Supporting Information

Identification of Factors Complicating Bioluminescence Imaging

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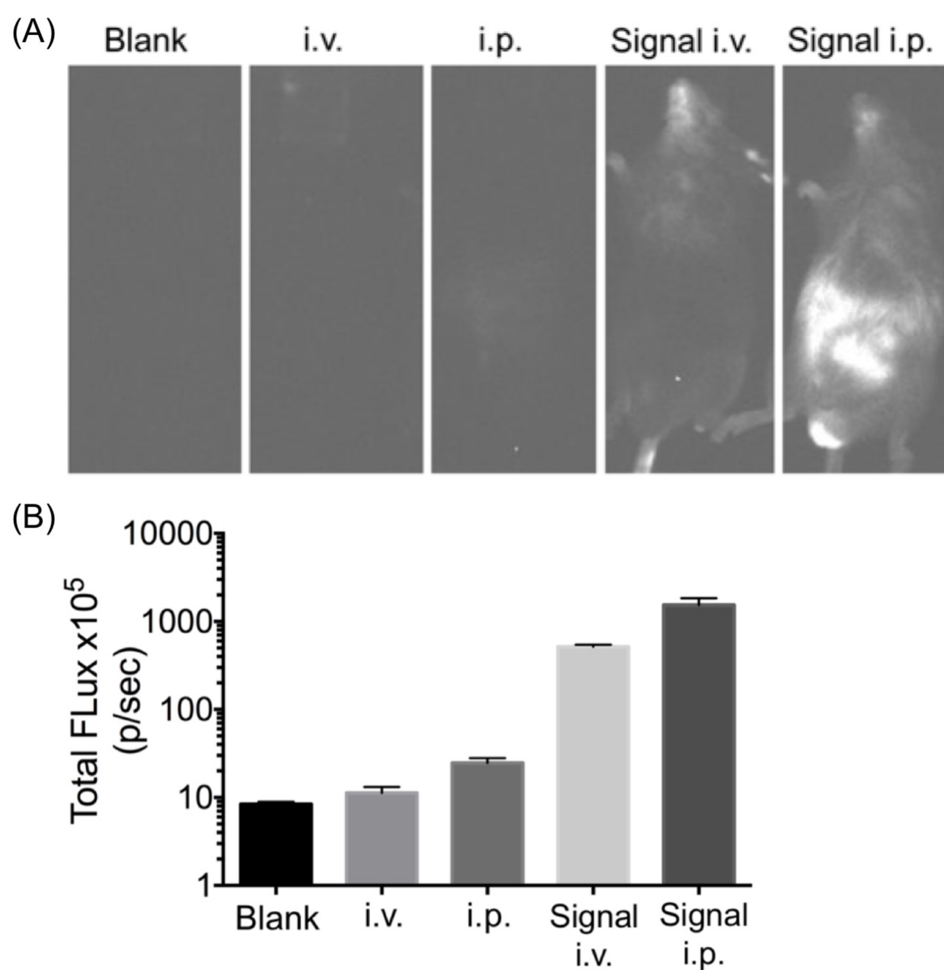

Figure S1. (**A**) Representative bioluminescence images of BALB/c mice (from left to right: blank, i.v. injection of DTZ, i.p. injection of DTZ, and i.v. or i.p. injection of DTZ after i.v. injection of Antares2-expressing HEK 293T cells). These are identical to the images in Figure 3B but are presented in grayscale. (**B**) Quantitative analysis of signals integrated over areas. The y axis is in a logarithmic scale. Data are presented as mean and s.d. of three biological replicates.

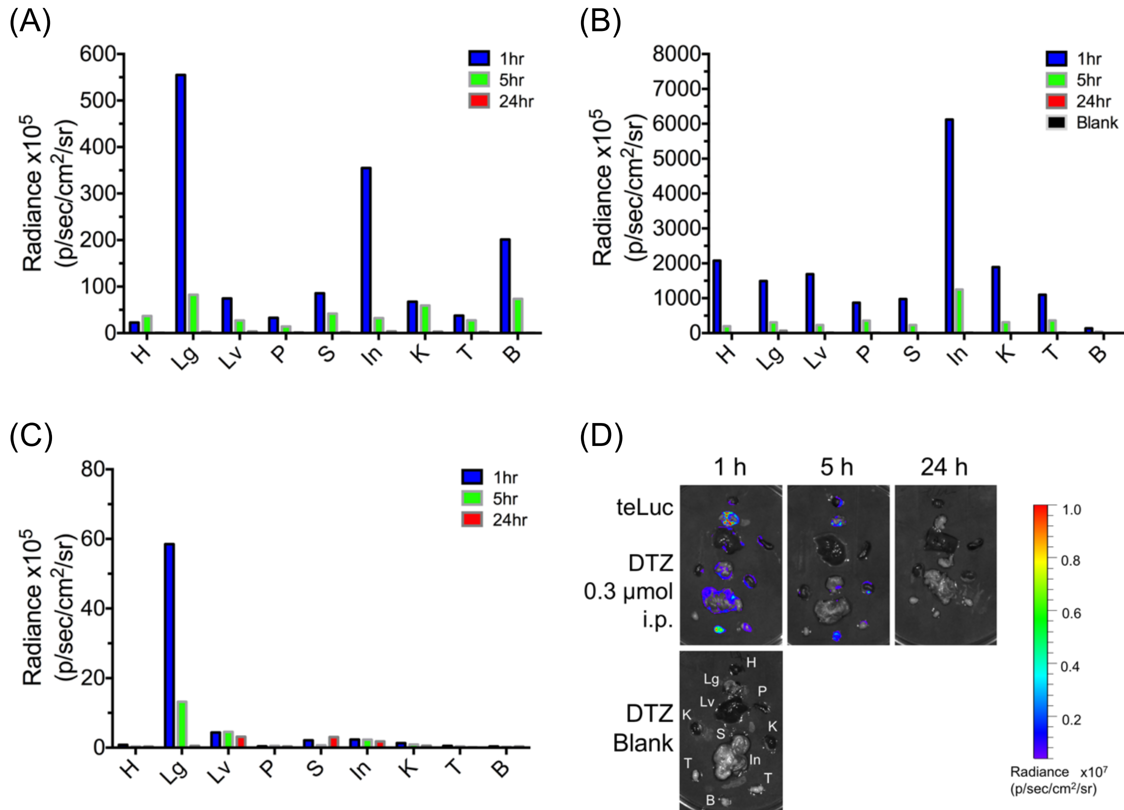

Figure S2. (**A-C**) *Ex vivo* bioluminescence of organs harvested from mice at 1, 5, 24 hours after i.v. injection of HEK293T cells expressing (**A**) teLuc, (**B**) Antares2, or (**C**) Akaluc (n = 1 for each group). Signals were integrated over the area of each organ. (**D**) Bioluminescence images of extracted organs from mice with i.v. injected teLuc-labeled HEK 293T cells (top row) or untransfected HEK 293T cells (bottom row). H: heart, Lg: lung, Lv: liver, P: spleen, S: stomach, In: intestine, K: kidney, T: testis, and B: bladder.

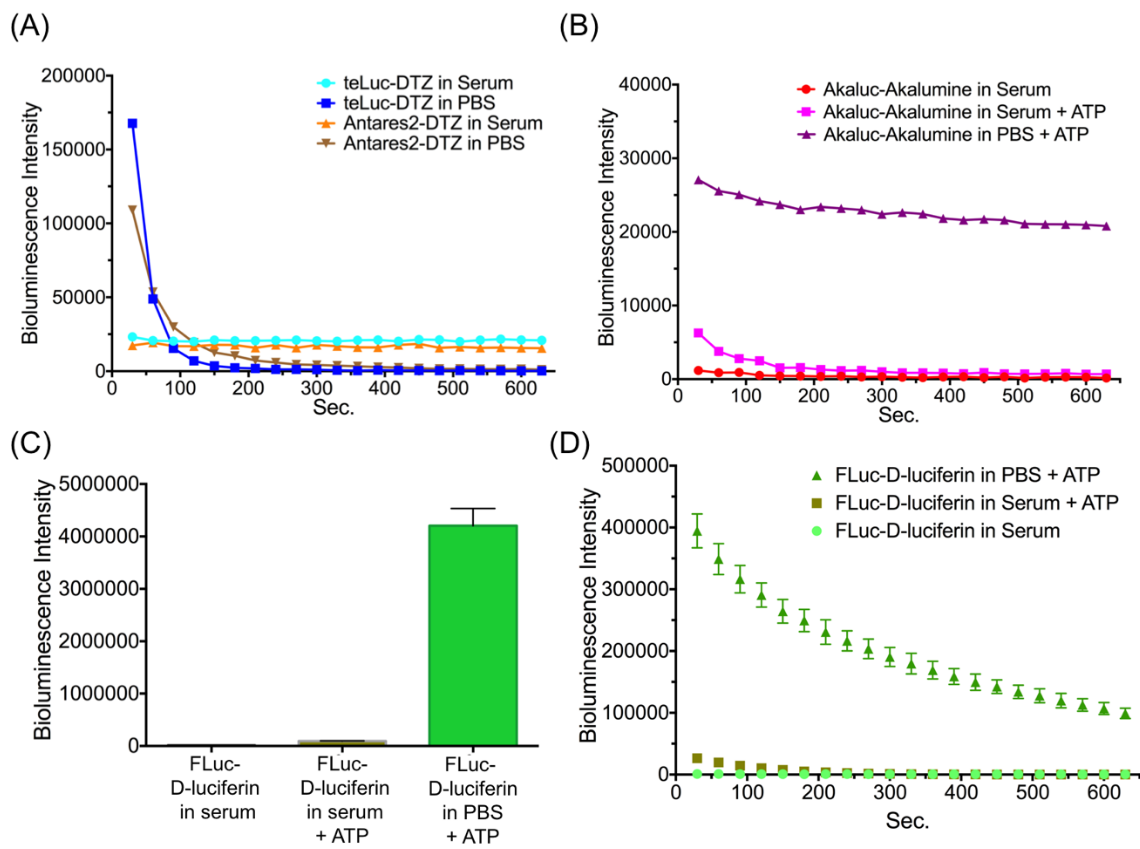

Figure S3. (**A**) Bioluminescence kinetics of purified 1 nM teLuc or Anatres2 in either PBS or mouse serum post injection of 25 µM DTZ. (**B**) Bioluminescence kinetic of purified 1 µM Akaluc in PBS or mouse serum post injection of 100 µM AkaLumine with or without 5 mM ATP. (**CD**) Bioluminescence intensities (**C**) and kinetics (**D**) of purified 1 µM FLuc in either PBS or mouse serum post injection of 100 µM D-luciferin with or without 5 mM ATP.

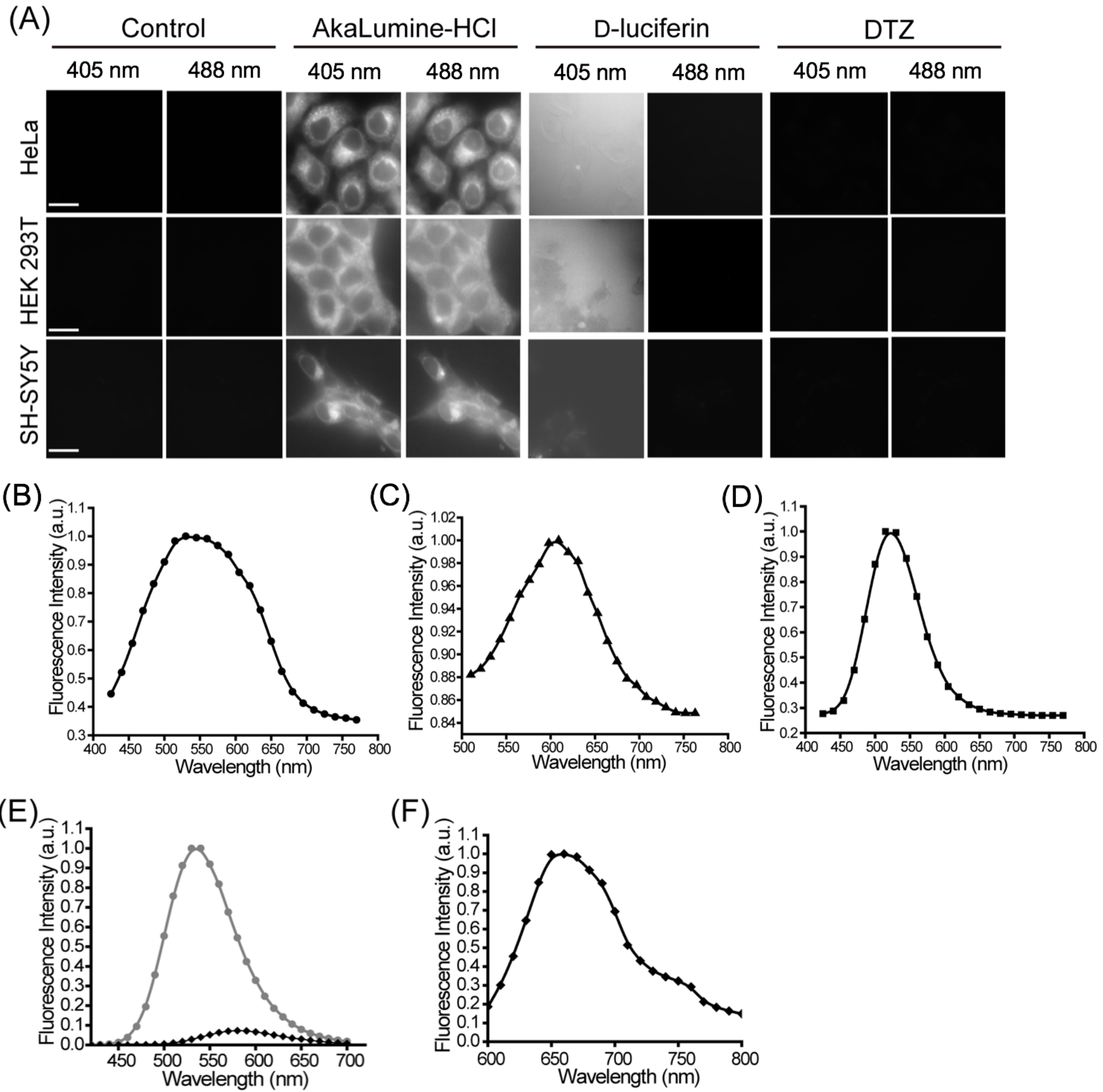

Figure S4. (**A**) Fluorescence imaging of live mammalian cells after incubation with indicated luciferins for 10 min. 100 μM AkaLumine-HCl, 200 μM D-luciferin, or 50 μM DTZ was added to cell lines, including HeLa, HEK 293T, and SH-SY5Y. These concentrations were chosen since each compound has different cell permeability and previous publications used these concentrations for corresponding assays.[1-4] Images were captured with 405-nm or 488-nm laser excitation, and emission was collected from 510 nm to 600 nm (scale bars: 20 μm). (**BC**) Emission spectra of AkaLumine-treated HEK 293T cells with 405 nm (B) or 488 nm (C) laser excitation. (**D**) Emission spectrum of D-luciferin-treated HEK 293T cell with 405-nm laser excitation. (**E**) Fluorescence emission of 1 mM D-luciferin (gray) or AkaLumine (black) in PBS with 400 nm excitation. (**F**) Fluorescence emission of 1 mM AkaLumine (black) in PBS with 580 nm excitation.

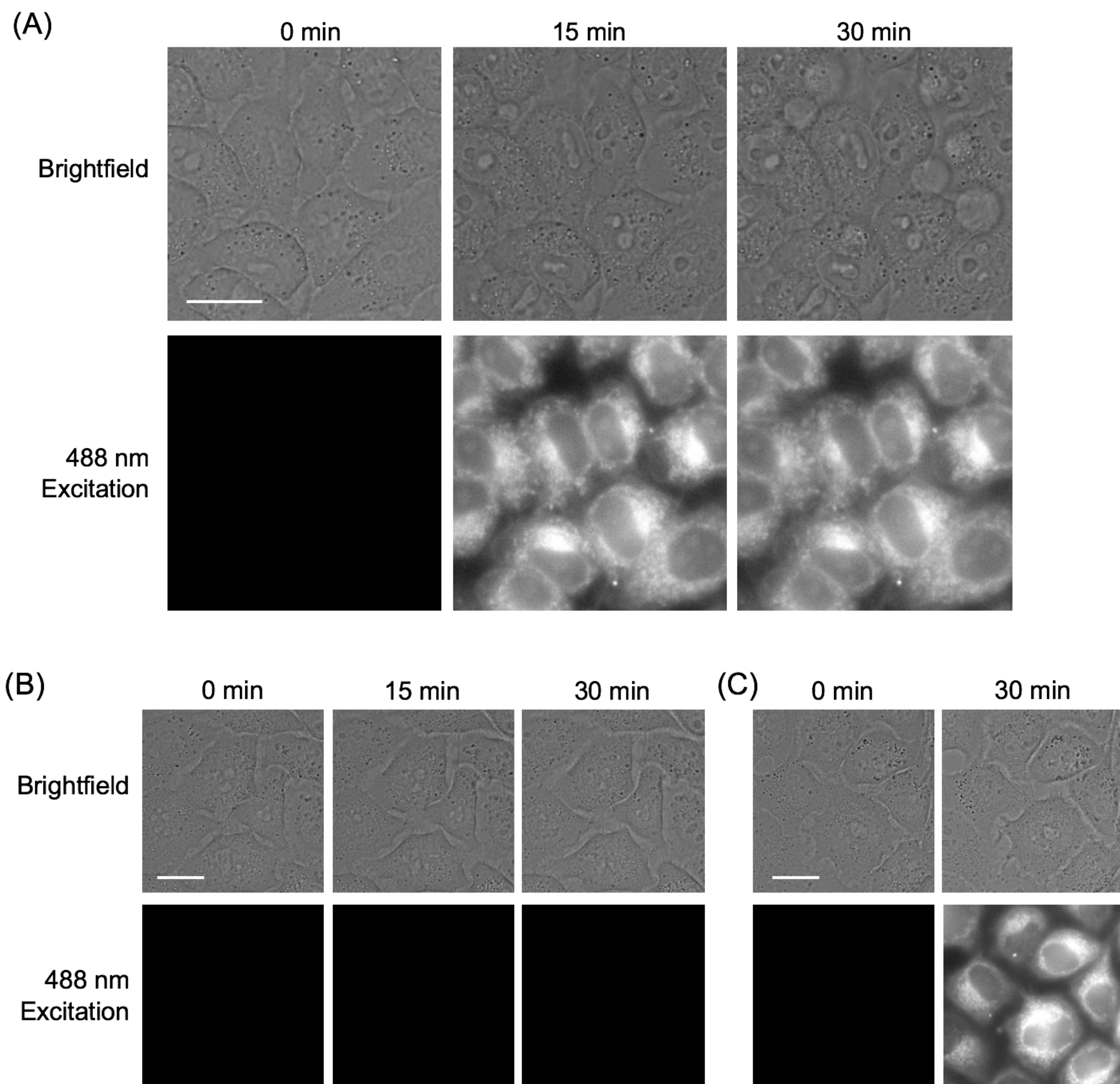

Figure S5. (**A**) Morphological changes of HeLa cells in response to addition of 100 µM AkaLumine at 0 min. Cells were illuminated under a Leica SPE confocal scanning microscope with a 10-mW 488-nm laser (10% output). The scanning line speed was 600 Hz for a 275 µm*275 µm area under a 63x objective. The acquisition interval was 5 s. The blebbing of cell membrane and the formation of extracellular vesicles were observed at 15 and 30 min. (**B**) HeLa cells in the absence of AkaLumine but otherwise treated the same as cells in panel A. (**C**) HeLa cells treated with 100 µM AkaLumine but in the absence of 488-nm excitation. The data suggest that AkaLumine may act as a photosensitizer in stained cells.

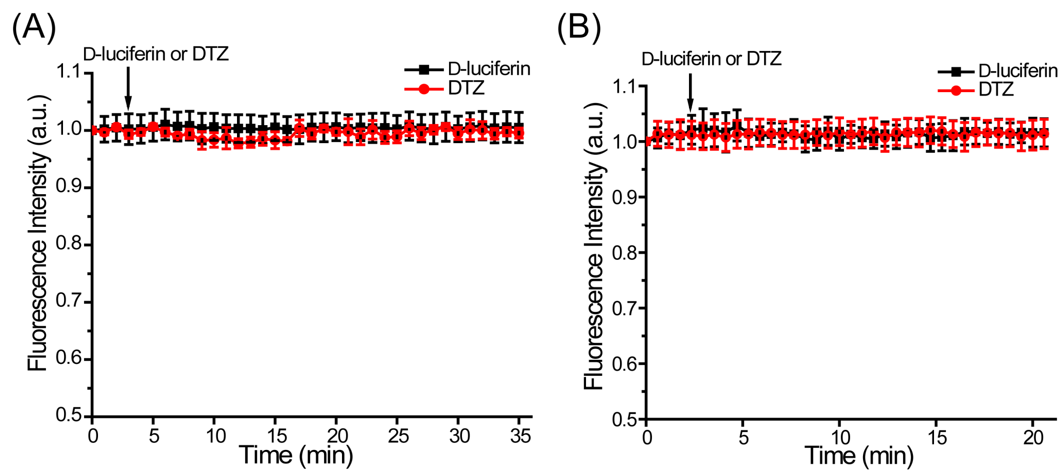

Figure S6. (**A**) PercevalHR fluorescence in HEK 293T upon treatment with D-luciferin or DTZ in the absence of FLuc or teLuc, showing no change in ATP occupancy. (**B**) pHRFP fluorescence in HEK 293T upon treatment with D-luciferin or DTZ in the presence of the corresponding luciferase, showing that the corresponding bioluminescence reactions do not affect intracellular pH.

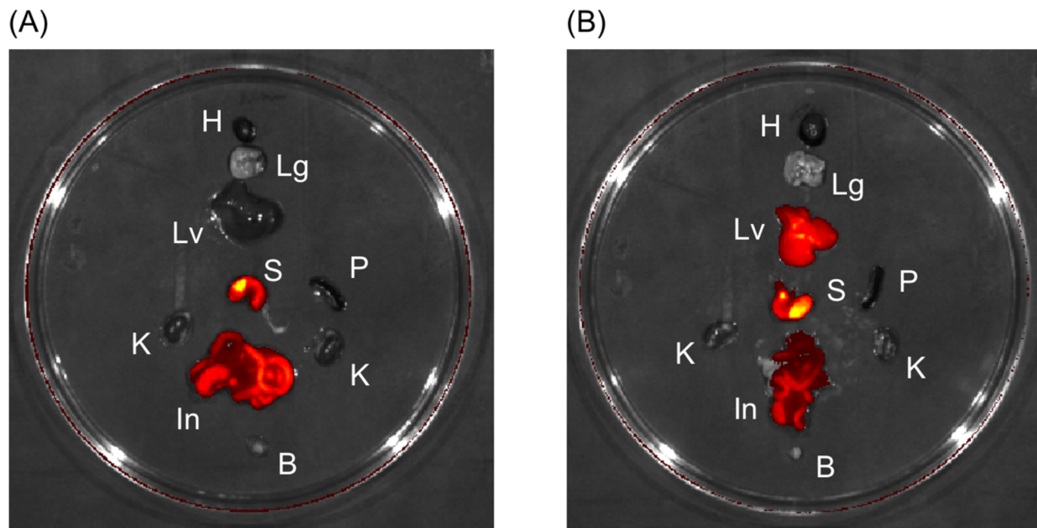

Figure S7. Fluorescence imaging (excitation: 605/25 nm bandpass; emission: 660/25 nm bandpass) of organs harvested from (**A**) an untreated BALB/c mouse, or (**B**) a BALB/c mouse i.v. injected with 1.5 µmol AkaLumine-HCl. Near-infrared (NIR) background fluorescence, mostly in the gastrointestinal tract, was observed for mice, because chlorophyll from plant-based diet (e.g., alfalfa) is fluorescence in this spectral range.[5] In addition to the background, the AkaLumine-injected mouse showed high NIR fluorescence in the liver. H: heart, Lg: lung, Lv: liver, P: spleen, S: stomach, In: intestine, K: kidney, and B: bladder.

References:

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(5) Inoue, Y., Izawa, K., Kiryu, S., Tojo, A., and Ohtomo, K. (2008) Diet and abdominal autofluorescence detected by in vivo fluorescence imaging of living mice, *Mol. Imaging 7*, 21-27.