Figures and figure supplements

Near-infrared dual bioluminescence imaging in mouse models of cancer using infraluciferin

Cassandra L Stowe et al
**Figure 1.** Crystal structure of Firefly luciferase in complex with a iLH$_2$ analogue. (a) Chemical structures of native D-Luciferin (LH$_2$) and the LH$_2$ analogue infraluciferin (iLH$_2$). (b) The crystal structure of Firefly luciferase (FLuc) in complex with the infraluciferyl-adenylate analogue 5'-O-[(N-dehydroinfraluciferyl)-sulfamoyl] adenosine (iDLSA) resolved to a 3.2 Å resolution (PDB ID: 6HPS). (c) The structure of FLuc in complex with the iLH$_2$ analogue iDLSA aligned to the reported structure of FLuc in complex with the LH$_2$ analogue 5'-O-[(N-dehydroluciferyl)-sulfamoyl] adenosine (DLSA) (PDB ID: 4G36) (Sundlov et al., 2012) based on the FLuc N-terminal domain (residues 1–436). The structure of FLuc in complex with iLH$_2$ is shown in red, and the structure of FLuc in complex with LH$_2$ is shown in orange. (d) The structure of FLuc in complex with luciferin analogue DLSA (PDB ID: 4G36) (orange) and the infraluciferin analogue iDLSA (red). Those residues within 4 Å of the substrate in each structure are highlighted in blue. (e) The table lists all residues within 4 Å of the both substrates, with those in orange or red only being found within 4 Å of DLSA and iDLSA respectively. (f) Highlights the same seven active site residues for both the aligned structures, with FLuc iLH$_2$ residues in blue and FLuc LH$_2$ residues in green. All analysis performed in PyMOL software (Schrodinger).

DOI: https://doi.org/10.7554/eLife.45801.003
Figure 1—figure supplement 1. Synthetic Scheme for the synthesis of iDLSA.
DOI: https://doi.org/10.7554/eLife.45801.004
Figure 2. Spectral unmixing of Firefly luciferase mutants in vitro. (a) Bioluminescence spectral unmixing of cells expressing colour-shifted Firefly luciferase (FLuc) mutants, for both in vitro assays and in vivo animal models, requires spectral bioluminescence imaging through a series of bandpass filters. Bioluminescence spectral imaging acquires spectral data, which can then be deconvoluted into its separate components. As depicted, spectral unmixing in vivo is significantly more challenging due to attenuation of bioluminescent signal that does not fall within the bio-optical window. (b) FLuc colour mutants FLuc_green and FLuc_red expressed in the B lymphoma Raji cell line were mixed in various proportions (0–100% of the total population). After addition of either D-luciferin (LH$_2$) or infraluciferin (iLH$_2$) plates were spectrally imaged using the IVIS Spectrum (Perkin Elmer). Unmixed green and unmixed red output images produced from library spectral unmixing using Living Image software (Perkin Elmer) for both substrates. (c) Percentage unmixed signal of FLuc_green and FLuc_red for each ratio of FLuc expressing cells tested when imaged with LH$_2$ and iLH$_2$. Signal adjusted to 100% populations. Mean and standard deviation plotted (n = 6 for both LH$_2$ and iLH$_2$).

DOI: https://doi.org/10.7554/eLife.45801.005
Figure 2—figure supplement 1. Bioluminescence spectra of x11 FLuc mutants FLuc_natural, FLuc_green and FLuc_red with LH2 analogues. (a) LH2, (b) iLH2, (c) CycLuc1 and (d) Aka-Lumine-HCL) in vitro, alongside the chemical structure of CycLuc1 and Aka-Lumine-HCL below the corresponding spectra). Spectra normalised to peak emission for each FLuc mutant with each substrate. Each point on the x-axis gives the midpoint of the 20 nm band pass filter used in spectral imaging.

DOI: https://doi.org/10.7554/eLife.45801.006
Figure 2—figure supplement 2. Spectral unmixing of different levels of expressed Fluc_green and Fluc_red in cells with LH2 and iLH2. The mammalian B Lymphoma Raji cell line was transduced with FLuc_green and FLuc_red, which was then sorted by flow cytometry using co-expressed marker genes to get cell populations with differing expression levels of the two luciferases enzymes. (a) The normalised median fluorescence intensity (MFI) of FLuc_green and FLuc_red for each cellular population (b) Differing combinations of low and high expression levels of FLuc_green and FLuc_red could be determined using spectral bioluminescence imaging and spectral unmixing with both LH2 and iLH2.

DOI: https://doi.org/10.7554/eLife.45801.007
Figure 3. Representative selection of bioluminescent images of Fluc mutants with iLH₂ in vivo. A representative selection of filter images from mice engrafted with the Raji B lymphoma cell line expressing either Fluc_green or Fluc_red for each of the in vivo tumour models when imaged with iLH₂ (a) subcutaneous (b) systemic and (c) intracranial (bandpass filters not shown are 600 nm, 640 nm, 680 nm, 720 nm, 760 nm, 800 nm).
DOI: https://doi.org/10.7554/eLife.45801.008
Figure 3—figure supplement 1. Example of all filter images for FLuc_green and FLuc_red acquired in each in vivo model with iLH₂. DOI: https://doi.org/10.7554/eLife.45801.009
**Figure 3—figure supplement 2.** Corresponding example of all filter images for FLuc_green and FLuc_red acquired in each in vivo model with LH₂. DOI: https://doi.org/10.7554/eLife.45801.010
Figure 4. Spectral characterisation of FLuc mutants with LH₂ and iLH₂ in vivo. The normalised spectra, and a plot of average radiance, for each FLuc mutant when expressed in the Raji B lymphoma cell line engrafted in each tumour model when imaged with LH₂ is shown, (a) subcutaneous (b) systemic and (c) intracranial, and when the same animals were imaged with iLH₂ is shown, (d) subcutaneous (e) systemic and (f) intracranial. Subcutaneous (n = 9), systemic (n = 10), intracranial (n = 7). T test used to establish statistical significance comparing average radiance values (systemic model when imaged with LH₂, p=0.0224). DOI: https://doi.org/10.7554/eLife.45801.011
Figure 5. Spectral unmixing of Firefly luciferase mutants in vivo. The Raji B lymphoma cell line expressing either FLuc_green or Fluc_red, were mixed in various proportions and engrafted in a systemic in vivo model. Cell mixtures ranged from 90:10 to 10:90 Fluc_green to Fluc_red, and each imaging session included one mouse engrafted with each mixture. Animals were spectrally imaged with D-Luciferin (LH$_2$) and infraluciferin (iLH$_2$) using the IVIS spectrum (Perkin Elmer) in separate imaging sessions. (a) An example of the unmixed Fluc_green, unmixed Fluc_red and composite output images when imaged with LH$_2$ and (b) iLH$_2$. Percentage of unmixed Fluc_green and Fluc_red signal for each cell mixture when imaged with (c) LH$_2$ (d) iLH$_2$, and (e) extracted bone marrow samples when analysed by flow cytometry (n = 4 per dilution condition).

DOI: https://doi.org/10.7554/eLife.45801.012
Figure 5—figure supplement 1. Representative flow cytometry plots of extracted bone marrow samples. Representative flow cytometry plots showing gating of extracted bone marrow sample from (a) an FLuc mutant Raji tumour model and from (b) a control mouse. (c) also representative flow cytometry plots from a mouse engrafted with each different mixture of mutant FLuc Raji cells (ratio given is Fluc_green: Fluc_red).

DOI: https://doi.org/10.7554/eLife.45801.013
Figure 6. Dual bioluminescence imaging of CAR T cell therapy using infrafuciferin. Mice were engrafted with the Raji B lymphoma tumour cell line expressing Fluc_green, and were subsequently treated with healthy human donor T cells engineered to express CD19 CAR and FLuc_red (except the tumour only control). Animals were then spectrally imaged after administration of iLH2 using the IVIS spectrum (Perkin Elmer). (a) The unmixed Fluc_green images, representing tumour burden, and unmixed Fluc_red images, representing CAR T cell homing, and the composite image are shown for day six post CAR T cell treatment. (b) The average radiance of signal classified as Fluc_green and Fluc_red is plotted for days 3, 4 and 6 post CAR T cell administration for the tumour only control and treatment (tumour + CAR T cells) animals. Three mice were randomly selected to receive CAR T cell therapy after engraftment was confirmed. Mean and standard deviation plotted. Radiance values from each femur are treated separately (tumour only = 2, treatment = 6).

DOI: https://doi.org/10.7554/eLife.45801.014