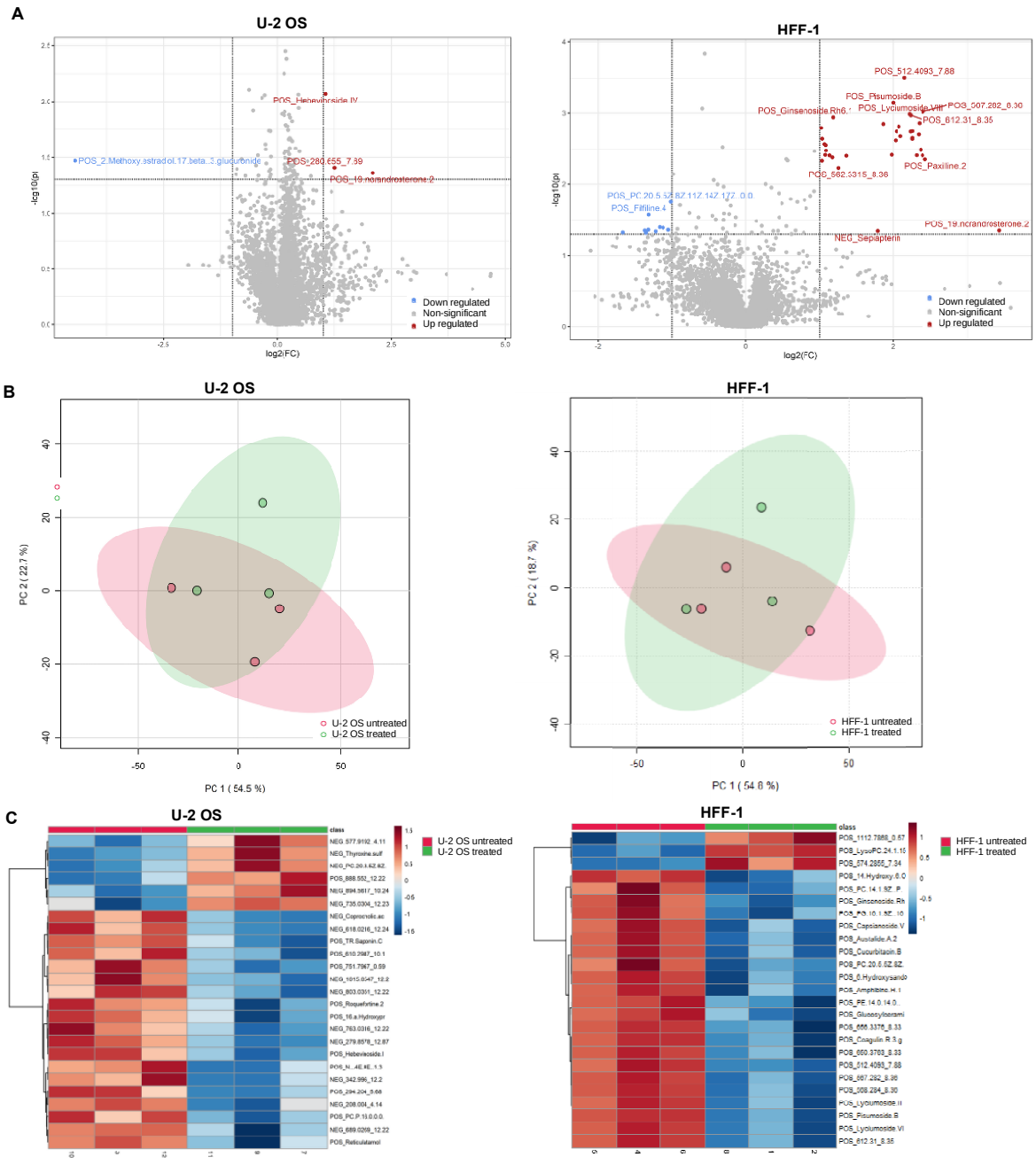
Supplementary Figure 4. Cell viability assay after loading Riboglow probe into U-2 OS cells. U-2 OS cells were loaded with Cbl-Cy5 for imaging experiments. Cell viability after loading was quantified. Live and dead U-2 OS cells were counted after defined intervals of time and displayed as percentages of cell survival following no treatment (dark gray with dots), bead loaded with Cbl-Cy5 (light gray), and treatment with Triton X-100 (stripes). Average cell sample sizes for each triplicate runs were 69, 190, and 158 cells, respectively. Average viability and SD are reported. Note that the “Triton X-100” values at 90 and 120 minutes yielded cell viabilities that were too low to be visible on the graph.



Supplementary Figure 5. Lifetime decay curves of Riboglow probes. Samples were collected for (A) HOS and (B) U-2 OS representative cells displayed in main figure 3A. For each cell measured, the ROI was defined as the whole cell, and the average lifetime decay curve was calculated in nanoseconds and displayed in a scatterplot (main figure 3B). The instrument response function (IRF) is shown here in blue for each graph.



Supplementary Figure 6. Bead loading efficiency of cell lines used in this study. (A) Representative images of U-2 OS and HFF-1 cells bead loaded with Cy5 probe and Nucblue nucleus stain. Scale bar = 100 μm. (B) Summary of percentage of cells bead loaded (teal) vs. not bead loaded (grey) for > 100 cells per cell line.



Supplementary Figure 7. Metabolomic analysis of Riboglow probe loaded in two cell types (U-2 OS vs. HFF-1). (A) Volcano plot of the differential analysis in MetaboAnalystR 4.0 for U-2 OS cells and HFF-1 cells treated (bead loaded with Cy5 probe) vs. normal (not bead loaded). (B) Principle component analysis (PCA) plot in MetaboAnalystR 4.0. showing scores plot for U-2 OS cells and HFF-1 cells treated (loaded with Cy5 probe) vs. normal (not bead loaded). (C) Heatmap shows top 25 T-test differential labeled metabolites in the statistical analysis function of MetaboAnalystR 4.0 for U-2 OS cells and HFF-1 cells treated (loaded with Cy5 probe) vs. normal (not bead loaded).

Supplementary Table 1

Sequence of Riboglow RNA A-tag previously developed.[1]

Name	Sequence
Riboglow 1x A-tag	5′- GGC CTA AAA GCG TAG TGG GAA AGT GAC GTG AAA TTC GTC CAG ATT ACT TGA TAC GGT TAT ACT CCG AAT GCC ACC TAG GCC ATA CAA CGA GCA AGG AGA CTC -3′

Supplementary Table 2

Comparison of fitting for data obtained in this study and previously reported fluorescence lifetimes for in vitro work of RNA tag (A-tag) in the presence of Cbl-4xGly-ATTO 590 at different probe and RNA concentrations.[1,2] Multiexponential reconvolution to either n=1 or n=2 variables was used in Sarfraz et al (2023)[2]. Values obtained in this study demonstrate clear reproducibility and the importance of fitting considerations.

Study	**Sarfraz et al. (2023)[2]**	**This Study**			
RNA and Probe	5 μM RNA; 0.5 μM probe	5 μM RNA; 5 μM probe		5 μM RNA; 0.5 μM probe	
Variable	n=1	n =1	n =2	n=1	n=2
Cbl-4xGly-ATTO 590 + A-tag	2.4 ns ± 0.2 ns	2.5 ns ± 0.2 ns	2.07ns ± 0.02 ns	2.6 ns ± 0.2 ns	2.04 ns ± 0.02 ns

Supplementary Table 3

Comparison of obtained and previously reported fluorescence lifetimes.[1,2] Values were measured in vitro unless otherwise indicated. Note that the lifetime was processed by tail-fitting in Braselmann et al (2018), whereas multiexponential reconvolution was used in Sarfraz et al (2023)[2,3] and in this study (number of variables for multiexponential reconvolution are listed as n below each lifetime). This different analysis method might explain the different lifetime values. See also Supplementary Figure 3.

Probe	Braselmann et al. (2018)[1]	Sarfraz et al. (2023)[2]	Sarfraz et al. (2023)[3]	This study
Cbl-5xPEG-ATTO 590	1.19 ns (tailfit)	-	-	0.95 ns ± 0.02 ns (n=2)
Cbl-5xPEG-ATTO 590 + A-tag	2.14 ns (tailfit)	-	-	1.24 ns ± 0.06 ns (n=2)
Cbl-Cy5	0.61 ns (tailfit)	-	0.57 ns ± 0.1 ns (n=2)	0.54 ns ± 0.02 ns (n=2)
Cbl-Cy5 + A-tag	1.04 ns (tailfit)	-	0.92 ns ± 0.1 ns (n=2)	0.80 ns ± 0.02 ns (n=2)
Cbl-4xGly-ATTO 590	1.44 ns (tailfit)	0.94 ns ± 0.10 ns (n=1)	-	1.05 ns ± 0.01 ns (n=2)
Cbl-4xGly-ATTO 590 + A-tag	2.98 ns (tailfit)	2.45 ns ± 0.20 ns (n=1)	-	2.03 ns ± 0.02 ns (n=2)
in U-2 OS cells: Cbl-4xGly-ATTO 590	-	1.40 ns ± 0.30 ns (n=3)	-	1.68 ns ± 0.20 ns (n=2)
in U-2 OS cells: Cbl-4xGly-ATTO 590 + A-tag	-	1.92 ns ± 0.20 ns (n=2)	-	-
in U-2 OS cells: Cbl-Cy5	-	-	0.55 ns ± 0.05 ns (n=2)	0.59 ns ± 0.05 ns (n=2)
in U-2 OS cells: Cbl-Cy5 + A-tag	-	-	0.83 ns ± 0.1 ns (n=2)	-

Supplementary Table 4

Statistical comparison of data listed in main figures 2B and 3B.

Reported value	P-value pair	P-value (symbol)
Average Lifetime (ns)	Cbl-5xPEG-ATTO 590: Cbl-5xPEG-ATTO 590 + A-tag	p = 0.0001 (****)
	Cbl-4xGly-ATTO 590: Cbl-4xGly-ATTO 590 + A-tag	p = 0.0001 (****)
	Cbl-Cy5: Cbl-Cy5+ A-tag	p = 0.0001 (****)
	HOS Cbl-5xPEG-ATTO 590: U-2 OS Cbl-5xPEG-ATTO 590	p = 0.0001 (****)
	HOS Cbl-4xGly-ATTO 590: U-2 OS Cbl-4xGly-ATTO 590	p = 0.05 (*)
	HOS Cbl-Cy5: U-2 OS Cbl-Cy5	p = 0.0001 (****)

Supplementary Table 5

Comparison of evaluated cell lines.[4–9]

Cell	Organism	Type	Morphology	Origin	Characteristics
U-2 OS[4]	Homo sapiens, human	cancer derived cell line	flat, epithelial-like	Bone	Commonly used in cancer research, osteosarcoma derived.
HOS[5]	Homo sapiens, human	cancer derived cell line	mixed, fibroblast and epithelial like cells	Bone	Commonly used in cancer research, osteosarcoma derived.
HFF-1[6]	Homo sapiens, human	Fibroblast	fibroblast	Skin; Foreskin	Non-cancerous, primary human fibroblasts, extensively used in cell biology and tissue culture research as a model for normal human cells. They are not transformed and exhibit normal growth and cellular behavior.
Adipose derived mesenchymal (AD-MSCs)[7,8]	Homo sapiens, human	mesenchymal stem cell	Spindle-shaped, fibroblast-like	Adipose tissue	Multipotent stem cells capable of differentiating into various cell types. They are often used in regenerative medicine and tissue engineering research.
Bone marrow derived (BM-MSCs)[8,9]	Homo sapiens, human	mesenchymal stem cell	spindle shaped, fibroblast-like	Bone	They are widely used in research involving hematopoiesis, immune system studies, and regenerative medicine.

MATERIALS AND METHODS

Riboglow Probes

Riboglow probes were a gift from Amy Palmer at CU Boulder, and stock solutions were prepared in phosphate buffered saline (PBS). Probe concentrations were determined from previously published extinction coefficients, namely 271,000 L mol^{-1} cm^{-1} for Cbl-Cy5 and 120,000 L mol^{-1} cm^{-1} for Cbl-4xGly-ATTO 590 and Cbl-5xPEG-ATTO 590.[1]

UV-Vis Absorbance

UV-Vis absorbance measurements were made using a Cary 60 UV-Vis Spectrophotometer. The spectrophotometer was blanked with 10 µL of 1X PBS, and samples of each of the three unbound probes (Cbl-Cy5, Cbl-5xPeg-ATTO 590, Cbl-4xGly-ATTO 590) were diluted using 1X PBS and loaded into a quartz cuvette and UV spectra obtained.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) measurements were carried out via a Waters Nano ITC. The ITC instrument was first cleaned 4 times with Ultrapure water (UPW) before sample preparation. Both the RNA ligand and the probe to be analyzed were diluted to a concentration of 10 µM and 350 µM respectively in RNA Buffer (100 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 40 mM HEPES at pH 7.4), and both solutions were degassed for 10 minutes. The Nano ITC was set to 25°C, and 300 µL of the RNA was drawn up into the syringe and deposited into the sample cell, ensuring no bubbles were injected. The burette was removed, cleaned with UPW, and used to draw up 50 µL of Cbl probe before being reinserted into the Nano ITC. The stirring rate was set to 400 RPM, the injection parameters were set to 22 injections of 1.6 µL at an interval of 180 seconds, and the machine was set to auto-equilibrate. Following a successful run, the data was analyzed in NanoAnalyze v 3.12.5, using one mode binding (independent) model.

DNA Preparation and RNA Purification

DNA preparation and subsequent RNA purification were conducted as outlined previously.[2] Paraphrased, *E. coli* cells transfected with the DNA plasmids of interest were lysed and the DNA was purified and amplified via PCR with the Q5 High Fidelity protocol (NEB). RNA was then transcribed with the T7 High Yield RNA Synthesis Kit (NEB), and Monarch® PCR & DNA Cleanup kits. Agarose gel electrophoresis was used to confirm products with a 1 kb Plus Ladder (NEB). DNA and RNA concentrations were determined on a BioTek Synergy H1 Microplate Reader using RNA nanodrop capabilities on a Take3 Multi-Volume plate. The sequence of the RNA used in this study is listed in Supplementary Table 2.

Mammalian Cell Culture

HOS, U-2 OS, HFF-1 cell lines were obtained from the Tissue Culture and Biobanking Shared Resource (Georgetown University) and Adipose Derived Mesenchymal stem cells (ADM-SC) and Bone Marrow derived stem cells (BMD-SC) were obtained from ATCC (PCS-500-012; PCS-500-011). Cells were passaged for up to 5 passages at 37°C and 5% CO_2 in 10% FBS (Gibco) and Dulbecco's modified eagle medium (DMEM, Gibco). For imaging, cells were seeded at 0.25×10^6 cells/dish in sterile 35 mm µ-dishes with a polymer coverslip (Ibidi) and incubated at 37°C and 5% CO_2.

Instrument Response Function (IRF)

A sterile 35 mm µ-dish with a polymer cover slip (Ibidi) containing a supersaturated solution of potassium iodide (KI) and Rhodamine B was utilized. The imaging process was conducted on an Abberrior STEDYCON microscope, utilizing a pulsed excitation laser line at 561 nm with 488/568 nm filter with high excitation power (see more details below for FLIM procedure). The fastest

acquisition setting was employed, capturing images within a fixed 512 × 512 pixel area. To obtain the decay curve, the fluorescence readout was measured using SymPhoTime 64 (Picoquant) for a duration of 30 seconds, while ensuring avoidance of saturation effects. The extraction of the Instrument Response Function (IRF) followed the instructions provided by Picoquant.[2]

Live cell and In Vitro FLIM Microscopy

35 mm µ-dishes containing adequately confluent cells (confluence of >70% verified by light microscopy) were washed with 1X phosphate buffered saline (1X PBS, Sigma) and bead loaded[10] with the appropriate probe in accordance with the procedure previously used.[1] Paraphrased, 3 µL of a 50 µM stock of the probe was added to the imaging dishes and loaded with glass beads, after which fresh media was immediately added. Cells were incubated for 10 min at 37°C and 5% CO_2 in 10% FBS (Gibco) and FluoroBrite™ DMEM (Gibco) and imaged within 3 hours of probe loading. In vitro measurements were collected for cobalamin free probes, ATTO-590-Biotin (Sigma) and Cy5-Azide (Sigma), and all three probes (Cbl-Cy5, Cbl-4xGly-ATTO 590, and Cbl-5xPEG-ATTO 590) both alone and in the presence of the RNA A-tag ligand. The probes were diluted in RNA buffer (100 mM KCl, 1 mM $MgCl_2$, 10 mM NaCl, 50 mM HEPES, pH 8) with 5 µM of purified RNA and a final concentration of probe at 5 µM. RNA concentrations of 5 µM and probe concentrations of 0.5 µM were also used to verify the concentration independence of FLIM. Samples containing RNA were incubated for 20–30 minutes at room temperature to allow binding of the probe to RNA to occur prior. 10 µL of the prepared probe/RNA solutions were added to 35 mm µ-dishes and imaged immediately afterwards. Each imaging dish containing either bead loaded cells or in vitro probe solutions was imaged within 1-2 hours of sample preparation using an Abberrior STED FLIM microscope (100x oil objective or 20x air objective) with a fixed imaging area of 512 × 512 pixels. Data was acquired using a PicoQuant Timeharp 260 card. Data per frame was acquired until a total threshold of 10^4 counts was reached with a diode pulsed laser of 40 MHz and excitation at 561 nm (Semrock Em01-R488/568 + SP01-633RU filter) or 640 nm (Chroma 675/50 ET Bandpass filter) for ATTO 590 and Cy5 respectively. Data was collected and analyzed using PicoQuant SymPhoTime 64 software. A false-color scale of the collected images was set based on a range of lifetime histograms for measured samples, and the average amplitude weighted lifetime images were extracted as outlined below.

In Vitro Crowding

In vitro FLIM was conducted using Cbl-Cy5 under 6 independent conditions in which dilution solution composition varied by either including Bovine Albumin (Thermo Scientific, 23210), nucleotides (New England N0446S), Ficoll or glycerol and varying the pH. Bovine Albumin at a concentration of 2mg/mL was supplemented with 5 µM of Cbl-Cy5 probe. A nucleotide mixture of 100 mM containing equal amounts of CTP, GTP, ATP, and UTP was prepared and supplemented with 5 µM of Cbl-Cy5 probe. Ficoll was dissolved in RNA buffer (100 mM KCl,1 mM $MgCl_2$, 10 mM NaCl, 50 mM HEPES, pH 8) to a stock concentration of 400 mg/mL. Using this solution, the Ficoll concentration was set to 150 mg/mL and the probe was diluted to get a concentration of 5 µM. For the glycerol condition, the probe was diluted in RNA buffer (100 mM KCl,1 mM $MgCl_2$, 10 mM NaCl, 50 mM HEPES, pH 8) and 50% glycerol to get a 50/50 mix and a probe concentration of 5 µM. For the low and high pH solutions, HCl or NaOH was added to RNA buffer (100 mM KCl,1 mM $MgCl_2$, 10 mM NaCl, 50 mM HEPES) to get the desired pHs of 7.5 and 8.5, respectively. The RNA buffers at 7.5 and 8.5 were used to dilute the probe concentration to 5 µM for low and high pH conditions. Two 10 µL drops of the prepared probe solutions were added to 35 mm µ-dishes, imaged immediately and analyzed using PicoQuant SymPhoTime 64 software as outlined below.

Multiexponential Fitting Analysis

Multiexponential fitting analysis of fluorescence decay curves acquired by the Abberrior STED FLIM microscope was conducted using SymPhoTime 64 reconvolution script (PicoQuant) and the

acquired IRF, as described previously.[2] Paraphrased, the photon arrival time at each pixel was summarized into a histogram of arrival times for a region of interest (ROI) which was defined as a whole cell for cellular measurements or as a square region approximately the size of a cell for in vitro measurements.[2] The acquired decay function at each pixel was then analyzed further to extract fluorescence lifetime values through multiexponential reconvolution fitting to n=2 parameters as guided by the residual map (Supplementary Figure 3), allowing for the determination of the average lifetime of the selected ROI. The resulting amplitude-weighted average lifetime was assigned to a false color scale for visualization.

Cell Viability Assay

Cell viability was measured in accordance with the procedure detailed in the Cell Viability Assay Kit (ab112120, Abcam). Briefly, live U-2 OS cells were grown in a 96-well plate and incubated at 37°C and 5% CO_2 for 24 hours to allow cells to adhere. The growth media was then aspirated away, and the cells were treated with 100 µL of media (untreated group), 100 µL XM Triton X-100 (cytotoxic control group) or 100 µL of media following the bead loading procedure (experimental group). 100 µL of the dye-loading solution was then added, and the cells were allowed to incubate for at least one hour before cell counts were taken. Fluorescence intensity of the dyed cells was monitored at Ex/Em= 360/450 nm using an EVOS M5000 microscope.

Bead Loading Efficiency

HOS, U-2 OS, HFF-1 cell lines were obtained from the Tissue Culture and Biobanking Shared Resource (Georgetown University) and Adipose Derived Mesenchymal stem cells (PCS-500-012) and Bone Marrow derived stem cells (PCS-500-011) were obtained from ATCC. Cells were passaged for up to 5 passages at 37°C and 5% CO_2 in 10% FBS (Gibco) and Dulbecco's modified eagle medium (DMEM, Gibco). All cells were grown to high confluency (~70%) then bead loaded with 50 µM Cy5 probe (Cy5-Azide, Sigma) as explained above. NucBlue stain was added to mark all cell nuclei. Cell images were collected on an EVOS M5000 microscope (Thermo Fisher) with DAPI and Cy5 LED light cubes and a 4x, 10x and 20x air objective. Images were exported as TIFFs and further processed in ImageJ using the manual Cell Counter plugin where number of cells containing the blue nuclear signal were counted and considered as total number of cells present, and cells with a Cy5 read out were considered as cells bead-loaded.

Metabolomics Sample Preparation and Collection

U-2 OS and HFF-1 cell lines were obtained from the Tissue Culture and Biobanking Shared Resource (Georgetown University). Cells were passaged for up to 5 passages at 37°C and 5% CO_2 in 10% FBS (Gibco) and Dulbecco's modified eagle medium (DMEM, Gibco). U-2 OS and HFF-1 cells were grown to high confluency (~ 10^7 cells) and some samples were bead-loaded with 50 µM Cy5 probe (as explained above) and others left un-loaded. The cell samples were washed three times with chilled 1X PBS (1X PBS, Sigma) and then lifted by gentle scraping using an 18 cm handle, 1.8 cm blade cell scraper (Falcon, 353085). The collected cells were transferred to 2 mL tubes and centrifuged at 1500 rpm at 4°C for 10 minutes. The supernatant was carefully removed, and the cell pellet was frozen at -80°C. The samples were resuspended in 50 µL of 1X PBS. 150 µL of extraction solution was added (3.5 mL water, 2.5 mL methanol, 4 mL IPA, 10 µL debrisoquine (1 mg/mL in ddH2O), 50 µL of 4-nitrobenzoic acid (1 mg/mL in methanol)). Samples were plunged into dry ice for 30 seconds and then heat shocked by plunging into a 37°C water bath for 90 seconds. This was repeated three times. The samples were vortexed and then incubated on ice for 20 minutes. 150 µL of chilled acetonitrile was added, samples vortexed and incubated at -20°C for 20 minutes. The samples were then spun at 15,493 x *g* for 20 minutes at 4°C, and the supernatant was transferred to a glass MS vial for UPLC-QTOF analysis.

UPLC-QTOF

A volume of 2 µL of each sample was injected onto a Waters Acquity BEH C18 1.7 µm, 2.1 x 50 mm column using an Acquity UPLC system coupled to a Xevo G2-S quadrupole-time-of-flight mass spectrometer with an electrospray ionization source (UPLC-ESI-QToF-MS) (Waters Corporation, Milford, MA). The mobile phases consisted of 100% water with 0.1% formic acid (solvent A), acetonitrile containing 0.1% formic acid (solvent B), and 100% isopropanol with 0.1% formic acid (solvent C). All solvents used were of LC-MS grade and were purchased from Fisher Scientific (Waltham, MA). The solvent flow rate was set to 0.4 mL/min with the column set at 40°C. The LC gradient was as follows: Initial: 95% A, 5% B; 0.5 minutes: 95% A, 5% B; 8.0 minutes: 2% A, 98% B; 9.0 minutes: 0% A, 11.8% B, 88.2% C; 10.5 minutes: 0% A, 11.8% B, 88.2% C; 11.5 minutes: 50% A, 50% B; 12.5 minutes: 95% A, 5% B; 13.0 minutes: 95% A, 5% B. The column eluent was introduced into the Xevo G2-S mass spectrometer by electrospray operating in either negative or positive electrospray ionization mode. Positive mode had a capillary voltage of 3.00 kV and a sampling cone voltage of 30 V. Negative mode had a capillary voltage of 2.00 kV and a sampling cone voltage of 30 V. The desolvation gas flow was set to 1000 L/hour and the desolvation temperature was set to 500°C. The cone gas flow was 25 L/hour and the source temperature was set to 120°C. The data were acquired in the sensitivity MS mode with a scan time of 0.300 seconds and an interscan time of 0.014 seconds. Accurate mass was maintained by infusing Leucine Enkephalin (556.2771 [M+H]+/554.2615 [MH]-) in 50% aqueous acetonitrile (1.0 ng/mL) at a rate of 10 µL/min via the Lockspray interface every 10 seconds. The data were acquired in centroid mode with a 50.0 to 1200.0 m/z mass range for TOF-MS scanning. An aliquot of each sample was pooled and used as a quality control (QC) which represented all metabolites present. This QC sample was run at the beginning of the sequence to condition the column and then injected throughout the batch to check mass accuracy, ensure presence of internal standard, and to monitor shifts in retention time and signal intensities. Data was further analyzed and plotted using MetaboAnalystR 4.0.

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