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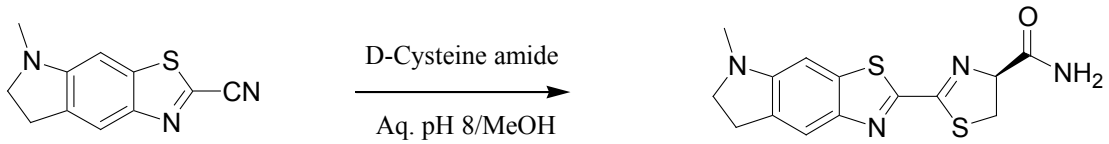
Experimental Section

Compounds. D-luciferin was purchased from Gold Bio. CycLuc1, CycLuc2, CycLuc1-amide, **1**, and D-cysteine amide were synthesized as previously described.[1,2]

CycLuc2-amide [(*S*)-4,5-dihydro-2-(6,7-dihydro-5-methyl-5H-thiazolo[4,5-f]indol-2-yl)thiazole-4-carboxamide]:



1 **2**

(*S*)-2-amino-3-mercaptopropanamide (D-cysteine amide, 7 mg, 0.0596 mmol) was dissolved in 50 mM aqueous sodium phosphate buffer (pH 8, 2 mL) and degassed using argon. This solution was added to **1** (10 mg, 0.049 mmol) in 2 ml of degassed methanol. The reaction was stirred for 2 h, and then diluted with sodium phosphate buffer and extracted with ethyl acetate (2 x 30 mL). The combined organic layers were washed with water (3 x 30 mL) and dried (Na_2SO_4), filtered and evaporated to afford CycLuc2-amide **2** as a yellow solid (12 mg, 81%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.71 (s, 1H), 6.72 (s, 1H and br s, 1H; overlapping), 5.68 (br s, 1H), 5.25 (t, 1H, J = 9.2 Hz), 3.75 (d, 2H, J = 10.0 Hz), 3.51 (t, 2H, J = 8.0 Hz), 3.10 (t, 2H, J = 8.0 Hz), 2.86 (s, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 173.5, 166.8, 153.7, 153.6, 146.5, 137.7, 132.9, 120.0, 96.1, 78.9, 55.8, 35.26, 35.18, 28.2. HRMS (ESI^+) Calcd for $C_{14}H_{15}N_4OS_2$: 319.0687, Found: 319.0677.

Plasmid Constructs

Wild-type and R218K mutant luciferase genes were prepared as previously described.[3,4] The L342A and R218K/L342A mutant luciferases were generated using the Q5 site-directed mutagenesis kit (NEB) using the WT or R218K luciferase, respectively, as template.

Enzyme Expression and Purification

Wild-type and mutant luciferases were expressed and purified as GST-fusion proteins from the pGEX6P-1 vector as previously described.[3] Briefly, JM109 cells were grown at 37 °C until the OD600 reached 0.5-1, induced with 0.1 mM IPTG, and incubated with shaking at 20 °C overnight. Cells were pelleted at 5000 rpm, then flash frozen in liquid nitrogen. The *E. coli* pellets from 1 L of culture were thawed on ice, resuspended in 25 mL lysis buffer (50 mM Tris [pH 7.4], 500 mM NaCl, and 0.5% Tween 20) containing 1 mM phenylmethylsulfonyl fluoride, and disrupted by sonification (Branson Sonifier). Dithiothreitol (DTT) was added at 10 mM, and the resulting cell lysate was clarified by centrifugation at 35,000 rpm for 60 min at 4 °C. The supernatant was batch-bound to immobilized glutathione (Thermo Scientific) for 1 hr at 4 °C, and the beads were washed with lysis buffer containing 10 mM DTT, followed by wash buffer (50 mM Tris [pH 8.1], 250 mM NaCl, and 10 mM DTT) and storage buffer (50 mM Tris [pH 7.4], 0.1 mM EDTA, 150 mM NaCl, 1 mM TCEP). Twenty units of PreScission Protease (GE Healthcare) were added, and incubation continued overnight at 4 °C to cleave the GST-fusion and elute the untagged enzyme. Protein concentrations were determined using Coomassie Plus (Thermo Scientific).

Purified Protein Luminescence Assays - IVIS

Luminescence assays were performed as previously described.[4] Briefly, luminescence was initiated by adding 30 μL of purified luciferase in enzyme buffer (20 mM Tris [pH 7.4], 0.1 mM EDTA, 1 mM DTT, and 0.8 mg/mL BSA) to 30 μL 2x substrate in substrate buffer (20 mM Tris [pH 7.4], 0.1 mM EDTA, 8 mM $MgSO_4$, and 4 mM ATP) in a black 96-well plate (Costar 3915).

Imaging was performed one minute after enzyme addition using a Xenogen IVIS-100 at a final enzyme concentration of 10 nM and final substrate concentrations ranging from 0.122 to 250 μM. Data acquisition and analysis was performed with Living Image® software. Data are reported as total flux (p/s) for each ROI corresponding to each well of the 96-well plate. The data were plotted using GraphPad Prism 6.0 and fit to a sigmoidal dose-response curve by non-linear regression. The cooled CCD in the IVIS has high quantum efficiency at the emission wavelengths of all substrates, and is the same detector used for *in vivo* imaging. However, these data do not reflect the initial rate and can be affected by product inhibition.

Luciferin Burst Kinetics Assays

Using a Promega GloMax-Multi Detection System, 50 μL of purified enzyme in enzyme buffer was rapidly injected into a white 96-well plate (Costar 3912) containing 50 μL of 2x substrate in substrate buffer to a final enzyme concentration of 0.2 nM and a final luciferin substrate concentration ranging from 0.122 to 250 μM. Measurements were taken every 0.5 s for 1 s pre-injection and 60 s post-injection. Data are reported as Relative Light Units (RLU) and were fitted to a sigmoidal dose-response curve by non-linear regression to determine apparent Km values (GraphPad Prism 6.0). Because the photomultiplier tube (PMT) in the GloMax has reduced sensitivity to red light, the photon emission of CycLuc1 and CycLuc2 are understated relative to D-luciferin. These data were not corrected for wavelength and were only used to determine apparent Km values.

ATP Burst Kinetics Assays

D-Luciferin was prepared at 400 μM in 20 mM Tris, [pH 7.4]. CycLuc1 and CycLuc2 were prepared at 40 μM in 20 mM Tris, pH 7.4. Separately, ATP was prepared in 20 mM Tris, pH 7.4 in two-fold serial dilutions from 16,000 μM down to 7.81 μM, with $MgSO_4$ present in two-fold excess at concentrations from 32,000 μM to 15.63 μM. WT and mutant luciferases were prepared at 0.4 nM in enzyme buffer lacking EDTA. In a white 96-well plate (Costar 3912), 25 μL of the luciferin solution and 25 μL of the ATP-Mg solution were combined. Using a Promega GloMax-Multi Detection System, 50 μL of luciferase solution was rapidly injected to achieve a final luciferase concentration of 0.2 nM, a final luciferin concentration of 100 μM for D-luciferin and 10 μM for CycLuc1 and CycLuc2 (for WT, R218K, and L342A luciferases), and a final ATP concentration ranging from 4,000 μM to 1.95 μM, with Mg in two-fold excess. For the R218K/L342A mutant, which has higher apparent Km values for the luciferins, final concentrations of 1 mM D-luciferin and 100 μM CycLuc1 and CycLuc2 were used. Measurements were taken every 0.5 s for 1 s pre-injection and 60 s post-injection. Data are reported as Relative Light Units (RLU). Dose-response curves were plotted with data at the peak of the burst (0.5 s post injection) using GraphPad Prism 6.0 and fit to a sigmoidal curve by non-linear regression to calculate the $K_{m(app)}$ for ATP with each luciferase/luciferin pair. Because the photomultiplier tube (PMT) in the GloMax has reduced sensitivity to red light, the photon emission of CycLuc1 and CycLuc2 are understated relative to D-luciferin. These data were not corrected for wavelength and were only used to determine apparent Km values.

Mice

FVB/N mice were purchased from the Jackson Laboratory. All procedures involving these mice were approved by the Institutional Animal Care and Use Committee (IACUC) at the University

of Massachusetts Medical School (protocol A-2714).

Construction of AAV-CMV-luc2

Mammalian codon-optimized luc2 luciferase from pGL4.1 (Promega) was mutated using a Quickchange mutagenesis kit (Agilent), and then cloned into the EcoRI-SalI sites of a pAAV-CMV plasmid.[5] The AAV-CMV-luc2 mutant plasmids were packaged into AAV serotype 9 by the University of Massachusetts Medical School Viral Vector Core.

AAV9-CMV-luc2 vector striatal injections

Three female FVB/NJ mice per luciferase mutant were purchased from the Jackson Laboratory. Striatal injections were performed at 6 weeks of age. A chemical hand warmer under a gel pad wrapped in paper towel was placed onto a stereotactic frame. Each mouse was anesthetized with 90 mg/kg ketamine and 4.5 mg/kg xylazine (ketamine/xylazine mix) before surgery. Once anesthetized, they were placed on a stereotactic frame and had the hair on their scalp removed by an application of super glue, followed by pulling the hair off as a single section. Three alternate betadine and 70% ethanol washes of the denuded area were performed with a new cotton-tipped applicator for each application. Sterilized forceps were used to raise the skin on the scalp, then sterilized surgical scissors were used to make a 1 cm incision anterior to posterior within the denuded area covering the bregma suture. The site of injection was acquired at the lateral edge of the striatum/cortex border (stereotactic coordinates: anterior 1 mm, lateral 3 mm and ventral 2 mm from bregma). The needle was left in place for one minute, then the mutant luciferase-containing AAV vector (1 μL of 2.5e12 GC/ml AAV9-CMV-Luc2 in PBS) was injected into the mouse at a rate of 125 nl/min using a micropump-controlled syringe (NanoFil, World Precision

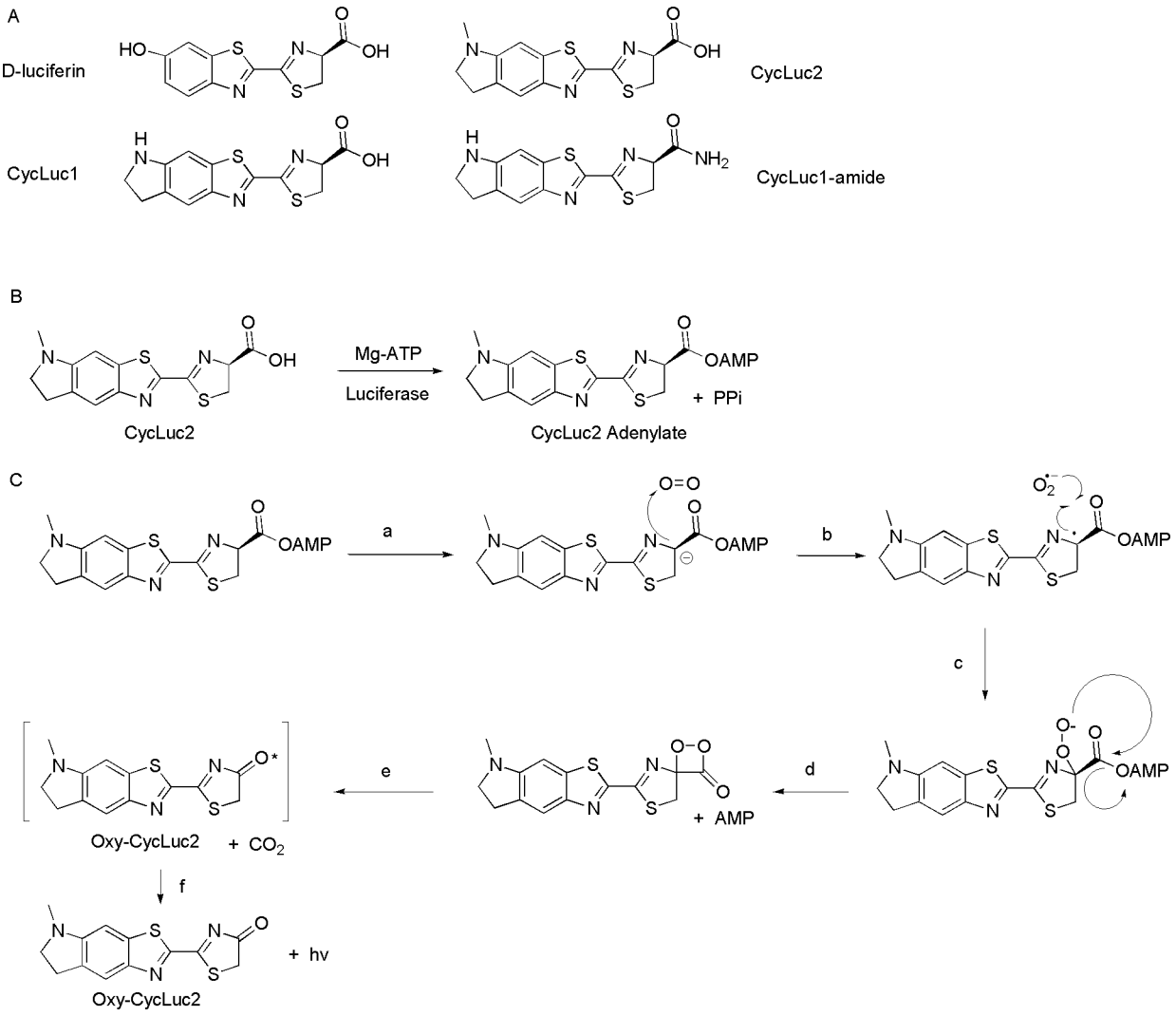
Instruments). After injection, the needle was left in place for one minute before withdrawing. The incision was sutured using 3 simple interrupted stitches of dissolving suture material. The mouse was then treated with 0.05 mg/kg buprenorphine i.p. before returning to its cage, and again every 6 hours for 48 hours post-surgery. The cages remained on a heat pad at 37 degrees Celsius until mice regained righting reflex. Bioluminescence imaging was performed at least 2 weeks after AAV delivery.

Preparation of luciferins for brain imaging

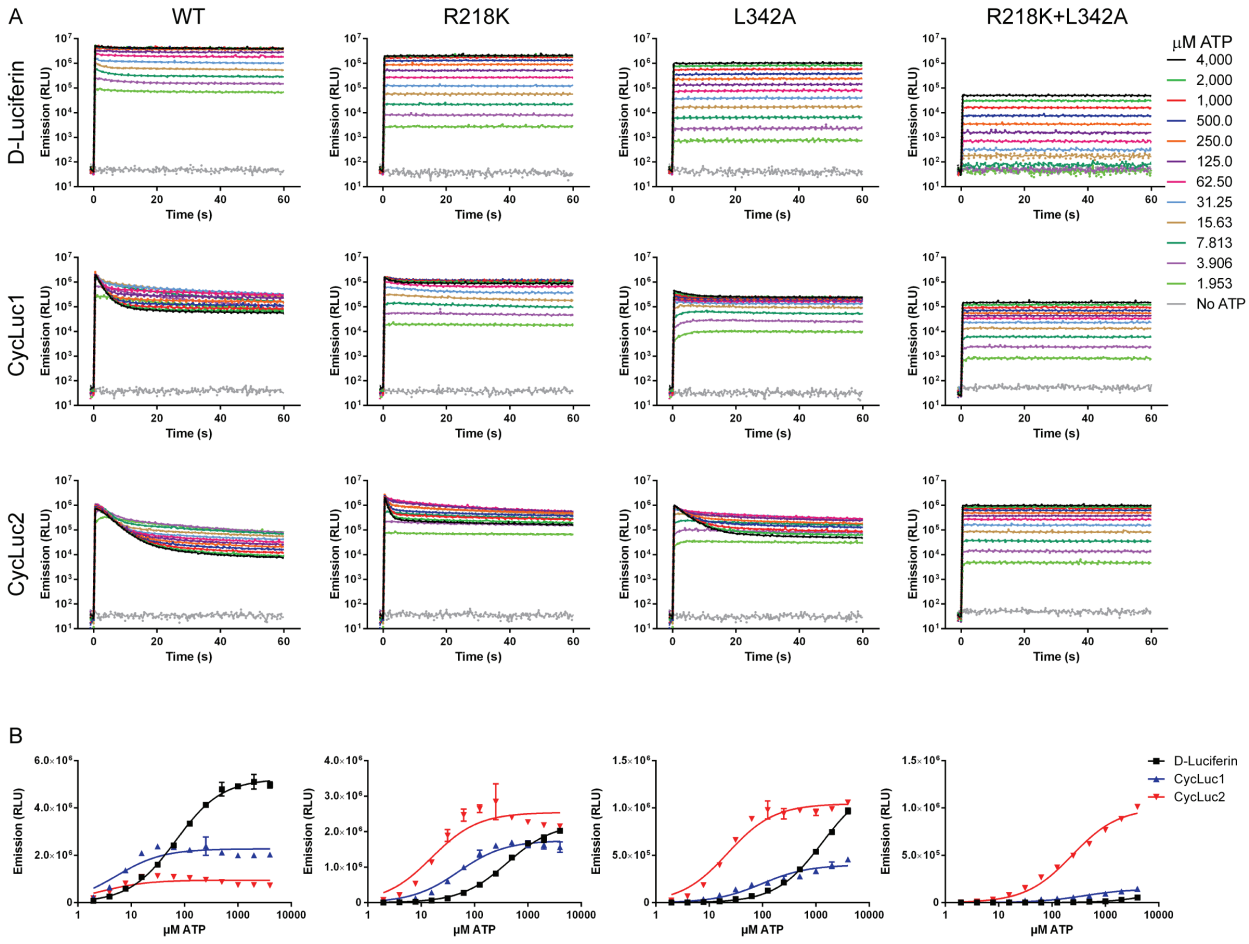
D-luciferin (100 mM), CycLuc1 (5 mM), and CycLuc2 (2.5 mM) were all prepared by dissolving dry compound directly into PBS. The respective amides were prepared by first dissolving dry compound into DMSO to a concentration of 50 mM, followed by dilution in PBS to a final concentration of 250 μM (CycLuc1-amide) or 100 μM (CycLuc2-amide). Once dissolved, all substrate stocks were filtered through a 0.2 μm filter (Millipore) prior to injection into mice.

Brain imaging

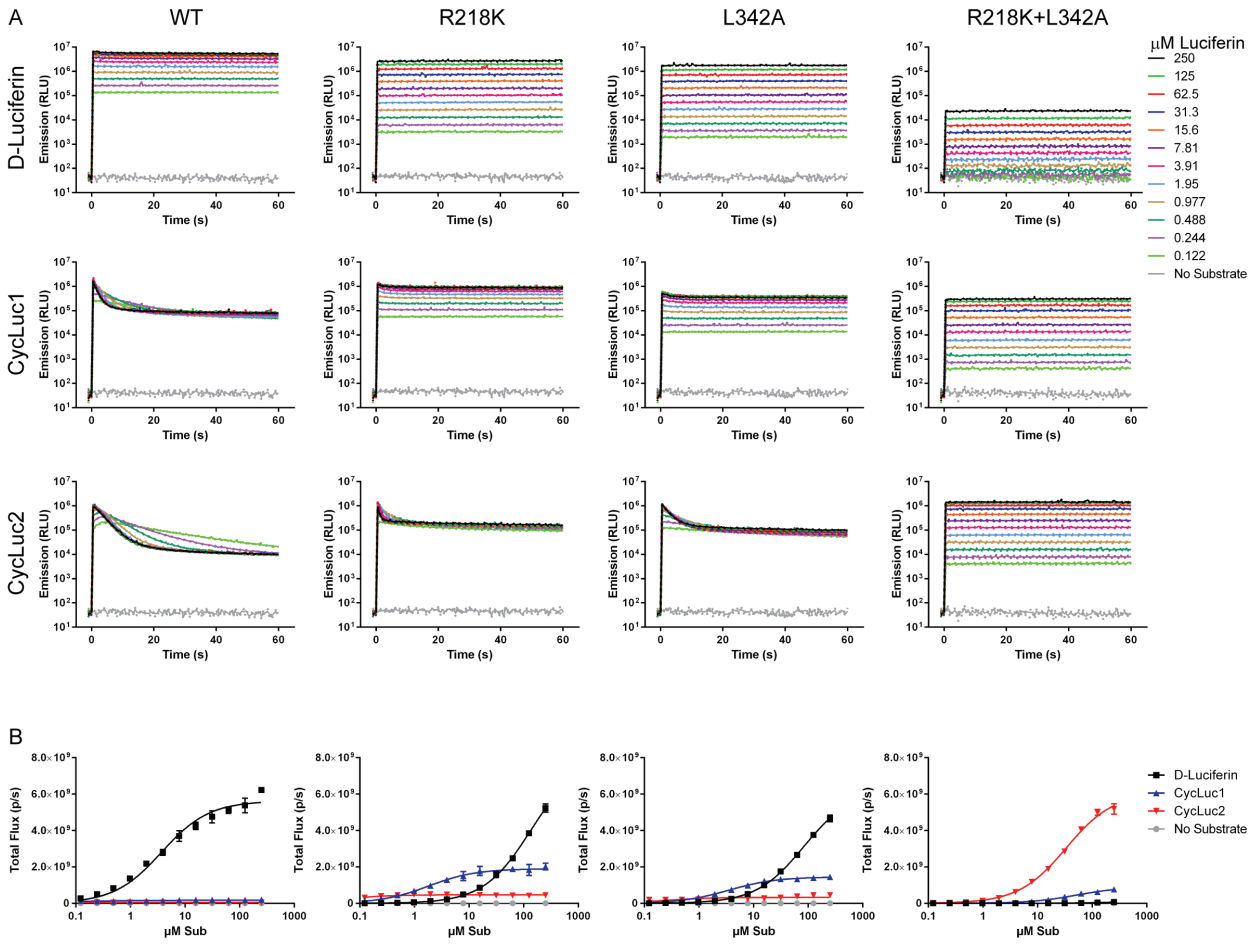
Mice were imaged in the University of Massachusetts Medical School Small Animal Imaging Core using an IVIS 100 imaging system. The animals were anesthetized with isoflurane (2% in 1 L/min oxygen), then injected i.p. with 4 μl of luciferin stock in PBS per gram of body mass. Images were acquired as 60 second exposures at 15 minutes after i.p. injection and analyzed using Living Image software. Regions of interest (ROIs) were drawn around the region covering the brain, and the total flux within each ROI was recorded. ROI sizes were identical across all images. Graphs and statistics were generated using GraphPad Prism 6.



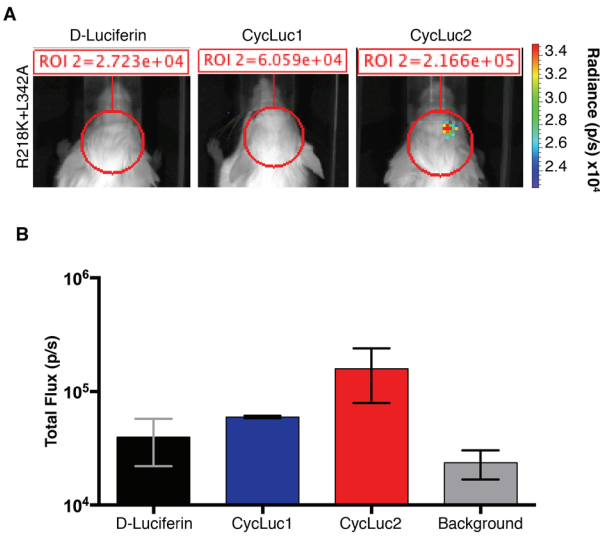
Supplementary Figure 1. Structures of D-luciferin, CycLuc1, CycLuc2, and CycLuc1-amide (A); Proposed mechanism of the luciferase reaction,[6] shown with CycLuc2 in place of D-luciferin: (B) Adenylation of the substrate is the key enzymatic chemistry required for bioluminescence. (C) Subsequent light emission chemistry from the adenylate is largely driven by the chemistry accessible to this intermediate within the enzyme pocket[6]: a) deprotonation of C4; b) single electron transfer to oxygen from the resonance-stabilized C4 anion; c) radical recombination with superoxide; d) displacement of AMP; e) decarboxylation of a dioxetanone; f) photon emission from an electronically-excited oxyluciferin.



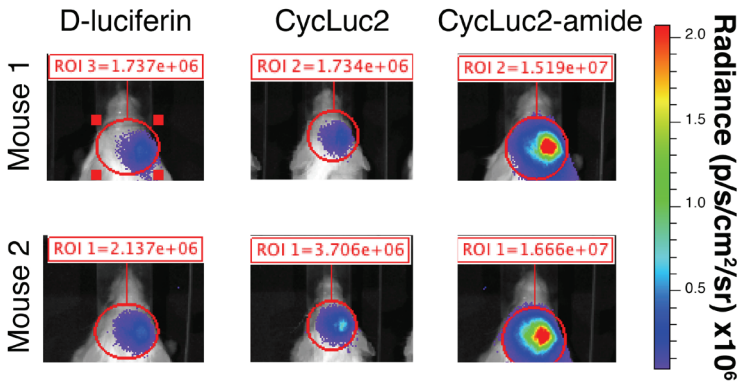
Supplementary Figure 2. Substrate selectivity of the mutant luciferases: role of [ATP]. A) Burst kinetic data for WT and mutant luciferases with D-luciferin, CycLuc1, and CycLuc2 and varying [ATP], measured after rapid injection as described in the Experimental Methods. B) Sigmoidal dose-response fit to peak photon emission (0.5s post-injection) for each luciferase/luciferin as a function of [ATP].



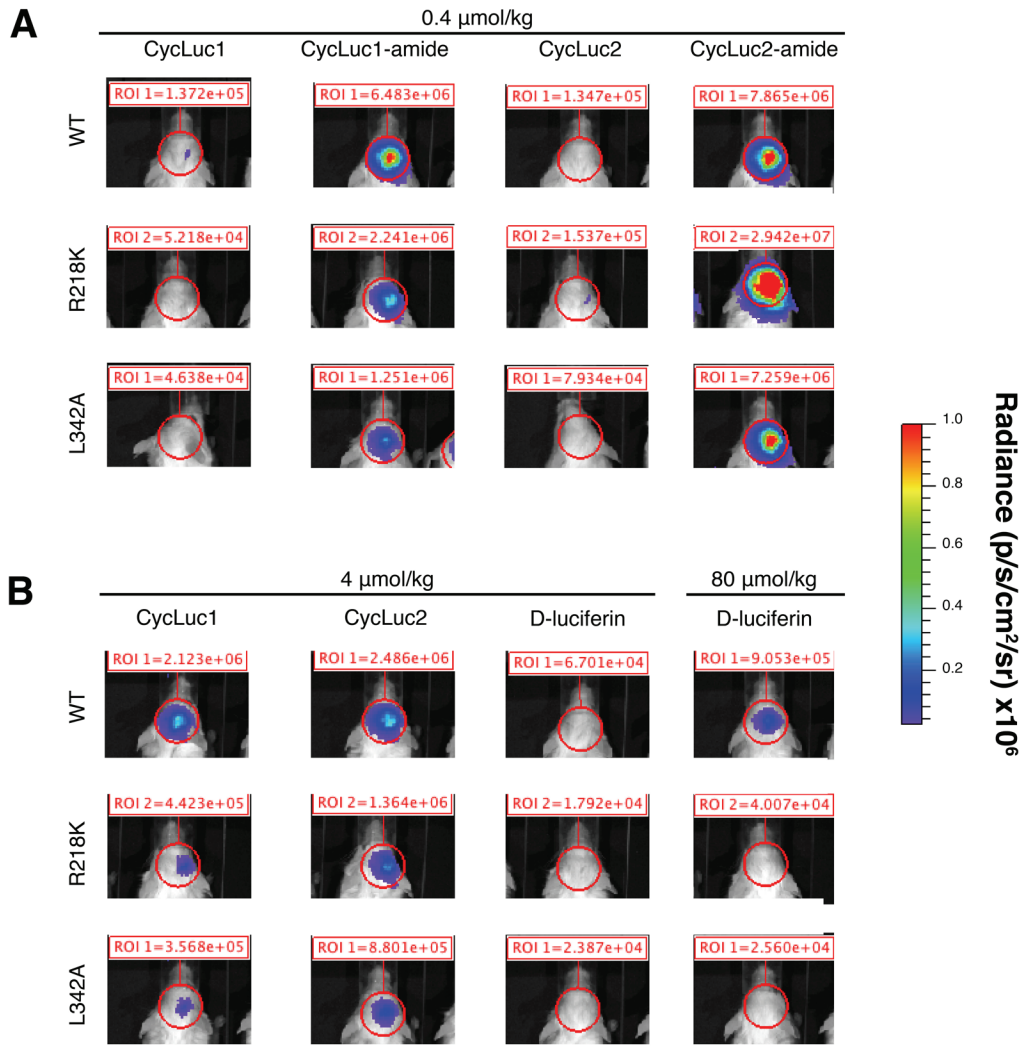
Supplementary Figure 3. Substrate selectivity of the mutant luciferases: role of [luciferin]. A) Burst kinetic data for WT and mutant luciferases with varying concentration of D-luciferin, CycLuc1, and CycLuc2 measured after rapid injection as described in the Experimental Methods. B) Sustained photon emission for each luciferase and luciferin as a function of [luciferin], measured on the IVIS as described in the Experimental Methods.



Supplementary Figure 4. Mouse imaging with R218K/L342A luciferase. A) Photon flux from a single mouse transduced in the brain striatum with AAV9-CMV-luc2(R218K/L342A) after i.p. injection with the indicated luciferin (D-luciferin at 400 µmol/kg, CycLuc1 at 20 µmol/kg, and CycLuc2 at 10 µmol/kg); B) Photon flux from n = 2 mice.



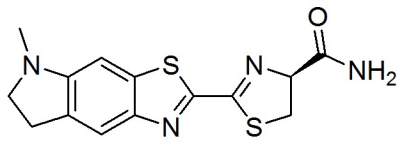
Supplementary Figure 5. Mouse imaging with wild-type (WT) luciferase. Photon flux from mice transduced in the brain striatum with AAV9-CMV-luc2 after i.p. injection with D-luciferin (400 µmol/kg), CycLuc2 (10 µmol/kg), or CycLuc2-amide (0.4 µmol/kg). Each mouse was separately imaged, on sequential days, with all three luciferins.



Supplementary Figure 6. Comparison of luciferin analogs at equimolar dose after i.p. injection with A) CycLuc1, Cycluc1-amide, CycLuc2, and CycLuc2-amide at 0.4 µmol/kg; and B) CycLuc1, CycLuc2, and D-luciferin at 4 µmol/kg. D-luciferin at 80 µmol/kg is also shown for reference. Radiance of all images is represented on the same scale bar to the right.

Supplementary References

(1) Reddy, G. R.; Thompson, W. C.; Miller, S. C. *J. Am. Chem. Soc* **2010**, *132* (39), 13586.
(2) Mofford, D. M.; Adams, S. T.; Reddy, G. S. K. K.; Reddy, G. R.; Miller, S. C. *J. Am. Chem. Soc.* **2015**, *137* (27), 8684.
(3) Harwood, K. R.; Mofford, D. M.; Reddy, G. R.; Miller, S. C. *Chem. Biol.* **2011**, *18* (12), 1649.
(4) Mofford, D. M.; Reddy, G. R.; Miller, S. C. *J. Am. Chem. Soc.* **2014**, *136* (38), 13277.
(5) Evans, M. S.; Chaurette, J. P.; Adams Jr, S. T.; Reddy, G. R.; Paley, M. A.; Aronin, N.; Prescher, J. A.; Miller, S. C. *Nat Methods* **2014**, *11* (4), 393.
(6) Mofford, D. M.; Reddy, G. R.; Miller, S. C. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111* (12), 4443.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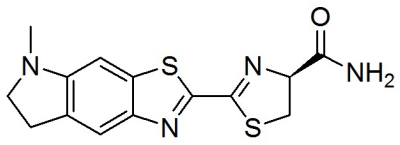
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