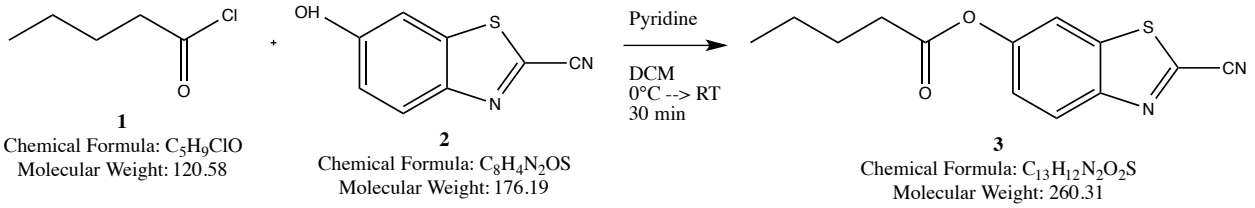
Annex

1.1 Synthesis

1.1.1 Synthesis of 2-cyano-1,3-benzothiazol-6-yl pentanoate (C_5-CBT)



1
Chemical Formula: C_5H_9ClO
Molecular Weight: 120.58

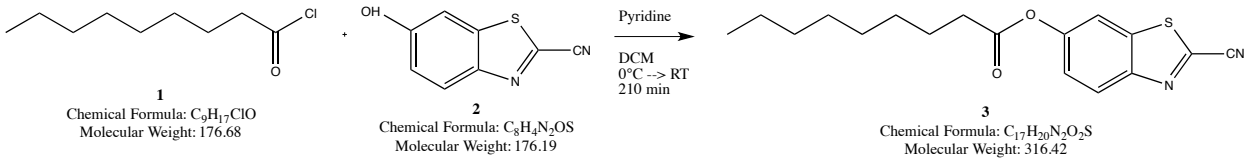
2
Chemical Formula: $C_8H_4N_2OS$
Molecular Weight: 176.19

3
Chemical Formula: $C_{13}H_{12}N_2O_2S$
Molecular Weight: 260.31

Figure 5-0-1 Synthesis of C5-CBT (Toya, Y. et al., Bull. Chem. Soc. Jpn, 65, 2604-2610 (1992))

To a 25 mL one neck round bottom flask 6-hydroxy-2-cyanobenzothiazole (hydroxy-CBT) (367 mg, 2.08 mmol) was added and suspended in 10 mL dichloromethane (DCM) under a nitrogen atmosphere and stirred at room temperature. Pyridine (350 µL, 342 mg, 4.32 mmol) was added to the flask and hydroxy-CBT dissolved to form a clear yellow solution. The flask was cooled in an ice bath and valeroyl chloride (348 µL, 352 mg, 2.92 mmol) was added dropwise to the reaction vessel. Upon addition of the valeroyl chloride, the yellow color of the solution disappears and a white slurry is formed, which progressively turns yellow. After 30 min the reaction was stopped and 8 mL $NaHCO_3$ was added. Extraction was performed in a separation funnel by washing with 10 mL DCM two times. Organic layer at the bottom of the separation funnel was collected and evaporated under reduced pressure on the rotary evaporator. The obtained material was stored in the freezer. The product was purified on a silica column using DCM only as eluant giving 30 fractions. Corresponding fractions were collected and the solvents were evaporated on the rotary evaporator to obtain 2-cyano-1,3-benzothiazol-6-yl pentanoate (C_5-CBT) as a white solid (456 mg, 1.75 mmol, 84% yield).

1.1.2 Synthesis of 2-cyano-1,3-benzothiazol-6-yl nonanoate (C_9-CBT)



1
Chemical Formula: $C_9H_{17}ClO$
Molecular Weight: 176.68

2
Chemical Formula: $C_8H_4N_2OS$
Molecular Weight: 176.19

3
Chemical Formula: $C_{17}H_{20}N_2O_2S$
Molecular Weight: 316.42

Figure 5-0-2 Synthesis of C9-CBT (Toya, Y. et al., Bull. Chem. Soc. Jpn, 65, 2604-2610 (1992))

To a 25 mL one neck round bottom flask 6-hydroxy-2-cyanobenzothiazole (hydroxy-CBT) (361 mg, 2.05 mmol) was added and suspended in 10 mL dichloromethane (DCM) under a nitrogen atmosphere and stirred at room temperature. Pyridine (330 µL, 324 mg, 4.1 mmol) was added to the flask and hydroxy-CBT dissolved with some small remaining precipitate. The flask was cooled in an ice bath and nonanoyl chloride (444 µL, 435 mg, 2.46 mmol) was added dropwise to the reaction vessel. Upon addition of the nonanoyl chloride, the solution becomes transparent. After 2 h, thin Layer Chromatography (TLC) indicated that some hydroxy-CBT was still present. An additional amount of 100 µL was added to the reaction vessel. After 1 h (3 h total) the reaction was complete and 8 mL $NaHCO_3$ was added. Extraction was performed in a separation funnel by washing with 10 mL DCM two times. Organic layer at the bottom of the separation funnel was collected and evaporated under reduced pressure on the rotary evaporator. The product was purified on a short silica column using DCM only as eluant. HPLC and Nuclear Magnetic Resonance (NMR) showed that the obtained product presented some impurities. The product was purified again on a silica column using petrol ether:ethyl acetate 29:1 mixture as eluant. Fractions were collected and evaporated on the rotary evaporator to

obtain 2-cyano-1,3-benzothiazol-6-yl nonanoate (C_9-CBT) as a white solid(330 mg, 1.04 mmol, 51% yield). Other fractions were also collected and evaporated on the rotary evaporator to give an additional amount of 90 mg of solid containing some impurities.

1.1.3 Synthesis of 2-[6-(pentanoyloxy)-1,3-benzothiazol-2-yl]-1,3-thiazole-4-carboxylic acid (C_5-Luciferin)

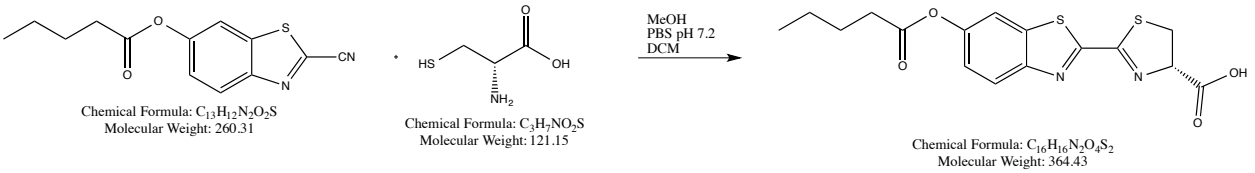


Figure 5-0-3 Synthesis of C5-Luciferin
(White, E. H., McCapra, F., and Field, G. F. (1963) The structure and synthesis of firefly luciferin. *J. Am. Chem. Soc.* 85, 337–343.)

To a 50 mL one neck round bottom flask 2-cyano-1,3-benzothiazol-6-yl pentanoate (C_5-CBT) (102 mg, 0.39 mmol) was added and dissolved in 0.4 mL DCM under a nitrogen atmosphere and stirred at room temperature. 2 mL of dry MeOH were added to the flask. D-cysteine (57 mg, 0.47 mmol) was dissolved in 2 mL PBS pH 7,2 and added to the reaction vessel. The solution became white upon addition of the D-cysteine and quickly turned transparent. 2 mL additional dry MeOH were added to help the aqueous and organic phases to mix. The solution turned transparent navy blue. After 30 min, the reaction was stopped and the solvents evaporated. The product was redissolved in 10 mL DCM. Extraction was performed in a separation funnel by adding 10 mL HCL 0,1 M and washing the water phase three times with 10 mL DCM. Organic layer at the bottom of the separation funnel was collected, dried over $MgSO_4$, filtrated and evaporated under reduced pressure to obtain 2-[6-(pentanoyloxy)-1,3-benzothiazol-2-yl]-1,3-thiazole-4-carboxylic acid (C_5-Luciferin) as a pink/orange solid (118 mg, 0.32 mmol, 83% yield).

1.1.4 Synthesis of methyl 2-[6-(pentanoyloxy)-1,3-benzothiazol-2-yl]-1,3-thiazole-4-carboxylate (C_5-Luciferin methyl ester)

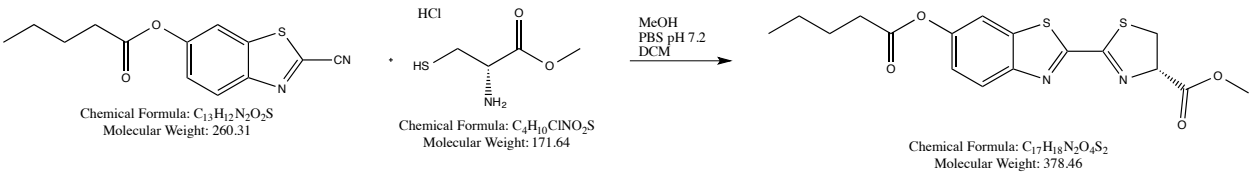


Figure 5-0-4 Synthesis of C5-Luciferin methyl ester
(White, E. H., McCapra, F., and Field, G. F. (1963) The structure and synthesis of firefly luciferin. *J. Am. Chem. Soc.* 85, 337–343.)

To a 50 mL one neck round bottom flask 2-cyano-1,3-benzothiazol-6-yl pentanoate (C_5-CBT) (107 mg, 0.41 mmol) was added and dissolved in 0.4 mL DCM under a nitrogen atmosphere and stirred at room temperature. D-cysteine methyl ester hydrochloride salt (86 mg, 0.49 mmol) was dissolved in 0.8 mL PBS pH 7,2 and 2 mL MeOH, and was added to the reaction vessel. The solution became white upon addition of the D-cysteine and some DCM remained in a separate phase. 2 mL additional dry MeOH were added to help the aqueous and organic phases to mix. After 1 h, the reaction was stopped and the solvents evaporated. The product was redissolved in 10 mL DCM. Extraction was performed in a separation funnel by adding 10 mL HCL 0,1 M and washing the water phase three times with 10 mL DCM. Organic layer at the bottom of the separation funnel was collected, dried over $MgSO_4$, filtrated and evaporated under reduced pressure. NMR showed that some C_5-CBT was still present. The reaction was performed using the previously obtained solid reacted with 70 mg D-cysteine methyl ester following the reaction and extraction

procedure described above to obtain 2-[6-(pentanoyloxy)-1,3-benzothiazol-2-yl]-1,3-thiazole-4-carboxylate (C_5-Luciferin methyl ester) as an orange/brown solid (115 mg, 0.30 mmol, 74% yield). NMR showed that some C_5-CBT traces were still present.

1.1.5 Synthesis of 2-[6-(nonanoyloxy)-1,3-benzothiazol-2-yl]-1,3-thiazole-4-carboxylic acid (C_9-Luciferin)

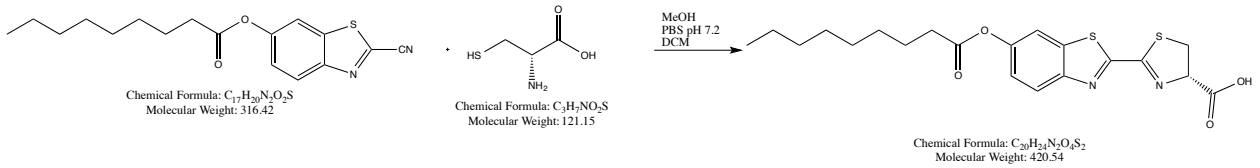


Figure 5-0-5 Synthesis of C9-Luciferin

(White, E. H., McCapra, F., and Field, G. F. (1963) The structure and synthesis of firefly luciferin. *J. Am. Chem. Soc.* 85, 337–343.)

To a 50 mL one neck round bottom flask 2-cyano-1,3-benzothiazol-6-yl nonanoate (C_9-CBT) (80 mg, 0.25 mmol) was added and dissolved in 0.4 mL DCM under a nitrogen atmosphere and stirred at room temperature. D-cysteine (39 mg, 0.32 mmol) was dissolved in 1.6 mL PBS pH 7,2 and 4 mL of dry MeOH, and added to the reaction vessel. The solution became clear white upon addition of the D-cysteine and some precipitation occurred after some time. After 40 min, the reaction was stopped and the solvents evaporated. The product was redissolved in 10 mL DCM. Extraction was performed in a separation funnel by adding 10 mL HCL 0,1 M and washing the water phase three times with 10 mL DCM. Organic layer at the bottom of the separation funnel was collected, dried over $MgSO_4$, filtrated and evaporated under reduced pressure on the rotary evaporator to obtain 2-[6-(nonanoyloxy)-1,3-benzothiazol-2-yl]-1,3-thiazole-4-carboxylic acid (C_9-Luciferin) as a pale yellow solid (95 mg, 0.23 mmol, 90% yield).

1.1.6 Synthesis of methyl 2-[6-(nonanoyloxy)-1,3-benzothiazol-2-yl]-1,3-thiazole-4-carboxylate (C_9-Luciferin methyl ester)

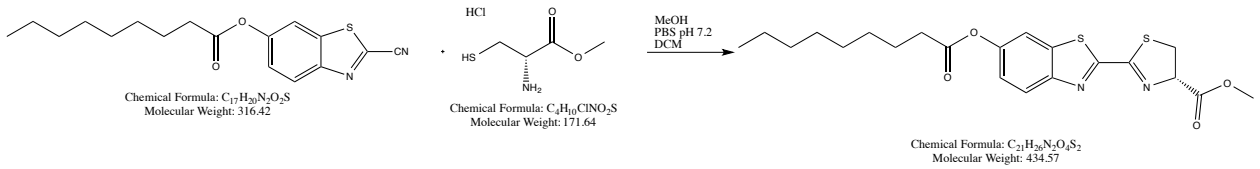


Figure 5-0-6 Synthesis of C9-Luciferin

(White, E. H., McCapra, F., and Field, G. F. (1963) The structure and synthesis of firefly luciferin. *J. Am. Chem. Soc.* 85, 337–343.)

To a 50 mL one neck round bottom flask 2-cyano-1,3-benzothiazol-6-yl nonanoate (C_9-CBT) (80 mg, 0.25 mmol) was added and dissolved in 0.4 mL DCM under a nitrogen atmosphere and stirred at room temperature. D-cysteine methyl ester hydrochloride salt (51 mg, 0.3 mmol) was dissolved in 0.8 mL PBS pH 7,2 and 4 mL MeOH, and was added to the reaction vessel. The solution is clear upon addition but rapidly forms some precipitate. After 1.5 h, the reaction was stopped and the solvents evaporated. The product was redissolved in 10 mL DCM. Extraction was performed in a separation funnel by adding 10 mL HCL 0,1 M and washing the water phase three times with 10 mL DCM. Organic layer at the bottom of the separation funnel was collected, dried over $MgSO_4$, filtrated and evaporated under reduced pressure. NMR showed that some C_9-CBT was still present. The reaction was performed again using the previously obtained solid reacted with 44 mg D-cysteine methyl ester following the reaction and extraction procedure described above to obtain methyl 2-[6-(nonanoyloxy)-1,3-benzothiazol-2-yl]-1,3-thiazole-4-carboxylate (C_9-Luciferin methyl ester) as an orange/brown solid (76 mg, 0.17 mmol, 69% yield). NMR showed that some C_9-CBT traces were still present.

1.2 Formulation for *in vivo* experiments

In order to use the prepared compounds for *in vivo* experiments, the probes must be dissolved in a formulation compatible with the existing animal protocol to minimize risks for the animals used in the experiments. The desired concentration is $2{,}68*10^{-2}$ M for luciferin in 20 µL DMSO or the equivalent amount.

1.2.1 Solubility in DMSO

A quantity of 1,24 mg (3,92 µmol) of C9-CBT, our most hydrophobic compound, was dissolved in 50 µL DMSO. Next, the product was dissolved in DMSO to a concentration a hundred times higher than the final concentration of $2{,}68*10^{-2}$ M needed for *in vivo* experiments. The product was not soluble at this concentration. Solubilization was obtained after adding 5 µL of DMSO to give a solution 1,79 M or 565 mg/mL. Thus, we observe that the solubility for our most lipophilic compound is sufficient in DMSO for our experiments.

	m C9-CBT (mg)	V DMSO (µL)	Final [] (mol L^{-1})	Soluble	After 2' at 6000 rpm
1	1,24	50	$7{,}8*10^{-2}$	YES	YES
2	8,48	10	2,68	NO	-
3	8,48	15	1.79	YES	YES

Table 0-1 Solubility of C9-CBT in DMSO

1.2.2 Solubility in DMSO/PBS

Attempts to obtain solutions of the probe in 100 µL PBS at the desired concentration of $2{,}68*10^{-2}$ M, using different percentages of DMSO were performed. Using DMSO in 24% or 48% percentage, the formation of a cloudy suspension was noted and it sedimented after centrifugation. The probe was not soluble in PBS even at a percentage of DMSO as high as 48%, already too high to be used for i.v. injection in mice.

	[] in DMSO (mol L^{-1})	V DMSO (µL)	V PBS (µL)	% DMSO	Final [] (mol L^{-1})	Soluble	After 2' at 6000 rpm
1	1,79	1,5	98,5	1,5	$2{,}68*10^{-2}$	NO	-
2	$9*10^{-1}$	3	97	3	$2{,}68*10^{-2}$	NO	-
3	$4{,}5*10^{-1}$	6	94	6	$2{,}68*10^{-2}$	NO	-
4	$2{,}2*10^{-1}$	12	88	12	$2{,}68*10^{-2}$	NO	-
5	$1{,}1*10^{-1}$	24	76	24	$2{,}68*10^{-2}$	Suspension	NO

6	$5{,}6*10^{-2}$	24	26	48	$2{,}68*10^{-2}$	Suspension	NO

Table 0-2 Solubility of C9-CBT in DMSO/PBS

1.2.3 Solubility in PEG, PEG/PBS and PEG/PBS/Tween20

10.85 mg of C9-CBT were dissolved in 255 µL PEG400 to obtain a 1,34 M stock solution. The probe was soluble in PEG400, but it was not possible to mix it with PBS or PBS/Tween20. Unfortunately, PEG400 alone is not suitable for injection in mice, and it would probably result in a precipitate immediately after injection.

	[] in PEG400 (mol L^{-1})	V PEG400 (µL)	V PBS (µL) [a]1%Tween20	% PEG400	Final [] (mol L^{-1})	Soluble	After 2' at 6000 rpm
Stock	$1{,}34*10^{-1}$	100	0	100	1,34	YES	YES
1.1	$2{,}68*10^{-2}$	100	0	100	$2{,}68*10^{-2}$	YES	YES
1.2	$5{,}36*10^{-2}$	50	50	50	$2{,}68*10^{-2}$	Suspension	NO
1.3	$1{,}34*10^{-1}$	20	80	20	$2{,}68*10^{-2}$	NO	-
2.1	$1.34*10^{-2}$	100	0	100	$1.34*10^{-2}$	YES	YES
2.2	$2{,}68*10^{-2}$	50	50	50	$1.34*10^{-2}$	Suspension	NO
2.3	$6{,}7*10^{-2}$	20	80	20	$1.34*10^{-2}$	Suspension	NO
3.1	$8{,}93*10^{-3}$	100	0	100	$8{,}93*10^{-3}$	YES	YES
3.2	$1{,}79*10^{-2}$	50	50	50	$8{,}93*10^{-3}$	Suspension	NO
3.3	$4.46*10^{-2}$	20	80	20	$8{,}93*10^{-3}$	Suspension	NO
4.1	$1{,}79*10^{-2}$	50	50[a]	50	$8{,}93*10^{-3}$	Suspension	NO
4.2	$1{,}79*10^{-2}$	50	150[a]	25	$8{,}93*10^{-3}$	Suspension	NO

Table 0-3 Solubility of C9-CBT in PEG, PEG/PBS and PEG/PBS/Tween20

1.2.4 Solubility in BSA

2,54 mg of C9-CBT were dissolved in 299 µL PBS/0,1%BSA to obtain a $2{,}68*10^{-2}$ M solution. The solution was then diluted with an additional volume of 299 µL PBS/0,1%BSA to obtain a $1.34*10^{-2}$ M solution. A third formulation was prepared by weighing 1,23 mg of C9-CBT and dissolving it in 435 µL of PBS/0,1%BSA. It was not possible to dissolve C9-CBT in PBS/0,1%BSA.

	m C9-CBT (mg)	V PBS/0.1%BSA (µL)	Final [] (mol L^{-1})	Soluble	After 2' at 6000 rpm

1	2,54	299	$2,68*10^{-2}$	NO	-
2	2,54	598	$1.34*10^{-2}$	Suspension	NO
2	1,23	435	$8,93*10^{-3}$	Suspension	NO

Table 0-4 Solubility of C9-CBT in BSA

1.2.5 Solubility in DMSO/PBS/Tween20 and DMSO/PBS/BSA

2,93 mg of C9-CBT were dissolved in 13,84 µL DMSO to obtain a $6,7 *10^{-1}$ M stock solution. It was not possible to dissolve C9-CBT in both systems.

	[] in DMSO (mol L^{-1})	V DMSO (µL)	V PBS (µL) [a] 0,1% BSA [b] 1% tween20	% DMSO	Final [] (mol L^{-1})	Soluble	After 2' at 6000 rpm
1	$6,7*10^{-1}$	5	5[a]	50	$3,35*10^{-1}$	NO	-
2	$6,7*10^{-1}$	5	5[b]	50	$2,68*10^{-2}$	NO	-

Table 0-5 Solubility of C9-CBT in DMSO/PBS/Tween20 and DMSO/PBS/BSA

1.2.6 Solubility in 1,3-propanediol

3,46 mg of C9-CBT were dissolved in 81,6 µL 1,3-propanediol to obtain a $1,34*10^{-1}$ M stock solution. It was not possible to dissolve C9-CBT in 1,3-propanediol.

	m C9-CBT (mg)	V 1,3-propanediol (µL)	Final [] (mol L^{-1})	Soluble	After 2' at 6000 rpm
1	3,46	81,6	$1,34*10^{-1}$	NO	-
2	-	Diluted 2x	$6,7*10^{-2}$	NO	-
2	-	Diluted 3x	$4,46*10^{-2}$	NO	-

Table 0-6 Solubility of C9-CBT in 1,3-propanediol

1.2.7 Solubility of C9-luciferin

Based on the previous results for C9-CBT and the impossibility to find a suitable formulation for intravenous injection of the CBT probes, solubilizing the C9-luciferin probe was performed in PBS/0,1%BSA and PEG:PG 1:1 formulations, compatible with tail vein injection.

2,69 mg of C9-luciferin were dissolved in 23,91 µL DMSO to obtain a $2{,}68*10^{-1}$ M stock solution. The obtained solution was diluted 100 times in either PBS/0,1%BSA or PEG:PG 1:1. It was possible to dissolve it in PEG:PG 1:1.

	[] in DMSO (mol L^{-1})	V DMSO (µL)	V (µL) [a] PBS/0,1% BSA [b] PEG:PG 1:1	% DMSO	Final [] (mol L^{-1})	Soluble	After 2' at 6000 rpm
1	$2{,}68*10^{-1}$	3	300[a]	1	$2{,}68*10^{-3}$	NO	-
2	$2{,}68*10^{-1}$	3	300[b]	1	$2{,}68*10^{-3}$	YES	YES
3	$2{,}68*10^{-1}$	3	150 [b]	2	$5{,}36*10^{-3}$	YES	YES

	[] in DMSO/PEG/PG (mol L^{-1})	V DMSO/PEG/PG (µL)	V PBS (µL)	% PEG:PG 1:1	Final [] (mol L^{-1})	Soluble	After 2' at 6000 rpm
4	$2{,}68*10^{-3}$	100	100	50	$1{,}79*10^{-3}$	YES	YES
5	$5{,}36*10^{-3}$	150	450	25	$8{,}95*10^{-4}$	Suspension	NO

Table 0-7 Solubility of C9-luciferin

100 µL of solution 2 obtained was added in 100 µL PBS and it was soluble. We tried to add 150 µL of solution 3 in 450 µL PBS, but it was not soluble. We selected formulation 4 that uses 1% DMSO in 100 µL PEG:PG 1:1 mixed with 100 µL PBS for a final injected volume of 200 µL, compatible with tail vein injection.

1.3 Supplementary figures

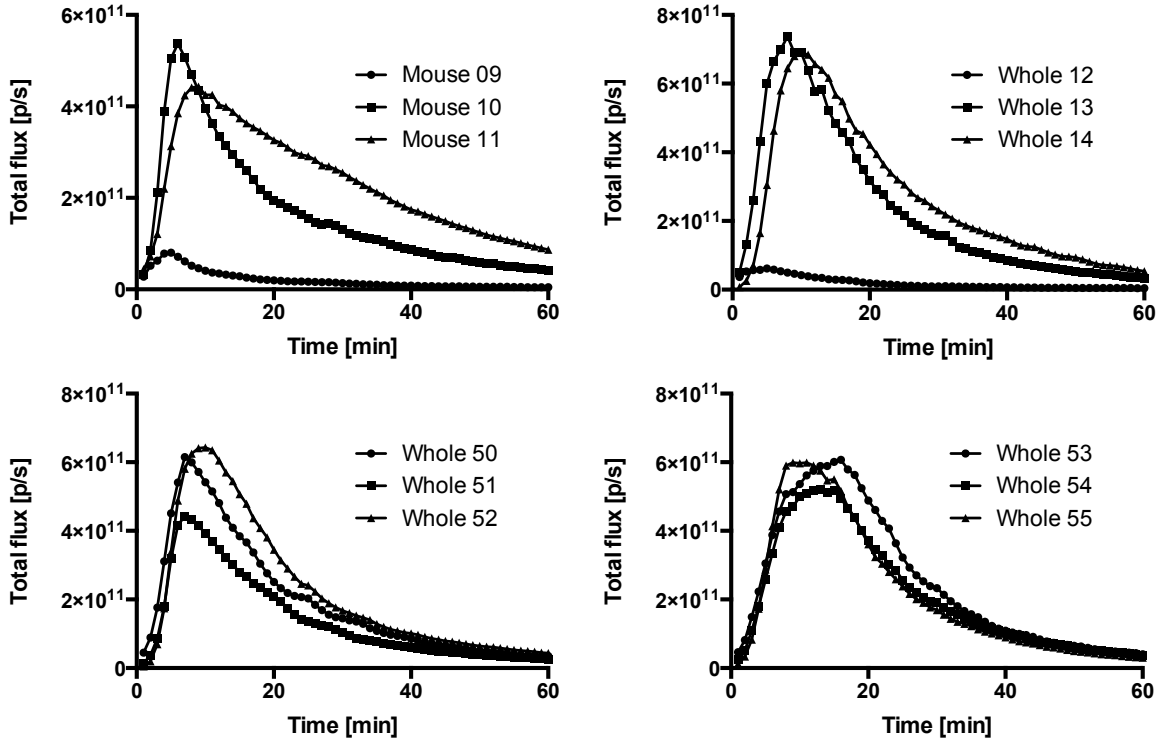


Figure 5-0-7 Kinetics of light emission for intraperitoneal injection of D-Luciferin *in vivo*

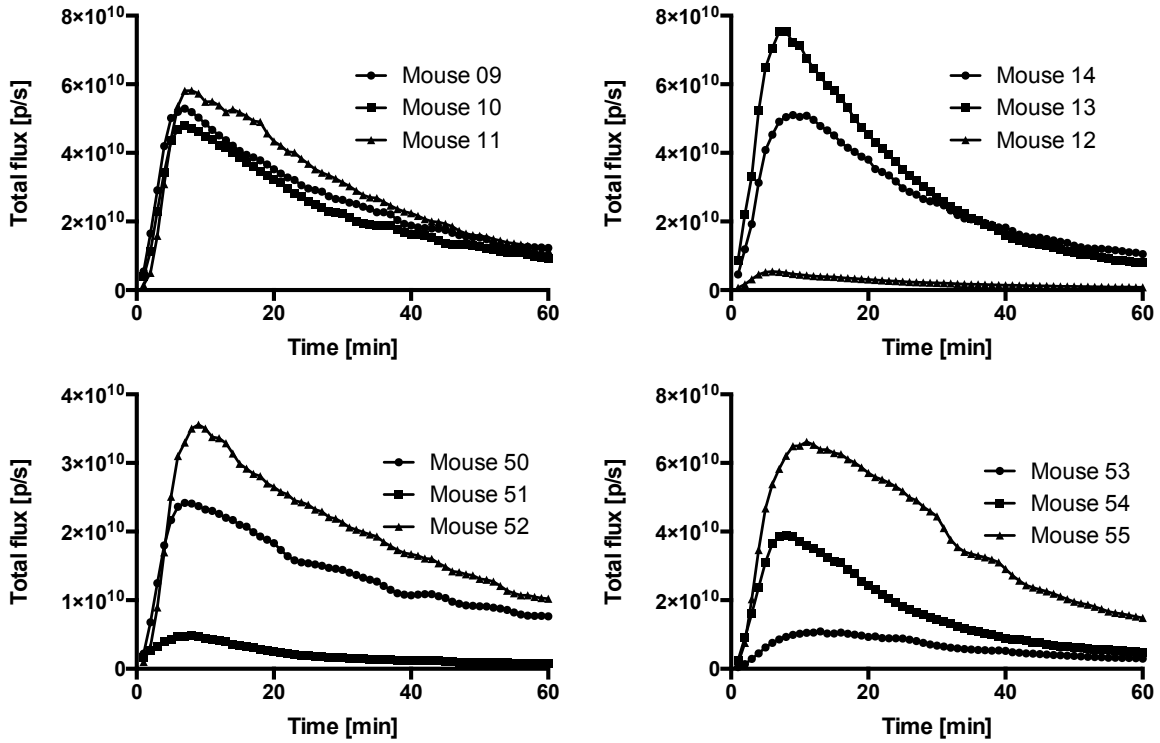


Figure 5-0-8 Kinetics of light emission for intraperitoneal injection of OH-CBT *in vivo*

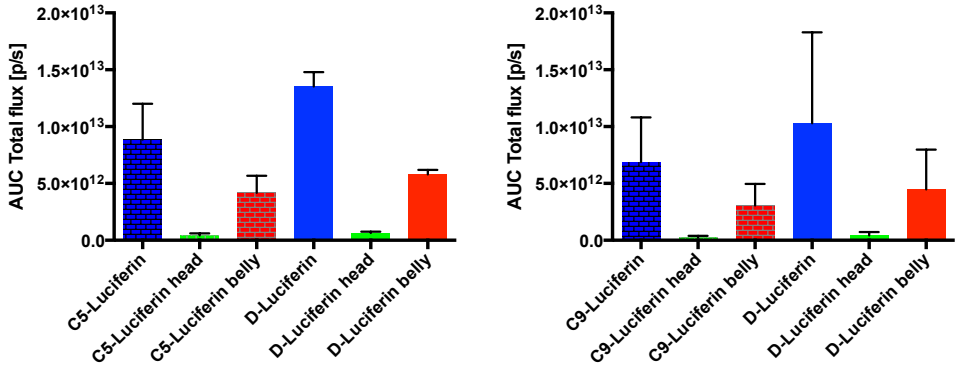


Figure 5-0-9 Contributions of ROIs to total luminescence for C5-luciferin (left) and C9-luciferin (right) (60 minutes)

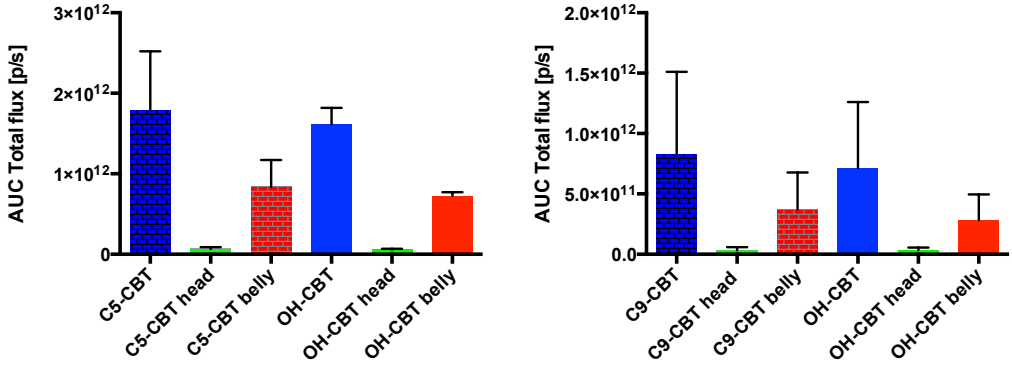


Figure 5-0-10 Contributions of ROIs to total luminescence for C5-CBT (left) and C9-CBT (right) (60 minutes)

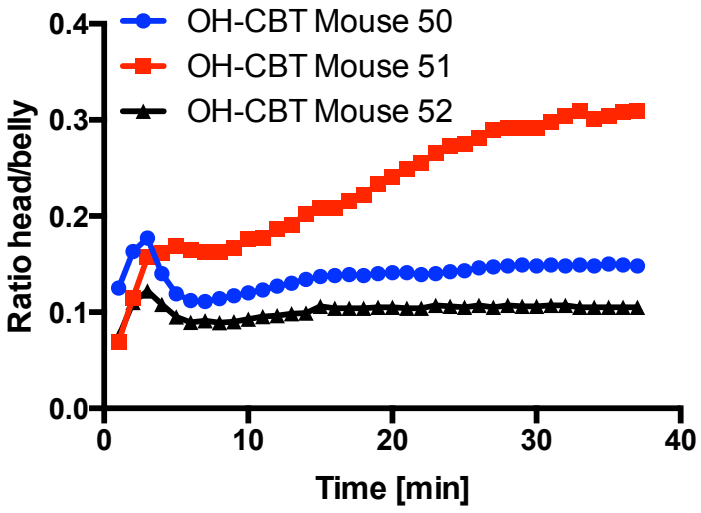


Figure 5-0-11 Evolution of the ROIs head to belly ratio for OH-CBT in mice 50, 51 and 52