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1 Abstract – English

Nowadays laboratory animals are still used as models in several areas of research, especially in the field of cancer research. For specific applications, mice are genetically modified to obtain models with a specific function. These mice, called transgenic mice, can be characterized genetically (e.g. polymerase chain reaction) and by phenotype (e.g. bioluminescence imaging incase the reporter gene was included within the transgenic mice). The expression and regulation of a gene can be monitored by reporter gene assays which are based on bioluminescence e.g. Firefly luciferase.

This master thesis investigates optimization of protocols for *ex vivo* activity measurements of Firefly luciferase in tissues of transgenic mice. Firstly, a homogenization protocol was developed and further optimized for different mouse tissues by employing organs from non-transgenic mice and spiking them with recombinant Firefly luciferase protein at different steps (before homogenization vs after homogenization). For this, various parameters were used and adjusted (buffer, homogenization time, centrifugation). After optimization, we applied the optimized protocol for tissues of transgenic mice expressing Firefly luciferase (Luc) as a reporter gene. Two transgenic mouse strains were therefore examined, Tg Sp-C-Luc and Tg Thy-1.2-Luc. As a last step, to reveal correlation in luciferase activity, our *ex vivo* results were compared with the genotypic and phenotypic status of *in vivo* analyses.

In our work, we show a successfully optimized homogenization protocol for detecting the activity of spiked recombinant Firefly luciferase protein. We observed that CCLR 1X (BSA) works best as a buffer. With the equipment we have used, the ideal homogenization time for brain tissue is 15 seconds, for lung, liver and skin 10 seconds, respectively. A centrifugation step is inevitable to obtain consistent conditions for the measurement.
The optimized *ex vivo* assays show measurable activity of Firefly luciferase for both transgenic strains. Generally speaking, these results correspond to the respective genotype and phenotypic status of *in vivo* 2D BLI imaging based phenotyping. However, in some cases, the correlation is not detectable because of probable outliers. Further experiments with more tissue samples could be done to obtain more significant results.

2 Abstract – Deutsch

Heutzutage werden Versuchstiere im Labor immer noch in den verschiedensten Bereichen der Forschung, vor allem im Bereich der Krebsforschung, eingesetzt. Mäuse werden für bestimmte Anwendungen gentechnisch verändert, um Modelle mit spezifischen Funktionen zu erhalten. Diese Mäuse, auch transgene Mäuse genannt, können genetisch (z.B. mit Hilfe der Polymerase-Kettenreaktion) oder phänotypisch (z.B. mit Biolumineszenz-Imaging, falls das Reportergen in die transgenen Mäuse zuvor eingebracht wurde) charakterisiert werden. Die Expression und die Steuerung eines Gens kann mit Hilfe von Reportergen Assays, die auf der Biolumineszenz basieren überwacht werden, wie es bei Firefly Luciferase der Fall ist.

In dieser Masterarbeit wird ein Protokoll entwickelt und optimiert, um die *ex vivo* Aktivität von Firefly Luciferase in Geweben von transgenen Mäusen zu untersuchen. Als Erstes wurde ein Protokoll zur Homogenisierung verschiedener Gewebe von Mäusen entwickelt und optimiert. Dafür wurden die Organe von nicht-transgenen Mäusen verwendet, mit rekombinantem Firefly Luciferase Protein versehen und in verschiedenen Arbeitsschritten untersucht (vor bzw. nach der Homogenisierung). Es wurden dazu einige Parameter verwendet und angepasst (Puffer, Dauer der Homogenisierung, Zentrifugieren). Nach der Optimierung haben wir dieses Protokoll für Gewebe transgener Mäuse, die Firefly Luciferase (Luc) als Reportergen exprimieren, verwendet. Es wurden zwei transgene Linien dafür untersucht, Tg Sp-C-Luc und Tg Thy-1.2-Luc. Als letzten Schritt haben wir unsere *ex vivo* Ergebnisse mit dem Genotyp und dem Phänotyp Status aus *in vivo* Untersuchungen verglichen, um einen Zusammenhang in der Aktivität des Luciferase Enzyms aufzeigen zu können.

Mit dieser Arbeit zeigen wir ein erfolgreich entwickeltes Homogenisierungsprotokoll, um die Aktivität von zugefügten rekombinanten Firefly Luciferase Protein nachweisen zu können. Wir stellten fest, dass CCLR 1X (BSA) die besseren Ergebnisse als Puffer liefert. Ausgehend von unserem gewählten Equipment beträgt die optimale Homogenisierungszeit für Gehirngewebe 15 Sekunden, bzw. 10 Sekunden für Lunge, Leber und Haut. Ein

Zentrifugierungsschritt ist unabdingbar, um gleichbleibende Bedingungen für die Messung zu erhalten.

Die optimierten *ex vivo* Assays zeigen messbare Aktivität des Luciferase Enzyms in beiden untersuchten transgenen Linien. Im Großen und Ganzen entsprechen diese Ergebnisse dem jeweiligen Genotyp und Phänotyp Status aus den *in vivo* 2D BLI basierten Untersuchungen. In einigen Fällen ist die Übereinstimmung jedoch auf Grund von möglichen Ausreißern nicht erkennbar. Es könnten weitere Versuche mit mehr Gewebeproben durchgeführt werden, um aussagekräftigere Ergebnisse zu erzielen.

3 List of Abbreviations

°C	celsius
BCA	bicinchoninic
BLI	bioluminescence imaging
BSA	bovine serum albumin
CCLR	cell culture lysis reagent
CD90	cluster of differentiation 90
DNA	deoxyribonucleic acid
dNTPs	nucleotides
GFP	green fluorescent protein
kDa	kilodalton
mg	milligram
ml	milliliter
mM	millimolar
MMCT	Laboratory of Macromolecular Cancer Therapeutics
MQ-water	milliQ-water
ng	nanogram
non-TG	non-transgenic
PCR	polymerase chain reaction
pg	picogram
rFFluc	recombinant firefly luciferase
RLU	relative light unit
RNA	ribonucleic acid
RPM	revolutions per minute
SP-C	surfactant associated protein C
Taq	thermophilic bacterium *Thermus aquaticus*
TG, Tg	transgenic
Thy-1	thymus cell antigen 1/ Thymocyte differentiation antigen 1
TRIS	tris(hydroxymethyl)aminomethane
wh	weak-hem
wt	wild-type
µl	microliter

4 Introduction

Animals are successfully used in research for many years now. It is still difficult and, in some cases, not yet possible to replace the use of animals with alternative methods. There are many areas where animals are utilized in research, e.g. to advance scientific understanding or as models to study disease.[1]

Today, the mouse is by far the most common research mammal in the world[2]. According to the German Federal Ministry of Food and Agriculture (BMEL), in Germany alone, 2 millions of vertebrates and cephalopods were used in animal experiments in 2014, whereas mice had a share of 63% of all animals used[3]. As a research model, the mouse (Mus musculus) combines all required criteria: it is relatively small and easy to keep and reproduces rapidly.

A major breakthrough happened in the 1990s and early 2000s. Scientists could perform an initial sequencing and analysis of the mouse and human genome[4,5]. Its genome has now been fully sequenced. A comparison of mouse genes with those of humans shows that mice are biologically very similar to humans. 95 percent of the genes in the mouse genome generally correspond to humans[6]. Thus, the murine model serves as an important experimental system for many areas of research.

4.1 Transgenic mice

In the 1900s spontaneous mutations within mouse colonies were observed. This was the start for the development and production of genetic mouse models.[7] Since spontaneous mutations are a relatively infrequent happening, it is very difficult to obtain mice with specific mutations of biomedical interest[8].

In the early 1980s, genetic engineering entered a new era. Papers were published describing the results of experiments in which DNA was introduced in mouse germ line by microinjection into fertilized mouse eggs[9–12]. The concept of ‘transgenic’ was mentioned in 1981 by Gordon and Ruddle to describe animals which were genetically manipulated by introducing exogenous genes into its genome[13]. The term ‘transgenic animal’ defines an animal, in which specific genes of interest are modified by splicing or inserting foreign DNA into its chromosomes[14]. Foreign genes, called transgenes, are either added (“knock-in”) or

'normal' genes are replaced by non-readable variants to determine its effects ("knock-out")[14,15].

Transgenic mice can be generated using several techniques. Besides various methods, e.g. retrovirus-mediated gene transfer or gen knockdown by RNA interference, the main two techniques are embryonic stem (ES) cell technology and DNA microinjection[13].

By the help of research with transgenic mice, scientists could more investigate the causes of genetic disorders and focus on the development of drugs for the treatment of pathologies like cancer.

4.2 Luciferase based transgenic mice

The expression and regulation of a gene can be monitored by reporter assays which are based on bioluminescence. The term bioluminescence is defined as light produced by a chemical reaction within a living organism, it is a type of chemiluminescence[16]. The purpose of bioluminescence in nature is quite diverse, e.g. as defense or camouflage, or to attract prey. It is used by terrestrial as well as by aquatic organisms, whereby it is more seen in animals that live in a marine environment.[17]

Compared to fluorescence, where a fluorophore absorbs the energy from a light source and emits light with a longer wavelength, light emission of bioluminescence is solely the result of a chemical reaction[17,18]. Using the example of Firefly luciferase, figure 1 shows the oxidation of D-Luciferin, the substrate of the reaction, to Oxyluciferin by the enzyme luciferase, that catalyzes the reaction in the presence of oxygen. This oxidative decarboxylation leads to light emission as a result of releasing photons when returning from the electronically excited state to the ground state.[17,19] The light can be generally measured in the range of 400-700 nm wavelength, whereby peak emission of Firefly Luciferase occurs in the area of 550-570 nm[17,20].

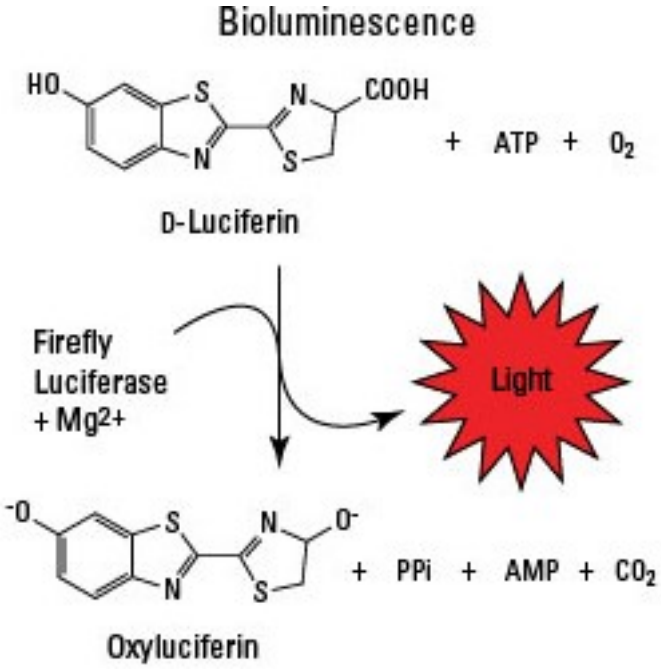


Figure 1. Bioluminescence. Light is emitted as a result of the reaction of Firefly Luciferase and Luciferin. Figure from reference [17].

4.3 Characterization methods for transgenic mice

4.3.1 Genotyping

The most precise way to characterize genetically modified mice is via genotyping. After gene transfer is done, the crucial step is to detect the success of the generation of specific mouse models. All forms of genotyping have in common the need of mouse DNA extraction and isolation. DNA can be obtained from several animal tissues. Invasive methods are for instance tail biopsy[21,22] and ear punches[23], whereas hair[24], stool[25], blood[26] and oral[27,28] samples represent noninvasive alternatives.

To identify transgenic mice, polymerase chain reaction, or PCR, is commonly used. Compared to other methods, PCR requires the least amount of DNA for determination of the genotype[29]. Since the introduced DNA is well known, primers can be used designed from these specific fragments.

PCR copies short fragments of DNA based on the natural DNA replication process and is controlled by heating and cooling. For DNA amplification are required: appropriate buffer,

dNTPs, primers and DNA polymerase. For the first step, the strands of the DNA template are separated by using a high temperature of around 94-95°C. This process is called 'denaturation'. Then, the temperature is lowered, so that the primer can bind on the complementary sequence on the template strand ('annealing'). This temperature has to be chosen depending on the primer. For the last step, termed 'extension', the temperature is adapted to the optimum function of DNA polymerase, e.g. thermostable *Taq* DNA polymerase, which begins to extend the primers. These steps have to be repeated several times to ensure an adequate amount of replicated DNA and is done by the help of a thermal cycler.[30,31] Figure 2 shows the exponential amplification of the target DNA.

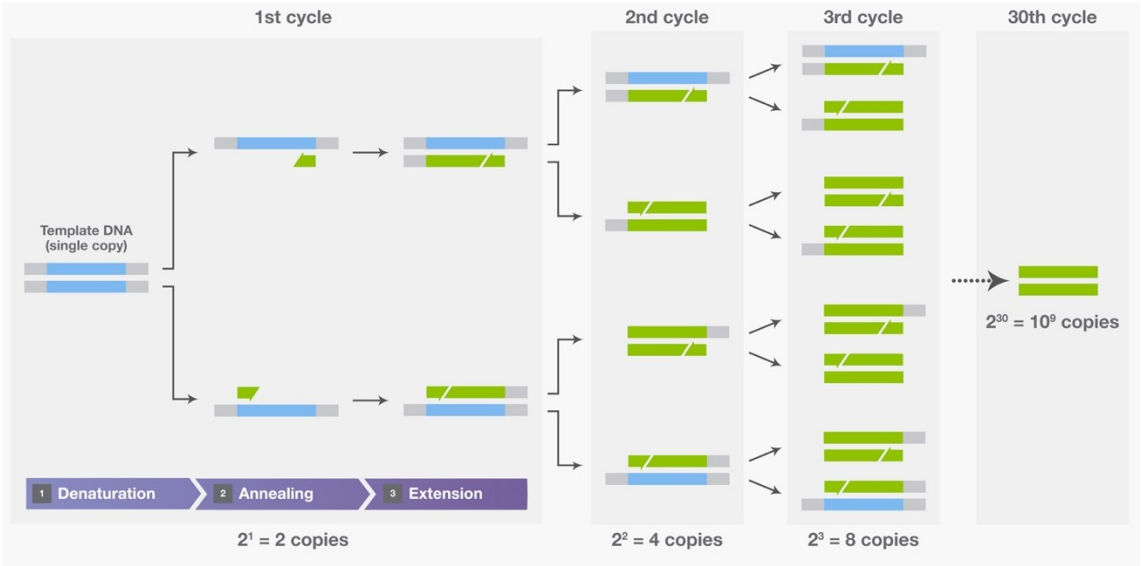


Figure 2. PCR. General steps – denaturation, annealing, extension – as shown in the first cycle, then exponential production of copies of the target DNA. Figure from reference [31].

The easiest, rapid and therefore most widely used method for analyzing PCR products is the use of standard agarose gel electrophoresis. DNA fragments are thereby separated on the basis of charge and size in an electric field[32]. Since the target DNA is known well, obtained PCR products can be determined by adding a molecular size marker such as DNA ladder in an additional lane on the gel[33]. For visualizing, the bands on the gel are either stained with a dye such as ethidium bromide, which intercalates between the base pairs of nucleic acid double helix and fluoresces under ultraviolet light, or DNA primers or nucleotides are labeled with fluorescent dyes prior to PCR[32,33]. Since ethidium bromide is a DNA intercalator and therefore a potent mutagen, it must be used with caution or better substituted

by other gel stain alternatives such as SYBR®[34,35]. Hence, the genetic state of mice can be determined by the presence or absence of the band of the target DNA.

Another approach to characterize transgenic mice via genotyping is Southern blotting. This technique is named after its inventor, Edwin Southern, who introduced it 1975 for the first time[36]. With Southern blot it is possible to detect the presence of a specific sequence of interest, like the target DNA, out of a DNA sample. The first step after DNA extraction from tissue sources and purification is to digest the DNA into fragments with restriction enzymes. Then, the fragments are separated by size and split into single strands using gel electrophoresis. Next, the DNA fragments are transferred from the gel to a nylon membrane ('blotting'). Once the transfer is complete, the membrane is then treated with a hybridization solution containing single stranded DNA that is complementary in sequence to the target DNA. This short piece of DNA, called probe, is labeled either with a radioactive molecule or a fluorescent dye. After washing off unhybridized probe, the radioactive or fluorescent signal can be detected appearing in a distinct band (Figure 3).[37–39]
Other than with PCR, where all DNA fragments have the same size after amplification and form clearly a band on the agarose gel, the fragments used for Southern blot appear more as a smear on the gel. This is the reason why the blotting has to be done after gel electrophoresis.

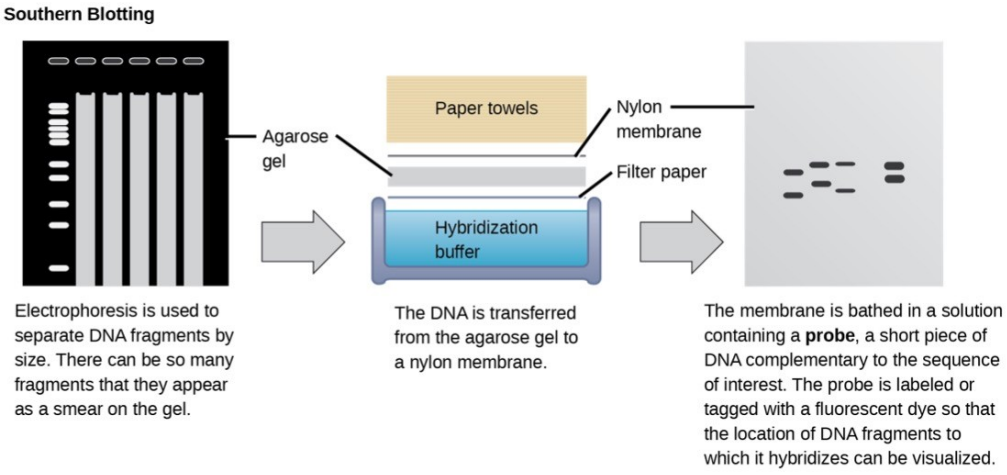


Figure 3. Southern Blot is used as a laboratory method to detect specific DNA molecules. DNA fragments are separated on a gel and transferred to a nylon membrane. The membrane is then bathed in a solution with a labeled probe to visualize the bands of DNA sequence of interest. Figure from reference [39].

4.3.2 Phenotyping

Besides genotypic characterization, it is possible to distinguish transgenic and non-transgenic mice on the basis of phenotypic differences. The term 'phenotype' means a set of observable characteristics of a mouse, e.g. appearance, development and behavior, as determined by its genes and the environment[40]. For differentiation of founder and offspring mice, coat color, growth rate or, as an example for optical imaging, fluorescence of a GFP gene can be used[21].

Apart from fluorescence, which requires incident light, *in vivo* bioluminescence imaging (BLI) is increasingly being utilized as an optical molecular imaging technique. BLI uses light emitted by enzyme-catalyzed reactions to visualize cellular and molecular processes and gene expression[19]. This powerful technique both is low cost and noninvasive, and has a high sensitivity due to low background signal and excellent signal-to-noise-ratio[41,42].

BLI can be separated into three main steps (Figure 4). First, a luciferase gene-based reporter construct is genetically engineered and transferred into a small animal, like a laboratory mouse. Second, the bioluminescent substrate is injected in the animal. Third, light signals are acquired and analyzed[19].

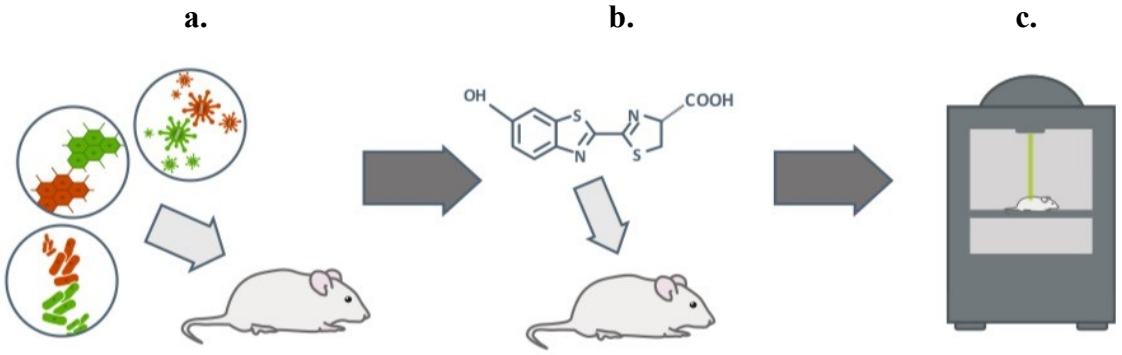


Figure 4. *in vivo* bioluminescence imaging. a. Injecting bioluminescent reporter genes into animal. **b.** Administering substrate to animal. **c.** Acquiring and analyzing imaging data. Figure from reference [19].

4.4 Firefly luciferase-based reporter transgenic mice

Luciferase-based reporter animals can be created using different enzymes. Except Renilla and Gaussia luciferases, Firefly luciferase, a monomeric protein from the North American

Firefly (*Photinus pyralis*) with a molecular weight of 61 kDa, is one of the most common used enzymes for various bioluminescent experiments[17]. As Firefly luciferase can be identified easily and is not present normally in mammalian tissue, it is routinely used for reporter gene assays[43]. Generally, reporter genes and their products can be used as markers, e.g. to study the regulation of gene expression or to investigate successfully transfected cells[44].

For luciferase reporter assays, a reporter construct consisting of a regulatory element like a promoter and the luciferase gene is genetically engineered and transferred into animal cells. Then, to quantify the activity of the regulatory element, the expression of the luciferase reporter gene is measured[17]. The intensity of bioluminescence signal directly correlates thereby with the amount of expressed luciferase enzyme and, hence, can be used to determine the activity of the promoter (Figure 5)[17,45].

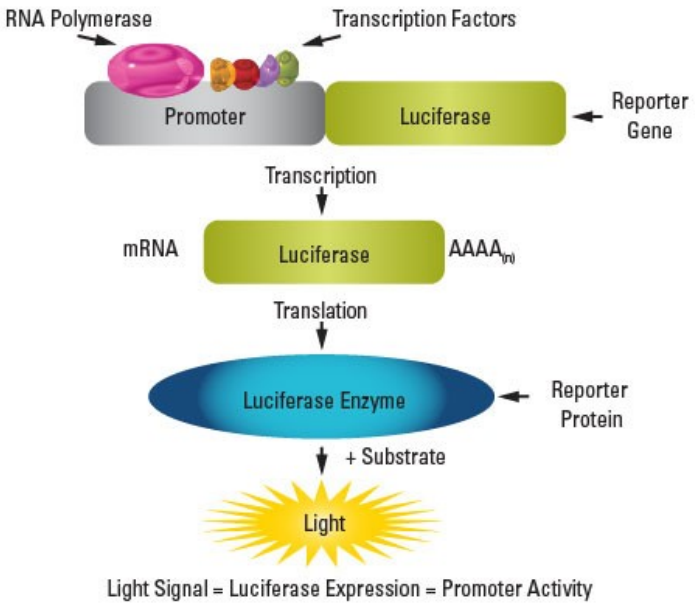


Figure 5. Luciferase reporter gene assay. Intensity of light signal correlates with the luciferase expression and promoter activity. Figure from reference [17].

For special research, mice are genetically engineered to express luciferase gene in a specific region of the body by using tissue specific promoters[46]. Cordonier et al.[47] reported in a journal article, that a real-time gene expression reporter mouse of *Socs3* (suppressor of cytokine signaling-3) was generated. In these *Socs3*-Luc mice the activity of luciferase, for

example in hypothalamic tissues, was detected, as a response to peripheral injection of lipopolysaccharide.

4.4.1 SP-C promoter driven luciferase

Pulmonary surfactant, produced and secreted by type II alveolar epithelial cells in lung, is a surface-active thin layer, which covers the alveolar epithelium. With over 90%, surfactant mainly consists of lipids, followed by proteins with 10%. Half of the protein amount consists of special surfactant proteins, called surfactant associated proteins with different functions. Surfactant protein A (SP-A) and surfactant protein D (SP-D), both hydrophilic proteins, are part of the innate immune system. They have domains for recognition of carbohydrates that enable them to interact with the surface of pathogens like bacteria and viruses. Hence, they facilitate phagocytosis by macrophages. The hydrophobic surfactant proteins B (SP-B) and C (SP-C) mainly help to distribute surfactant on the alveolar surface and, thus, decrease surface tension. They are essential for a normal pulmonary function and prevents alveolar collapse during respiration, especially during expiration.[48–50]

In terms of the amino acid sequence, mature human SP-C shows a lot of similarity with the surfactant protein C of mice[50]. On the basis of this, further research on functions and necessity of SP-C and possible pulmonary diseases due to dysfunction or lack of SP-C can be done on laboratory mice. Glasser et al.[51] summarizes in his review article, that there are numerous studies on surfactant proteins, that show the essential role of these proteins. The importance was revealed by transgenic mice studies, as well. It is known that SP-C is probably only expressed in type II cells[50], therefore the SP-C gene can be utilized as a tissue specific promoter in transgenic mice. Luciferase gene is ligated to the promoter and serves as a reporter gene.

4.4.2 Thy-1.2 promoter driven luciferase

Thy-1, also known as CD90, is a cell surface glycoprotein and a member of the immunoglobulin superfamily of proteins. In 1964, it was identified on mouse T lymphocytes by Reif and Allen[52], and originally named theta θ, but years later it was renamed Thy-1[53]. Human Thy-1 protein shows about 66% similarity of murine Thy-1[54]. However, there are differences between these two species. While human *THY1* gene is mapped to chromosome

11q22.3, the murine *thy1* gene is mapped to chromosome 9 with two alleles that encode the proteins Thy-1.1 and Thy-1.2. The only distinction of these two murine proteins is the amino acid at position 89, arginine and glutamine, respectively.[55]

Thy-1 is expressed on many cells of the immune and nervous systems, e.g. on neurons or thymocytes and T-cells, but also on fibroblasts and endothelial cells[56]. The various functions of Thy-1 include T-cell activation, cell proliferation, migration and apoptosis, to name a few. It functions as a cell adhesion molecule as well and is involved in many signaling cascades.[57] *THY1* may plays a role as a tumor suppressor in various types of cancer, such as nasopharyngeal carcinoma, Lung et. al [58] reported in an article.

Its promoter can be used as a brain specific expression promoter to generate brain models. Luciferase gene is ligated to the promoter and serves as a reporter gene, similar to SP-C promoter driven luciferase. There are numerous Thy1-related studies. To name a different method of expression, Feng et al.[59] for example, generated transgenic mice, which expressed YFP (yellow fluorescent proteins) under the control of neuro-specific Thy1-promoter.

4.5 *ex vivo* assays

Compared with *in vitro* studies, for what specific cells are isolated and purified from their regular biological environment, *ex vivo* means “out of the living”. The tissues or organs are not created artificially, but directly taken from a living organism. *ex vivo* experiments are carried out with little to none modifications of the natural conditions.[51] In present study, we worked with *ex vivo* assays, since we harvested tissues and organs from transgenic mouse strains and examined them.

Other *ex vivo* studies have been done as well. Colin et al.[60] investigated the interference of haemoglobin in the *ex vivo* luciferase assay. His group observed, that haemoglobin can cover the detection of luciferase activity, which leads to decreased obtained values. They recommend removing haemoglobin from tissue samples and therefor represent different methods.

In another study, El-Amouri et al.[61] demonstrated *Gaussia* luciferase-based systems as a potential tool to evaluate the biodistribution of proteins or agents by systemic delivery. The researchers used, besides other methods, *ex vivo* assays to reveal biodistribution of *Gaussia* luciferase (Gluc) in peripheral organs and brain tissue. They observed, that Gluc is mostly taken up by kidney/bladder and stomach/intestine but could not cross the blood-brain barrier.

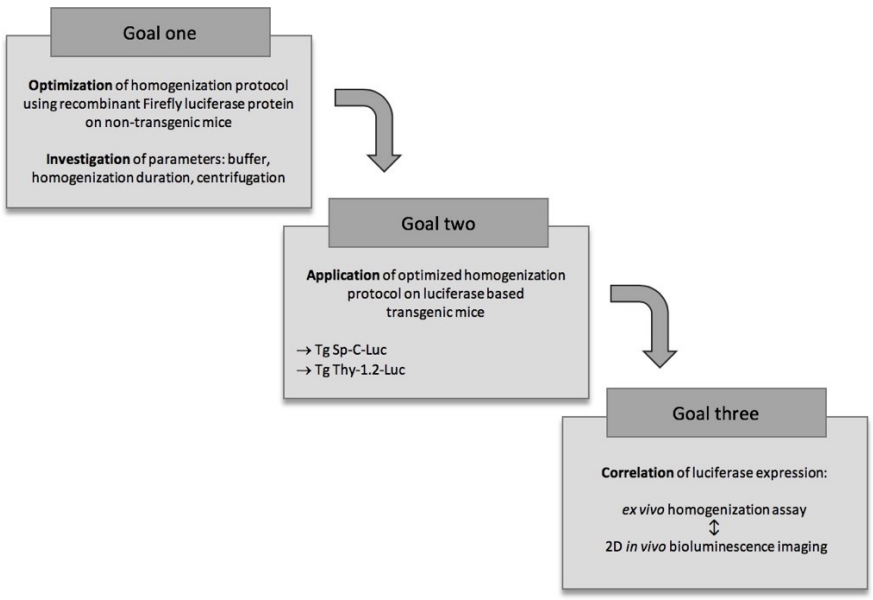
In an article from 2003, Yoo et al.[62] reported the investigation of circadian rhythms and the correlation between the circadian pacemaker in SCN (suprachiasmatic nucleus, located in anterior hypothalamus) and peripheral oscillators found in peripheral organs. They generated transgenic mice containing PERIOD2::Luciferase fusion protein as a reporter. Due to luciferase expression measured in tissues *ex vivo*, they could demonstrate that peripheral tissues can maintain a sort of circadian rhythm without the control of SCN.

5 Goals

This thesis mainly focuses on optimization of *ex vivo* assays for detection of luciferase activity in organs of Firefly reporter based transgenic mice. The first goal was to optimize a homogenization protocol for different mouse tissues by employing organs from non-transgenic mice and spiking them with recombinant Firefly luciferase protein at different steps (before homogenization vs after homogenization). The aim was to investigate various parameters like buffer, homogenization time and centrifugation. Quantification was performed by using a recombinant Firefly luciferase standard curve.

Second goal was the application of this optimized homogenization protocol for tissues of transgenic mice expressing Firefly luciferase (Luc) as a reporter gene. The aim was to characterize luciferase expression in different organs of transgenic mice. Two transgenic mouse strains were investigated, Tg Sp-C-Luc and Tg Thy-1.2-Luc.

Finally, the correlation of luciferase expression by homogenization assay with the genotype and phenotypic status by 2D *in vivo* BLI was examined. The results of *in vivo* and *ex vivo* experiments were compared briefly.



6 Materials and Methods

6.1 Equipment and devices

The following equipment and reagents were used in general.

- BCA Protein Assay Kit with Reagent A and Reagent B (REF 23225, Pierce™, Thermo Scientific)
- Bovine Serum Albumin (REF 23209, Thermo Scientific)
- Cell Culture Lysis Reagent 5X (REF E153A, Promega)
- Cell Culture Microplate 96 Well, PS, F-Bottom, White (REF 655098; Greiner Bio-One)
- Cell Culture Microplate 96 Wells, PS, F-Bottom, Clear, without lid (REF 655160; Greiner Bio-One)
- Centrifuge Tube 15ml CT-15 (Cat. -No.: E1415-0200, Starlab)
- Filter Tips, different sizes (Nerbe plus GmbH)
- Gloves XS (Cat. -No.: SG-T-XS, Starlab, Starguard touch)
- Handle for surgical blades (SI-Line SI-11-0304, No. 4)
- LBL substrate (Luciferase Assay Reagent-buffer + Luciferin; prepared and frozen at -80°C by a member of the MMCT laboratory)
- Lid for Cell Culture Microplate 96 Wells, Clear (REF 656172; Greiner Bio-One)
- Microcentrifuge tubes, different sizes (Eppendorf Research Plus)
- Micropipettes (Eppendorf Research Plus)
- MilliQ-H_2O (Sartorius arium®pro)
- Precellys Lysing Kit CKMix (REF P000918-LYSK0-A, Bertin Technologies)
- Surgical blades, sterile (No. 21, REF 11-0210, Schreiber)
- Tweezers, disposable and sterile, white, 4x120mm (Rotilabo®, KI.05.1, Carl Roth)

The following devices were used for this thesis.

- Freezer -80°C (Revco ExF, Thermo Fisher Scientific)
- Incubator, CO_2, Heracell 150i, Thermo Scientific
- Micro centrifuge Micro Star 17R (Cat. -No.: 521-1647, Thermo Fisher Scientific, VWR)
- Plate reader (Tecan Infinite 200Pro)
- Plate shaker (ThermoMixer C, Eppendorf)
- Precellys® 24 homogenizer (Cat. Number EQ03119-200-RD000.0, Bertin Instruments)
- Table cooling plate Para Cooler (Thomas medical, www.thomas-medical.at)

6.2 Mice

Table 1. Mice used for protocol optimization. (#: not determined)

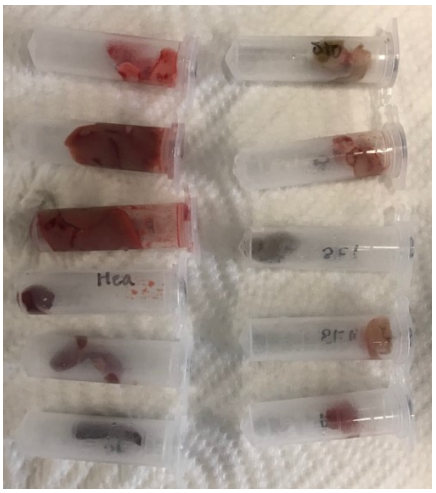
Protocol optimization			
Mouse Strain	***Genotype***	***Type***	***Mouse ID***
non-TG	-	B6 Albino	AGE 127
non-TG	-	#	AGE 298
non-TG	-	#	AGE 426
non-TG	-	BALB/c	AGE 272
non-TG	-	BALB/c	AGE 275
non-TG	-	BALB/c	AGE 274
non-TG	-	BALB/c	MCT 93 (09)
non-TG	-	BALB/c	MCT 92 (08)
non-TG	-	BALB/c	MCT 152 (15)
SP-C	hem	-	AGE 317 (40)
SP-C	wh	-	AGE 350 (68)
SP-C	wt	-	AGE 419 (18)
SP-C	wt	-	AGE 422 (02)
SP-C	wt	-	AGE 424 (04)
SP-C	wt	-	AGE 479 (85)
SP-C	wt	-	AGE 428 (09)
SP-C	wt	-	AGE 425 (05)

Table 2. Mice used for application of optimized protocol on transgenic mice and control mice (non-TG).

Application of optimized protocol			
Mouse Strain	***Genotype***	***Type***	***Mouse ID***
non-TG	-	B6 Albino	MCT 83
non-TG	-	B6 Albino	MCT 84
non-TG	-	B6 Albino Janvier	MCT 77 (05)
non-TG	-	B6 Albino Janvier	MCT 78 (06)
non-TG	-	B6 Albino Janvier	MCT 79 (07)
SP-C	hem	-	AGE 309 (51)
SP-C	hem	-	AGE 313 (46)
SP-C	hem	-	AGE 316 (39)
SP-C	hem	-	AGE 312
SP-C	wh	-	AGE 364 (78)
SP-C	wh	-	AGE 342 (60)
SP-C	wh	-	AGE 416 (15)
SP-C	wh	-	AGE 362 (76)
SP-C	wt	-	AGE 456 (40)
SP-C	wt	-	AGE 469 (24)
SP-C	wt	-	AGE 457 (41)
Thy-1.2	hem	-	AGE 296 (08)
Thy-1.2	hem	-	AGE 295 (07)
Thy-1.2	hem	-	AGE 375
Thy-1.2	hem	-	AGE 368 (61)
Thy-1.2	wt	-	AGE 291 (03)
Thy-1.2	wt	-	AGE 446 (30)
Thy-1.2	wt	-	AGE 447

6.3 Organs

a



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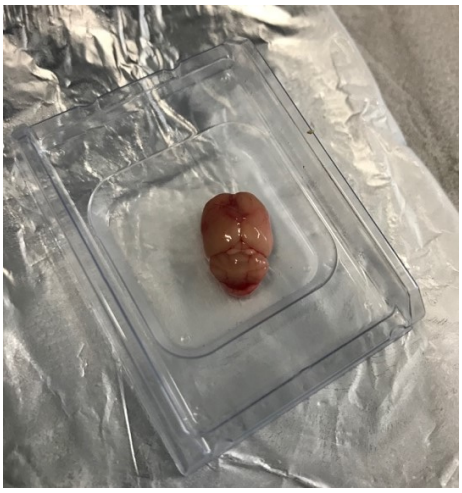
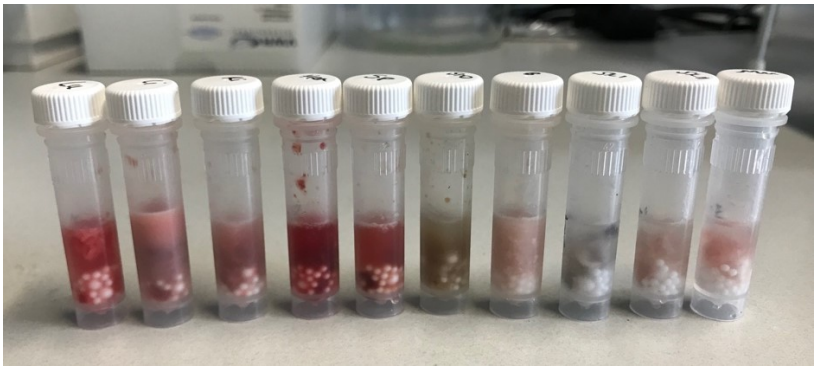


c



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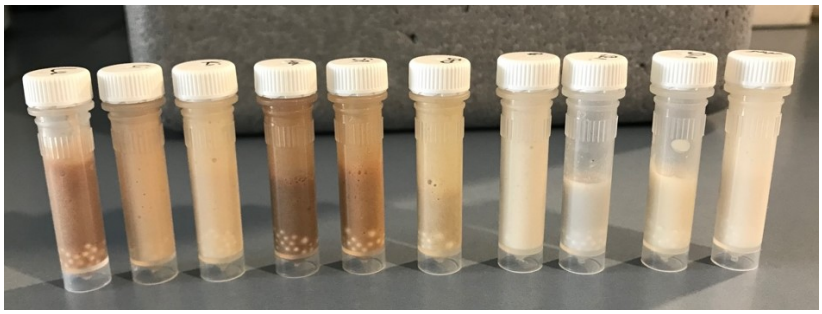
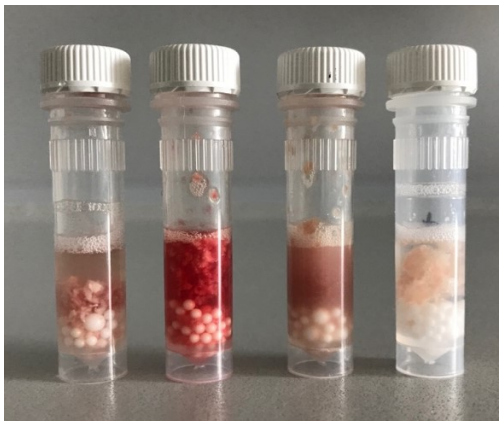


e



f

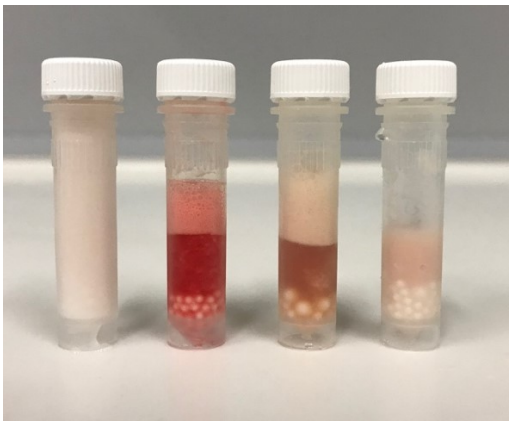


Figure 6. Organ harvesting and processing.

a. Harvested mouse tissues. Left side from above: lung, liver part I, liver part II, heart, kidney, spleen. Right side from above: stomach, brain, skin part I, skin part II, muscle. **b.** Sample of a mouse brain before shredding it on the cooling plate. **c, d.** Representative samples of shredded mouse tissues in lysing kit tubes with added buffer before (c) and after (d) homogenization. From left to right: lung, liver, kidney, heart, spleen, stomach, brain, skin part I, skin part II, muscle. Pictures taken during homogenization protocol optimization. **e, f.** Representative samples of shredded mouse tissues in lysing kit tubes with added buffer before (e) and after (f) homogenization. From left to right: brain, lung, liver, skin. Pictures taken during application of optimized homogenization protocol.

6.4 Firefly luciferase assay and BCA protein assay

6.4.1 Recombinant Firefly luciferase standard curve

For estimating protein concentration in unknown samples, it is essential to include a standard curve each time the protein assay is performed[63]. Similarly, for estimating the amount of luciferase in an unknown sample, a standard curve of different amounts of recombinant Firefly luciferase and their respective luminescence is plotted. Therefore, a standard curve of recombinant Firefly luciferase was carried out for every homogenization assay. This standard curve was used to estimate the amount of luciferase protein produced in different organs for non-transgenic mice (spiked with recombinant Firefly luciferase) and transgenic mice based on luciferase reporter gene.

Materials:

- Stock C (100 ng/µl) (Recombinant Firefly luciferase in CCLR 1X)
- Cell Culture Lysis Reagent 5X
- Bovine Serum Albumin (BSA)
- MilliQ-water (MQ-water)
- LBL substrate
- Cell Culture Microplate 96 Well, PS, F-Bottom, White

Method:
At first, BSA was mixed with MQ-water resulting in a solution of 1,25 mg/ml of BSA. Then, 4 volumes of BSA (1,25 mg/ml) were mixed with 1 volume of CCLR 5X to receive CCLR 1X with a BSA concentration of 1 mg/ml.
A series of dilutions was produced, starting with dilution 1, which was made of stock C (100 ng/µl). Stock C was prepared by Katharina Thekla Müller and stored at -80°C. Hence, a 1:10 dilution series was made (Table 3), whereas dilution 8 was used as a blank. Every solution was mixed carefully and vortexed. 10 µl of each dilution was pipetted in triplicates in wells. Luminescence was measured with Tecan plate reader after automatic injection of LBL and values were expressed as RLUs.

Table 3. Serial dilutions of recombinant Firefly luciferase.

Dilution ID	Volume from stock C (100 ng/µl)	1X CCLR (BSA 1mg/ml)	Final volume	Concentration	Volume used for rFFluc assay	Amount of enzyme in 10 µl	measured y/n
1	2 µl	18 µl	20 µl	10 ng/µl	10 µl	100 ng	n
1:10 dilution series							
2	5 µl from 1	45 µl	50 µl	1 ng/µl	10 µl	10 ng	y
3	5 µl from 2	45 µl	50 µl	0,1 ng/µl	10 µl	1 ng	y
4	5 µl from 3	45 µl	50 µl	0,01 ng/µl	10 µl	0,1 ng	y
5	5 µl from 4	45 µl	50 µl	0,001 ng/µl	10 µl	0,01 ng	y
6	5 µl from 5	45 µl	50 µl	0,1 pg/µl	10 µl	0,001 ng	y
7	5 µl from 6	45 µl	50 µl	0,01 pg/µl	10 µl	0,1 pg	y
8	-	50 µl	50 µl	0	10 µl	0	y

The standard curve was calculated with the obtained results. Figure 7 shows an example of a standard curve. All standard curves of recombinant Firefly luciferase assay used for application of optimized homogenization protocol can be find in the appendix.

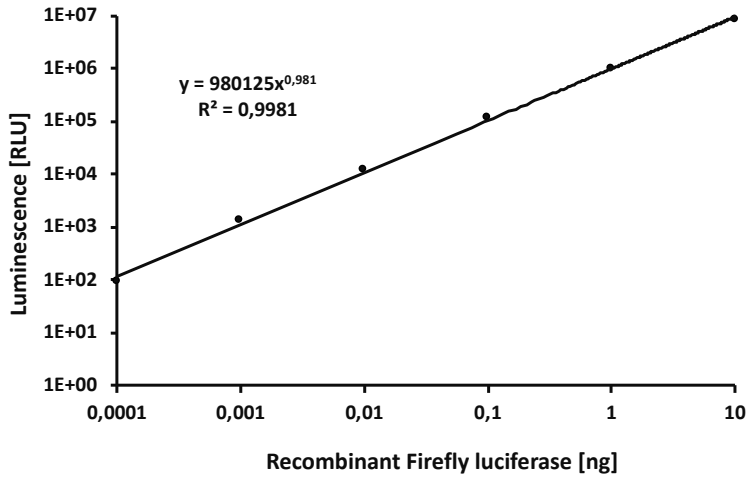


Figure 7. Representative standard curve of recombinant Firefly luciferase for estimation of amount of luciferase in unknown samples.

6.4.2 BSA standard curve

A BCA protein assay is an approved method for quantification of total protein. This protein assay was carried out in addition to the homogenization assay in the part of application of optimized homogenization protocol. For this, the BCA assay was adapted to required conditions. A BSA standard curve was carried out each time the BCA protein assay was performed.

Materials:

- BCA Protein Assay Kit: Reagent A, Reagent B
- Bovine Serum Albumin (BSA)
- Cell Culture Lysis Reagent 5X
- MilliQ-water (MQ-water)
- Cell Culture Microplate 96 Wells, PS, F-Bottom, Clear, without lid
- Lid for Cell Culture Microplate 96 Wells, Clear

Method:
At first, CCLR 5X was mixed with MilliQ-water to obtain CCLR 1X. According to manufacturer's user guide, required amount of BCA working reagent (WR) was prepared by mixing 50 parts of BCA Reagent A with 1 part BCA Reagent B resulting in a clear green liquid[64]. A series of dilutions was made for a BSA standard curve (Table 4). First, MQ-water was pipetted in microcentrifuge tubes, then BSA (2 mg/ml) and finally CCLR 1X was added and mixed thoroughly. 20 µl of each dilution was pipetted in triplicates in wells. 200 µl of WR was added to each well and the plate was mixed on a plate shaker for 30 seconds. The plate was covered and incubated at 37°C for 30 minutes. After incubation, the plate was cooled to room temperature and absorbance was measured at 562 nm on the plate reader. A BSA standard curve was calculated with the obtained results (Figure 8). All BSA standard curves of BCA assay used for application of optimized homogenization protocol can be find in the appendix.

Table 4. Samples of BSA standard curve.

Sample ID	MilliQ-water (µl)	BSA 2mg/ml from Kit (µl)	CCLR 1X (µl)	BSA (µg/well)
A	-	50,0	50,0	20
B	10,0	40,0	50,0	16
C	20,0	30,0	50,0	12
D	30,0	20,0	50,0	8
E	40,0	10,0	50,0	4
F	45,0	5,0	50,0	2
G	50,0	-	50,0	-

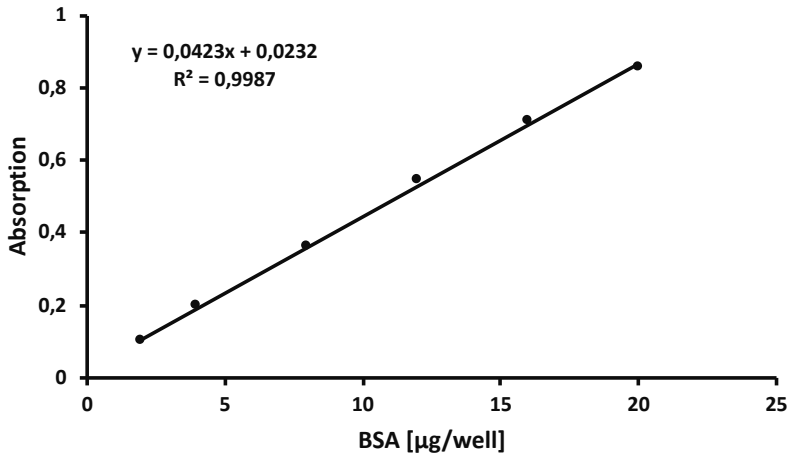


Figure 8. Representative BSA standard curve employed for protein estimation.

6.5 Optimization of organ homogenization with recombinant Firefly luciferase

6.5.1 Addition after homogenization

Materials:

- Surgical blades size 21
- Handle No. 4 for surgical blades
- Tweezer
- Precellys Lysing Kit CKMix
- Cell culture microplate 96 well, PS, f-bottom, white

Method:

All steps of the homogenization process were performed under cool conditions if possible. Required organs were excised from euthanized mice in animal house and transferred into 2 ml microcentrifuge tubes. The tubes were placed on ice all the time. If not needed immediately, organs were frozen and stored at -80°C. The following organs were used for the homogenization optimization process: lung, liver, kidney, heart, spleen, stomach, brain, skin and muscle, whereas lung and brain were of special interest, since Tg Sp-C-Luc mice express firefly luciferase in lung and Tg Thy-1.2-Luc mice in brain, respectively.

The organs were weighted and therefor transferred into a clean microcentrifuge tube, the weight was noted. To facilitate homogenization, the organs were minced by scalpel into smaller pieces on the cooling plate at -23°C. Approximately 200 mg of each organ was transferred into a lysing kit tube of 2 ml. In each lysing kit tube 500 µl of TRIS buffer 250 mM was pipet. All filled lysing kit tubes were weighted to check the balance. After this, the organs were homogenized with Precellys 24 homogenizer with the following setting: 6500-2x45-15, whereat 6500 means speed, homogenized two times for 45 seconds with a pause of 15 seconds in between.

After homogenization had been completed, the homogenate was let set down for 20 to 30 minutes and transferred into fresh microcentrifuge tubes. 10 µl of each organ homogenate (25 µl was used in the first experiment) were pipet two times in triplicates into the wells of a white 96-well plate. To measure the activity of Firefly luciferase in homogenized organs,

either 5 µl recombinant Firefly luciferase (0,2 ng/µl) was added in wells or 5 µl CCLR 1X (as recombinant Firefly luciferase is dissolved in that buffer) in the other wells (4 µl each were used in the first experiment). Luminescence was measured well-wise with Tecan plate reader. A schematic overview of the performed workflow can be seen in figure 9.

6.5.2 Addition after homogenization – including a centrifugation step

A centrifugation step was included. All steps were done as described above in 6.5.1. After homogenization had been completed, the foamy liquid of each sample was completely transferred into clean microcentrifuge tubes to separate the beads from the liquid. Cell debris was removed by microcentrifugation. A Micro Star centrifuge by Thermo Fisher was used. The following adjustment was used:

Speed	13300 RPM
Degrees	4°C
Duration	10 minutes

After that, the clear supernatant was used for luminescence measurement. 5 µl recombinant Firefly luciferase (0,2 ng/µl) was added after homogenization directly into the wells.

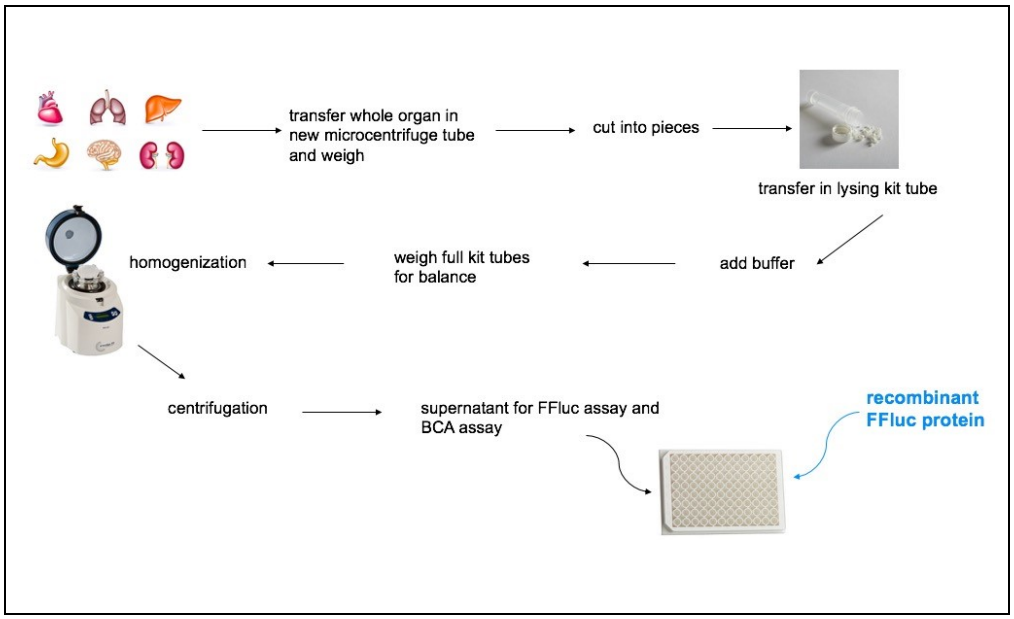


Figure 9. Schematic overview of the performed workflow. 5 µl of recombinant Firefly luciferase is added after organ homogenization and centrifugation directly into wells.

6.5.3 Addition before homogenization

After weighting and cutting the organs as described in 6.5.1, tissues were transferred in lysing kit tubes. 500 µl of TRIS 250 mM was added to each organ sample tube as well as 5 µl recombinant Firefly luciferase (0,2 ng/µl). Then, after homogenization by Precellys 24 homogenizer, tissue lysate was transferred in new microcentrifuge tubes and centrifuged at the beginning with at least the same settings as described above. The clear supernatant was used for measuring luciferase activity. A schematic overview of the performed workflow can be seen in figure 10.

6.5.3.1 Buffer optimization

For the first experiments, TRIS 250 mM was used as a buffer. During the optimization process, CCLR 1X and CCLR 1X with a BSA concentration of 1 mg/ml, named "CCLR 1X (BSA)", was tested as well. For all kind of buffer, 500 µl was used as a defined volume.

6.5.3.2 Centrifugation optimization

In the final phase of optimization, luciferase activity was measured in samples without centrifugation as well as in samples which were centrifuged before measurement. The adjustment for centrifugation was constantly set up at 13300 RPM at 4°C for 10 minutes, as described in 6.5.2.

6.5.3.3 Duration optimization

Different homogenization duration was tested. The first setting was, as described in 6.5.1, 6500-2x45-15. The samples were homogenized two times for 45 seconds. The following durations were also examined: 30, 20, 15, 10 and 5 seconds. A homogenization run was performed two times.

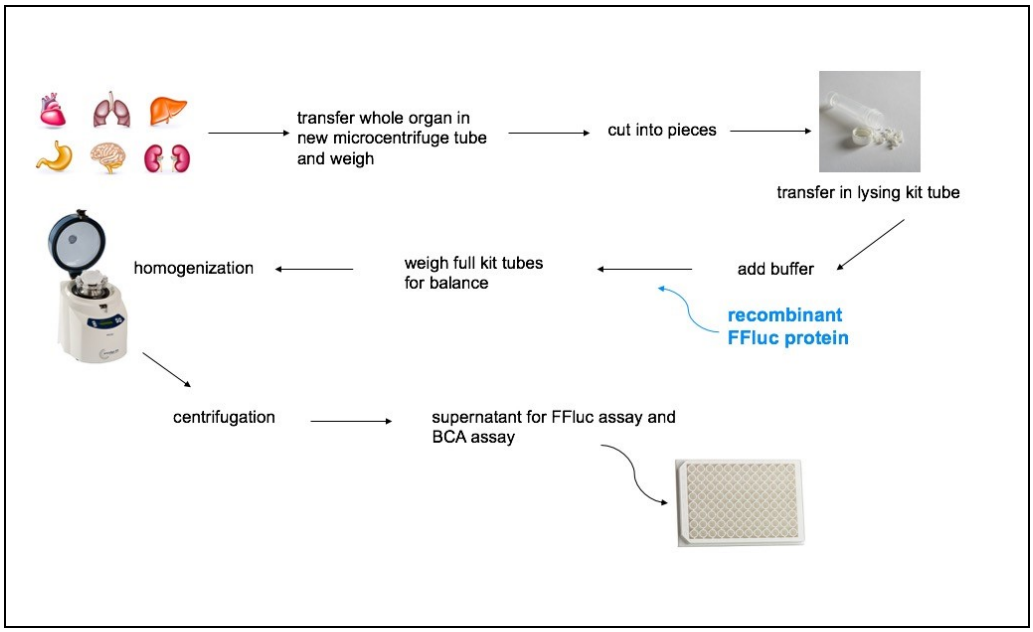


Figure 10. Schematic overview of the performed workflow. 5 µl of recombinant Firefly luciferase is added before organ homogenization.

6.6 Optimized homogenization and luciferase assay protocol

6.6.1 SP-C promoter driven luciferase

Materials:

- Surgical blades size 21
- Handle No. 4 for surgical blades
- Tweezer
- Precellys Lysing Kit CKMix
- Cell culture microplate 96 well, PS, f-bottom, white

Method:

All steps were performed under cool conditions if possible. Required organs of Tg Sp-C-Luc mice were excised from euthanized mice in animal house by a co-worker under currently valid guidelines and transferred into 2 ml microcentrifuge tubes and placed on ice. The organ of interest for SP-C was lung. In addition, brain, skin and liver were used as well.

The organs were weighted and therefor transferred in a fresh microcentrifuge tube. To facilitate homogenization, the organs were minced by scalpel into smaller pieces on the cooling plate at -23°C. Approximately 200 mg of each organ was transferred into a lysing kit tube of 2 ml. In each lysing kit tube 500 µl of CCLR 1X (BSA) was pipet. After weighting the tubes for checking the balance, the organs were homogenized with Precellys 24 homogenizer with different settings. Lung, skin and liver were homogenized with the following setting: 6500-2x10-15, whereby 6500 means speed, homogenized two times for 10 seconds with a pause of 15 seconds in between. Brain was homogenized two times for 15 seconds.

After this, the homogenate was let set down for 20 to 30 minutes. The foamy liquid of each sample was then completely transferred into new microcentrifuge tubes to separate the beads from the liquid. Cell debris was removed by microcentrifugation. Adjustment can be seen in 6.5.2.

After centrifugation, the clear supernatant was used for luminescence measurement. Subsequent, quantification of total protein was done with BCA assay. For every measurement, a standard curve of recombinant Firefly luciferase and a BSA standard curve was made as described in 6.4.1 and 6.4.2. This standard curve was used to estimate the amount of luciferase protein produced in different organs.

6.6.2 Thy-1.2 promoter driven luciferase

Experiments with Tg Thy-1.2-Luc mice were done similar to the method as described above for Tg Sp-C-Luc mice. Organ of interest was primarily the brain. Lung, skin and liver were used as well. All working steps are seen in 6.6.1.

7 Results and Discussion

7.1 Optimization of organ homogenization with recombinant Firefly luciferase protein

7.1.1 Effect of BSA on enzyme stability

Proteins are known as a large class of complex biological macromolecules. Many are unstable and need an environment similar to their native one. When it comes to protein concentration, dilute protein solutions are more affected by inactivation and loss. Since the binding to the storage vessel is low, it is usual practice to add a special carrier protein, such as bovine serum albumin (BSA).[65] Hence, BSA stabilizes small amounts of enzyme.
We first used TRIS 250 mM as a buffer, since this was applied according a previous protocol of a member of the MMCT laboratory. During the optimization process we switched to CCLR 1X and CCLR 1X (BSA). As mentioned before, the reason was obvious. On top of that, recombinant Firefly luciferase was already stored in CCLR 1X (BSA) at -80°C.
We analyzed liver, brain and skin. All steps were processed as described in 6.5.3, except of the buffer, here we used CCLR 1X and CCLR 1X (BSA) and compared the results. Firefly assay was performed for luciferase expression in different organs of non-transgenic mice, which were processed and spiked with one ng of recombinant Firefly luciferase protein (rFFluc). Luminescence was then converted into the corresponding amount of rFFluc by using the rFFluc standard curve. Figure 11 shows that BSA has a distinct effect on enzyme stability. As one ng of recombinant Firefly luciferase (rFFluc) protein was added to each sample, it can be seen that in absence of BSA the values are lesser then the expected one ng rFFluc. According to this, we used CCLR 1X (BSA) for further experiments.

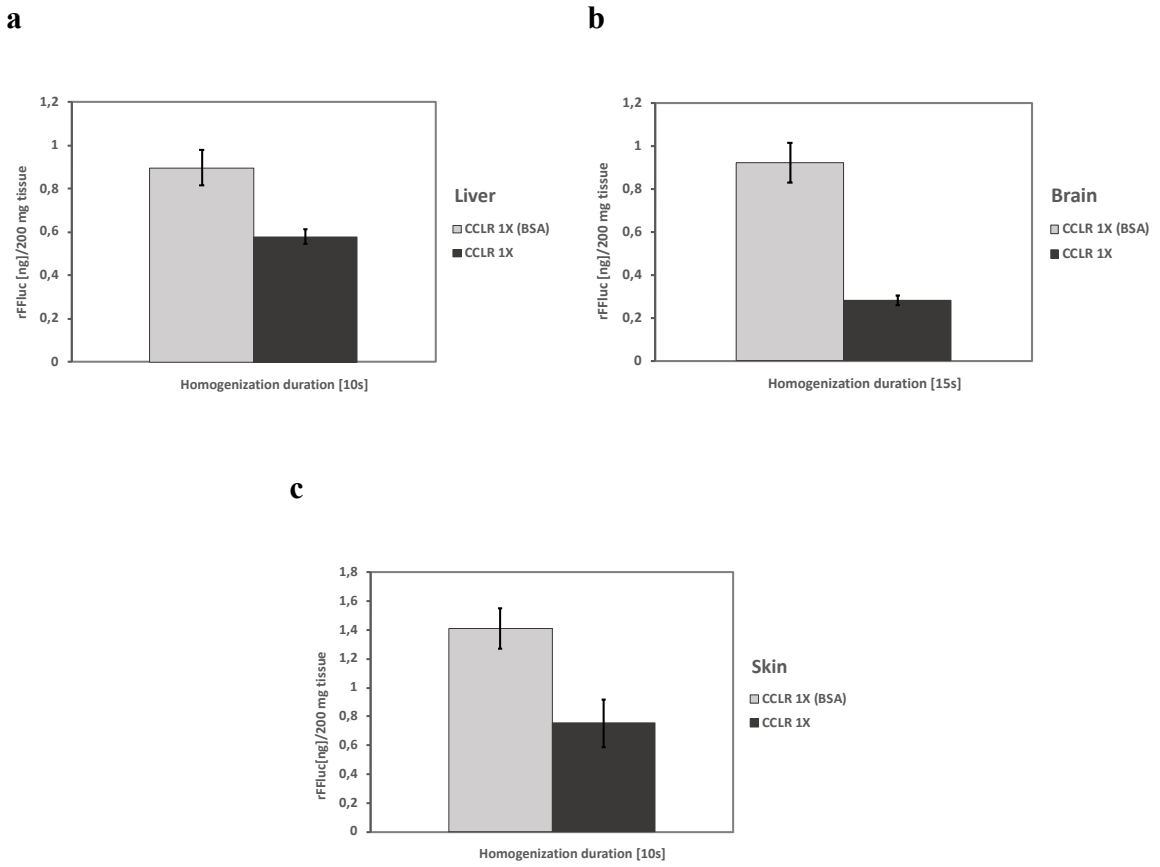


Figure 11. Effect of BSA on organ lysis in CCLR buffer. Firefly assays based estimation of luciferase expression in samples from different organs (**a.** liver, **b.** brain, **c.** skin) of non-transgenic mice, which were processed as described (6.5.3) and spiked with 1 ng of recombinant Firefly luciferase protein (rFFluc). Luminescence was measured by Firefly assay and converted into corresponding amount of rFFluc by using the rFFluc standard curve. The data presented is the average of two independent experiments ± SD.

Mice lungs we used for this work had an average weight of 200 mg. Hence, we defined 200 mg as our sample weight. Since the results mentioned above show clearly the effect of BSA on enzyme stability for liver, brain and skin, we decided not to examine lungs, because this would implicate an unacceptable disproportional high utilization of laboratory mice.

7.1.2 Impact of centrifugation

Homogenization of animal tissue at a sufficient speed causes the forming of air bubbles. This cannot be avoided at some point, except of using ice cold buffer and working as quickly as possible. The frothing is seen clearly in figure 12, even if homogenization time was short.

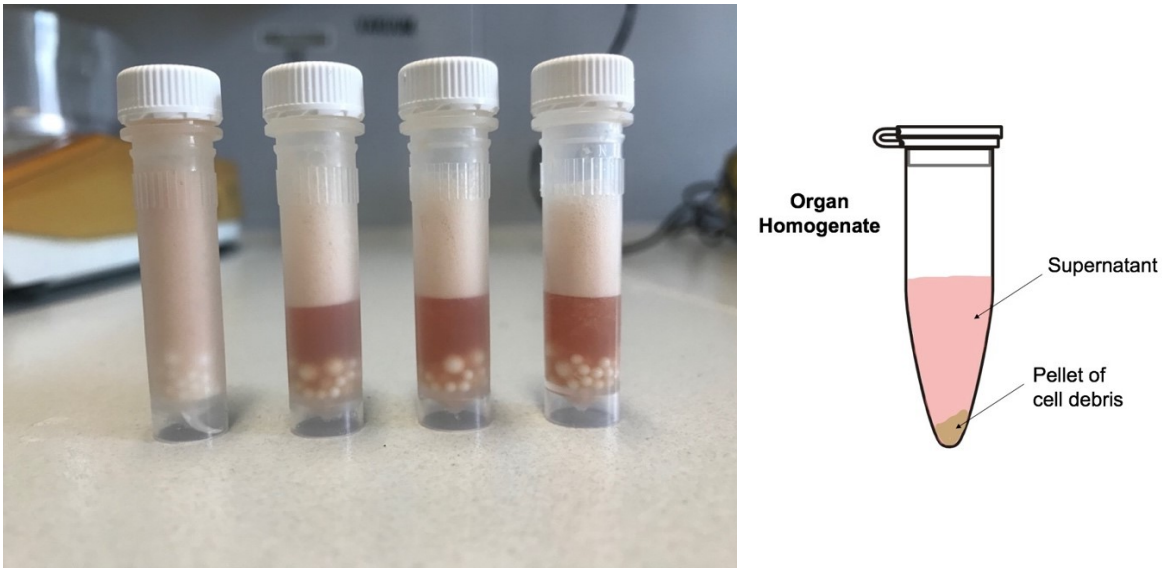


Figure 12. Frothing. Left: Samples of homogenized liver after different homogenization duration. From left to right: sample homogenized for 30, 20, 15 and 10 seconds, each two times. **Right:** Schematic figure of an organ homogenate after centrifugation.

The major difficulty was to pipet a defined volume of tissue homogenate from the lysing kit tube into the wells of our plate. Since the homogenate was too foamy and contained too much air to provide comparable results, we decided to include a centrifugation step to our workflow. In this way, the foam was eliminated. Moreover, cell debris was separated from the liquid and formed a pellet. We analyzed the supernatant of centrifuged samples and the foamy samples which weren't centrifuged. Firefly assay was performed for luciferase expression in different organs of non-transgenic mice, which were processed and spiked with one ng of recombinant Firefly luciferase protein (rFFluc). Luminescence was then converted into the corresponding amount of rFFluc by using the rFFluc standard curve. Figure 13 shows the relevant impact of centrifugation. It can be seen that the values of not centrifuged samples are lesser than the values of samples which were centrifuged.

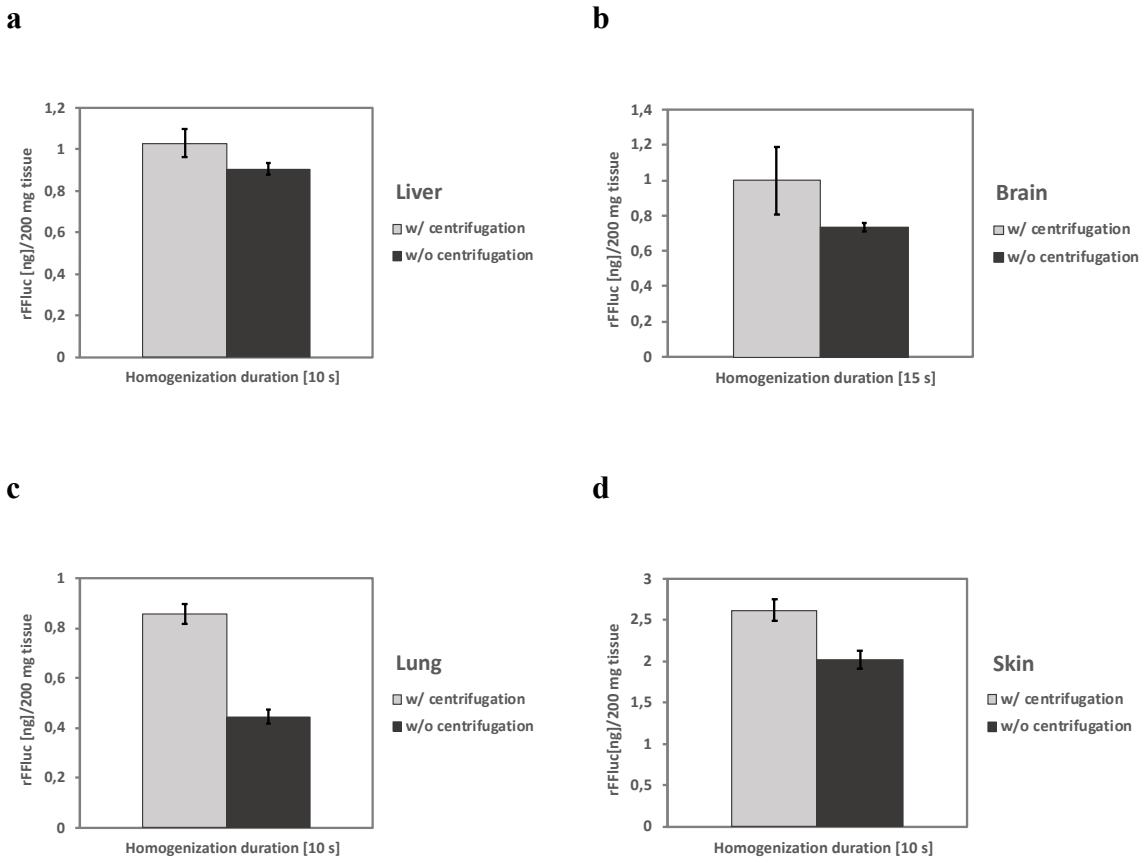


Figure 13. Impact of centrifugation. Firefly assays based estimation of luciferase expression in samples from different organs (**a**. liver, **b**. brain, **c**. lung, **d**. skin) of non-transgenic mice, which were processed as described (6.5.3) and spiked with 1 ng of recombinant Firefly luciferase protein (rFFluc). Luminescence was measured by Firefly assay and converted into corresponding amount of rFFluc by using the rFFluc standard curve. (w/): with centrifugation, (w/o): without centrifugation. The data presented is the average of two independent experiments ± SD.

7.1.3 Effect of homogenization duration on luciferase activity

Although detergent-based lysis methods have become the norm[66], we used a mechanical method. Since we worked with animal tissue, the best way to provide accurate cell lysis and protein extraction, is to go for physical disruption. It is important to choose an efficient method for disrupting the tissue that releases the protein from the inner compartments into the buffer[67]. Since Firefly luciferase is not secreted[68], this step is inevitable. One of the most widely used method for disrupting soft tissues is homogenization[67].

Prior to homogenization, organs of non-transgenic mice were cut into smaller pieces on a cooling plate. For the crucial homogenization step we used Precellys lysing kit CKMix with a tube volume of 2 ml. This kit is composed of 1,4 mm and 2,8 mm ceramic beads, which are perfect to homogenize soft and hard tissue[69]. Homogenization was carried out with

Precellys 24 device. Firefly assay was performed for luciferase expression in different organs of non-transgenic mice, which were processed and spiked with one ng of recombinant Firefly luciferase protein (rFFluc) prior homogenization.

During the first experiments of homogenization at setting 6500-2x45-15 and using TRIS as buffer, according to a previous protocol of MMCT, we couldn't detect any RLUs. We figured out that the tissue was homogenized too long, so Firefly luciferase protein might have been denaturized. Subsequently we tested different homogenization durations on liver. Figure 14 shows the results as RLUs when tissue, in this experiment liver, is homogenized for different duration. Recombinant Firefly luciferase protein (rFFluc) was added to samples before homogenization.

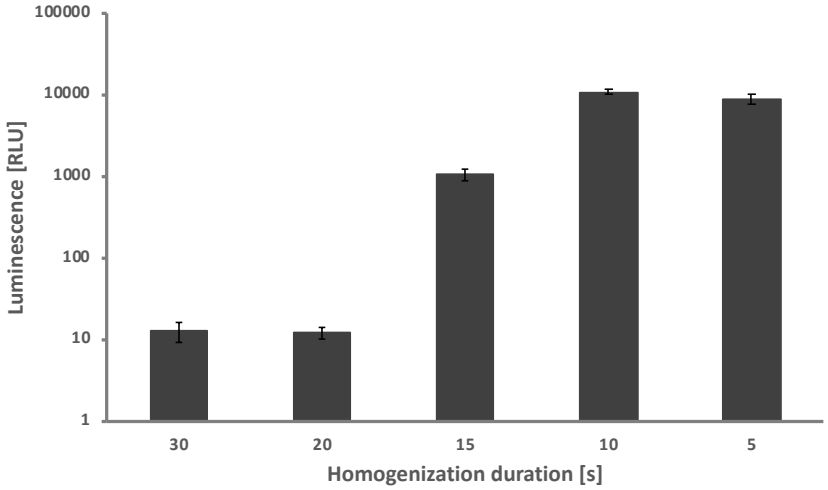
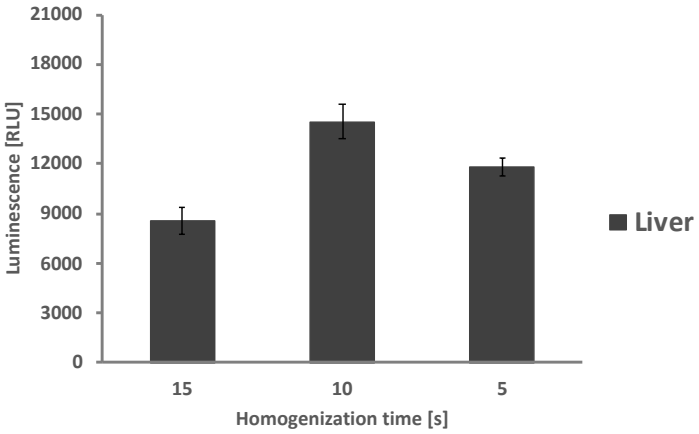


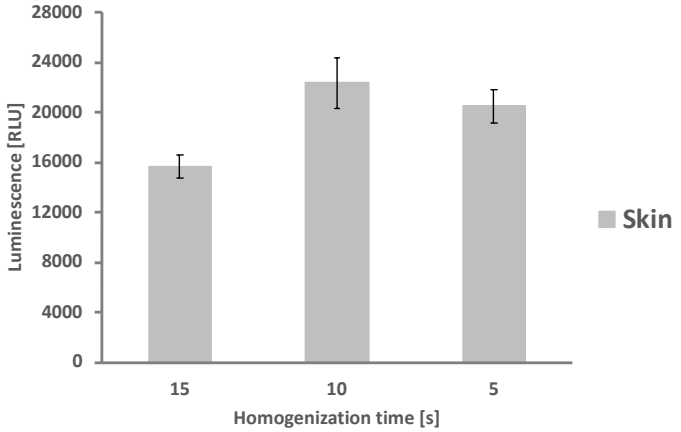
Figure 14. Effect of homogenization duration on luciferase activity. Firefly assays based luciferase expression in samples from liver of non-transgenic mice which were processed as described (6.5.3) and spiked with 1 ng of recombinant Firefly luciferase protein (rFFluc). Luminescence was measured by Firefly assay. Data presented as means ± SD. The graph represents average of two independent experiments.

When tissue was homogenized for 30 or 20 seconds, we could not detect any luminescence. This was the same we observed before, when we chose 45 seconds in the beginning. An acceptable signal was observed for tissue which was homogenized for 15 seconds and less. As a result, we decided to select these durations for analyzing every tissue of interest. For liver, skin and lung we determine 10 seconds as the best homogenization time, for brain 15 seconds, respectively (Figure 15).

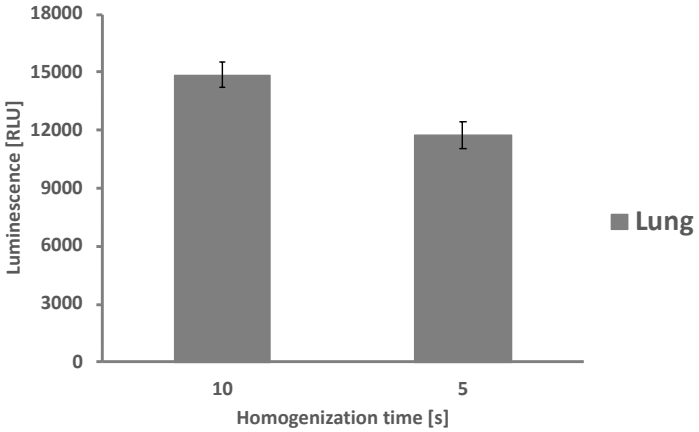
a


21000
18000
15000
12000
9000
6000
3000
0
Luminescence [RLU]
15
10
5
Homogenization time [s]
Liver

b


28000
24000
20000
16000
12000
8000
4000
0
Luminescence [RLU]
15
10
5
Homogenization time [s]
Skin

c


18000
15000
12000
9000
6000
3000
0
Luminescence [RLU]
10
5
Homogenization time [s]
Lung

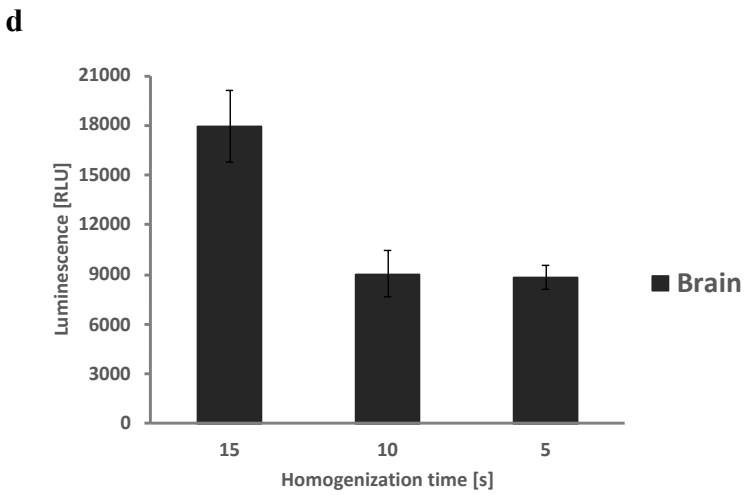


Figure 15. Effect of homogenization duration on luciferase activity. Firefly assays based luciferase expression in samples from different organs (**a**. liver, **b**. skin, **c**. lung, **d**. brain) of non-transgenic mice, which were processed as described (6.5.3) and spiked with 1 ng of recombinant Firefly luciferase protein (rFFluc). Luminescence was measured by Firefly assay. Results are expressed as means ± SD (n=3). Highest RLUs measured for (**a**) liver at 10 seconds, (**b**) skin at 10 seconds, (**c**) lung at 10 seconds and (**d**) brain at 15 seconds.

In general, a longer homogenization time can decrease the activity of an enzyme by denaturation due to heat. Since brain is characterized as a soft tissue, similar to liver, there is no adequate explanation why rFFluc activity is much higher when brain tissue is homogenized for 15 than for 10 seconds. There may be some structures in brain tissue that protect the Firefly luciferase enzyme from denaturation.

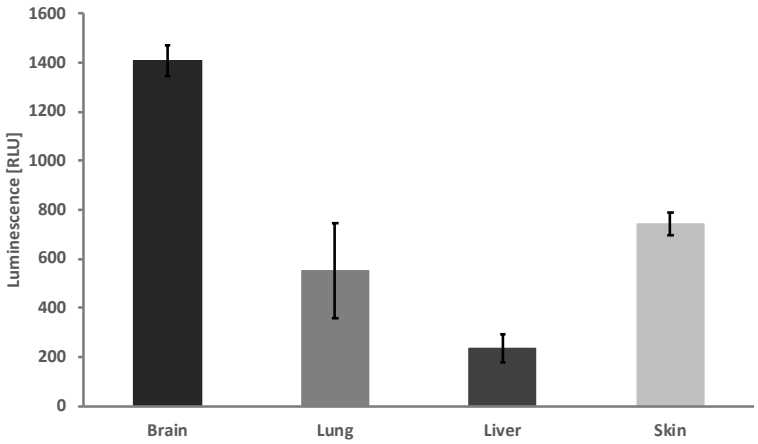
7.2 Application of optimized homogenization protocol for luciferase based transgenic mice

After optimizing organ homogenization using different parameters, we adapted our protocol to transgenic mice. Required tissues, like brain, lung, skin and liver, were removed from euthanized Tg Sp-C-Luc mice and Tg Thy-1.2-Luc mice, respectively. The organs were reduced to small pieces with a scalpel on a cooling plate at -23°C. The cut-up tissues were transferred into the lysing kit tubes, then 500 µl CCLR 1X (BSA) buffer was added. Homogenization was performed by Precellys 24 homogenizer two times for different duration: brain 15 seconds, lung, skin and liver 10 seconds each. The obtained organ homogenate was transferred into new microcentrifuge tubes and centrifuged for 10 minutes at 4°C. The clear supernatant was used for luminescence measurement and BCA assay.

Before the experiments with luciferase based transgenic mice, first we investigated the background values for firefly assay with non-transgenic mice. The applied workflow corresponds to the one described above. In general, we observed the highest RLU for brain, followed by skin, lung and liver (Figure 16). Thus, different organs showed different background RLUs.

An overview of the workflow of application of optimized homogenization protocol for luciferase based transgenic mice can be seen in figure 17.

a



b

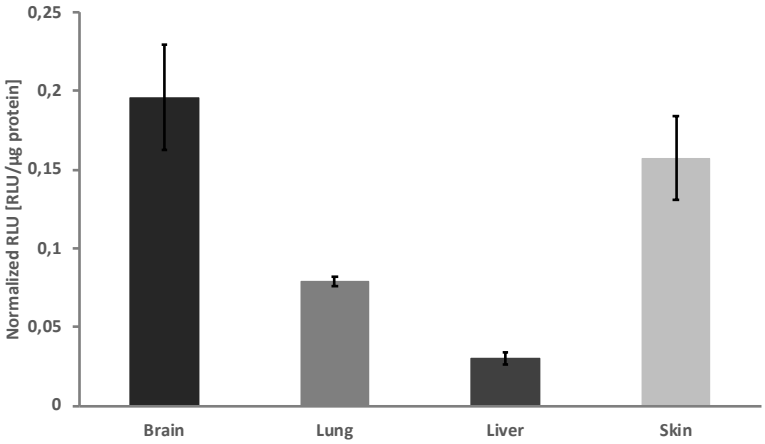


Figure 16. Luminescence of non-transgenic mice i.e. without luciferase expression. (**a**). Luciferase activity was normalized according to the amount of protein per sample (500 µl) (**b**). Data are represented as means ± SD (n=3).

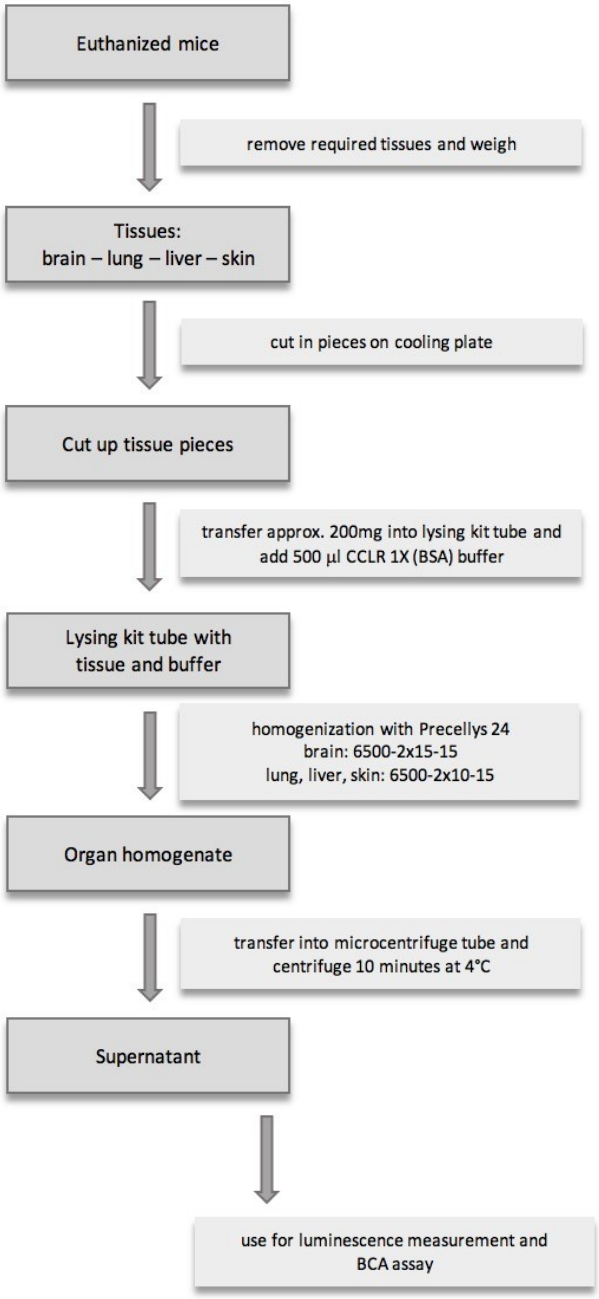


Figure 17. Schematic workflow of optimized homogenization protocol.

7.2.1 Transgenic mice with SP-C promoter driven luciferase

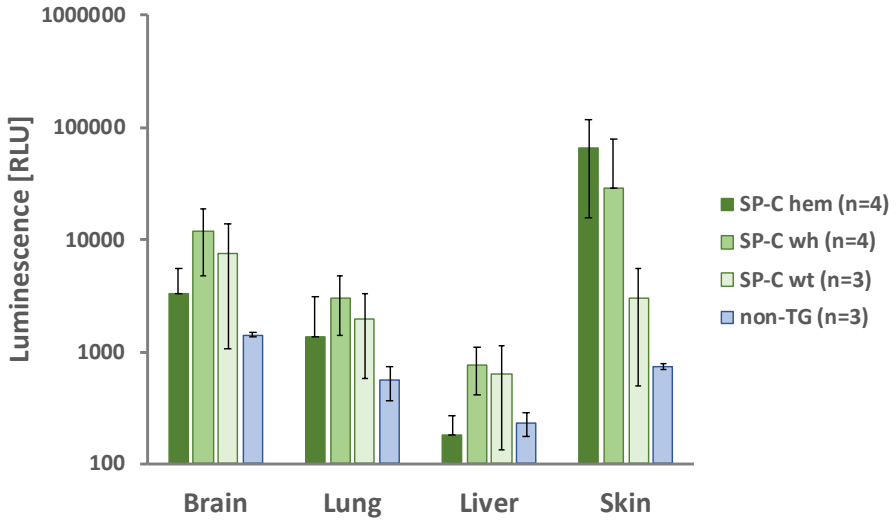
For this work we defined brain, lung, liver and skin as organs we want to analyze. We focused on brain and lung as specific organs of interest for our transgenic mice strains and liver as a reference tissue, where no activity of luciferase or at least the lowest RLU value

should be seen. In addition, we tested skin as well, since a signal has been detected in some mice with BLI by another member of the MMCT laboratory.

In general, Firefly luciferase reporter signals are almost higher than the signals of non-transgenic mice, but luminescence measured in our SP-C experiments correspond to the RLUs of non-transgenic mice regarding intensity between the different tissues. The lowest signals were observed for liver, as expected, followed by lung. However, in terms of Tg Sp-C-Luc, we expected the highest measured luminescence in lung, because of the tissue specific activity of the promoter. Interestingly, the values for skin are the highest on average, especially for SP-C hem and SP-C wh (Figure 18). This corresponds with the results of the other MMCT members' observation.

The luminescence measured for skin is more than 48-fold higher than for lung in SP-C hem mice and more than 9-fold higher in SP-C wh mice, respectively (Figure 18). Some studies already have suggested the presence of surfactant proteins outside the lung[70,71].

a



b

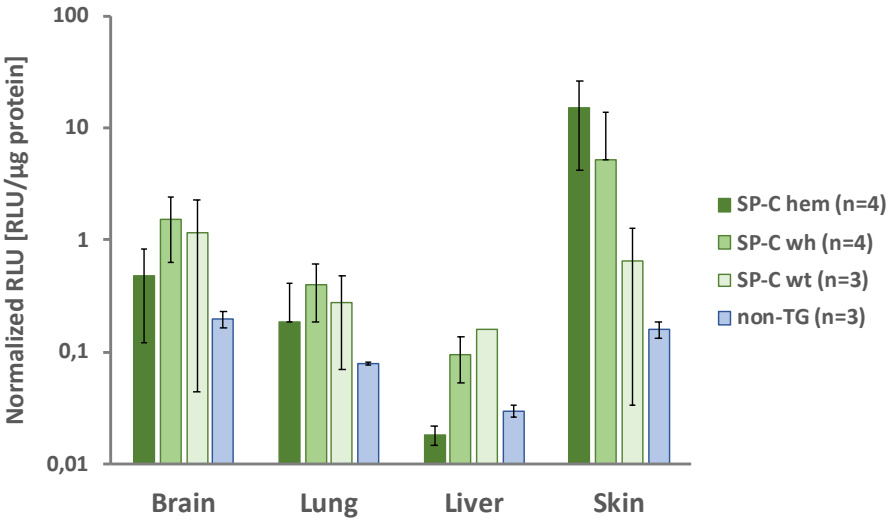


Figure 18. **Transgenic Sp-C-Luc mice experiments.** Luminescence of different tissues of transgenic Sp-C-Luc mouse strains was measured per well (10 µl). Logarithmic presentation: **a.** absolute values, **b.** normalized RLU. Luciferase activity was normalized according to the amount of protein per sample (500 µl). Dark green columns represent SP-C hem, lighter green columns represent SP-C wh, light green columns represent SP-C wt and blue columns represent non-TG. n = number of animals. Results are expressed as means ± SD.

In figure 19 and 20, an overview of all examined Tg Sp-C-Luc mice can be seen in detail. Figure 19 represents luminescence measured individually for each animal in one graph, including the average values of three non-transgenic mice as a background. Figure 20 shows normalized RLUs. Tg Sp-C-Luc mice which do not correspond to the respective genotype are marked as outliers with a red asterisk. Missing columns are marked with a red pound because protein amount could not be determined since the signal was out of the measure capacity. A detailed description of the obtained results and correlation of *ex vivo* luciferase assay with *in vivo* bioluminescence imaging and genotype will be discussed in 7.3.1.

The obtained values of examined Tg Sp-C-Luc mice can be seen separately for each mouse in detail in figure 24 (appendix).

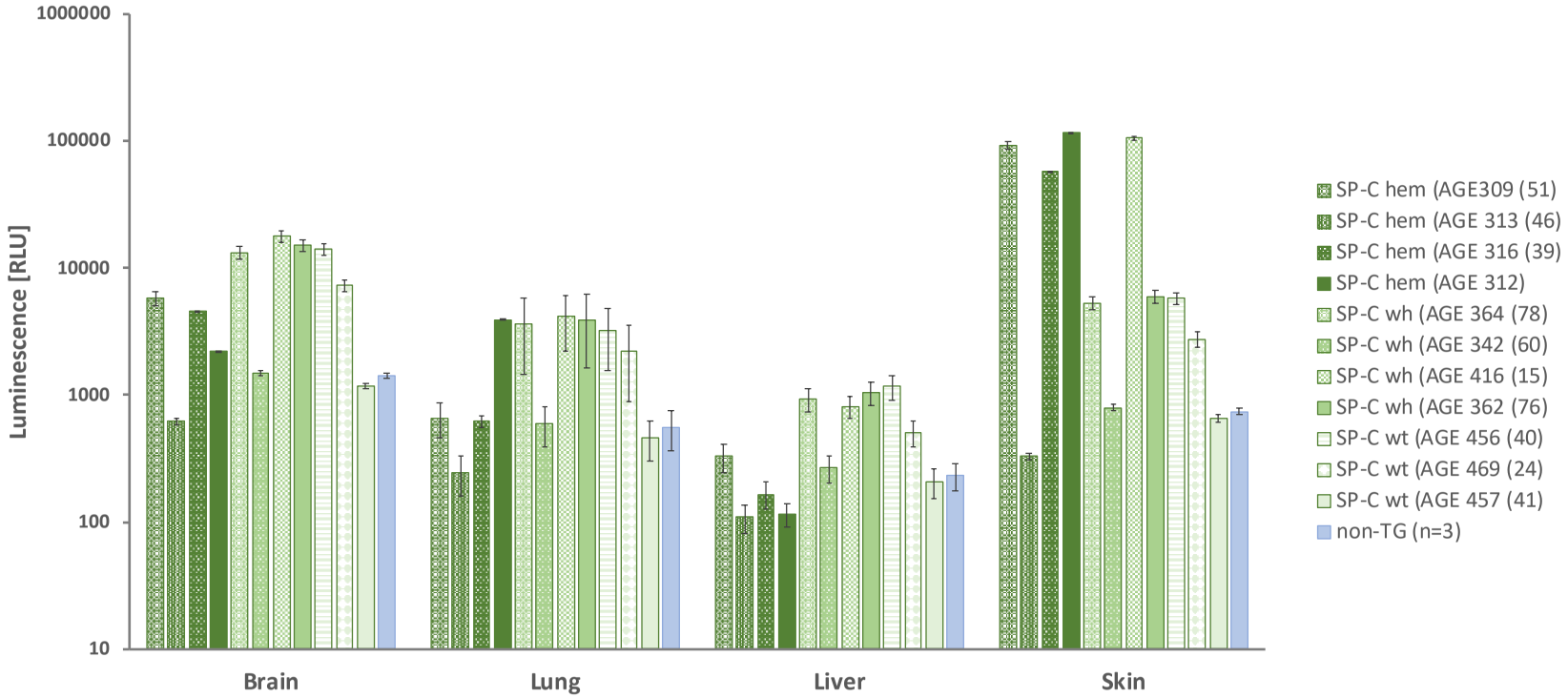


Figure 19. Overview of transgenic Sp-C-Luc mice experiments. Luminescence of different tissues measured from transgenic SP-C mouse strains, more detailed presentation. Non-TG values are expressed as mean ± SD (n=3).

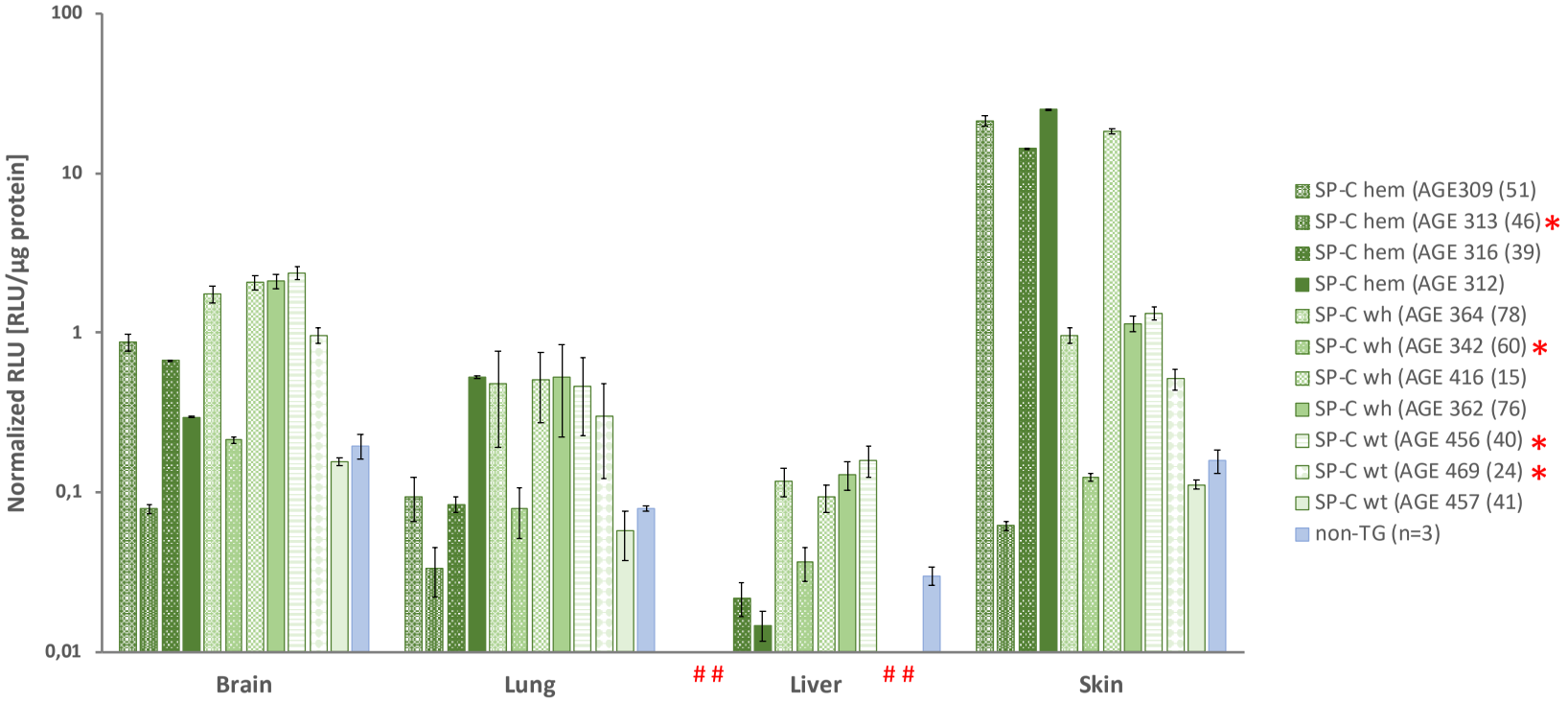


Figure 20. Overview of transgenic Sp-C-Luc mice experiments. Normalized luminescence of different tissues measured from transgenic SP-C mouse strains, more detailed presentation. Luciferase activity was normalized according to the amount of protein per sample (500 µ l). #: Protein amount could not be determined since signal was out of measure capacity. Outliers are marked with a red asterisk (*). Non-TG values are expressed as mean ± SD (n=3).

7.2.2 Transgenic mice with Thy-1.2 promoter driven luciferase

In our Thy-1.2 experiments we observed a similar distribution of RLUs of the different tissues as seen in the results of the RLUs of non-transgenic mice. The highest signal was detected for brain, followed by skin, lung and liver. Since Thy-1.2 promoter driven luciferase is specific for brain tissue, its activity is the highest measured in brain (Figure 21). This confirms our expectation.

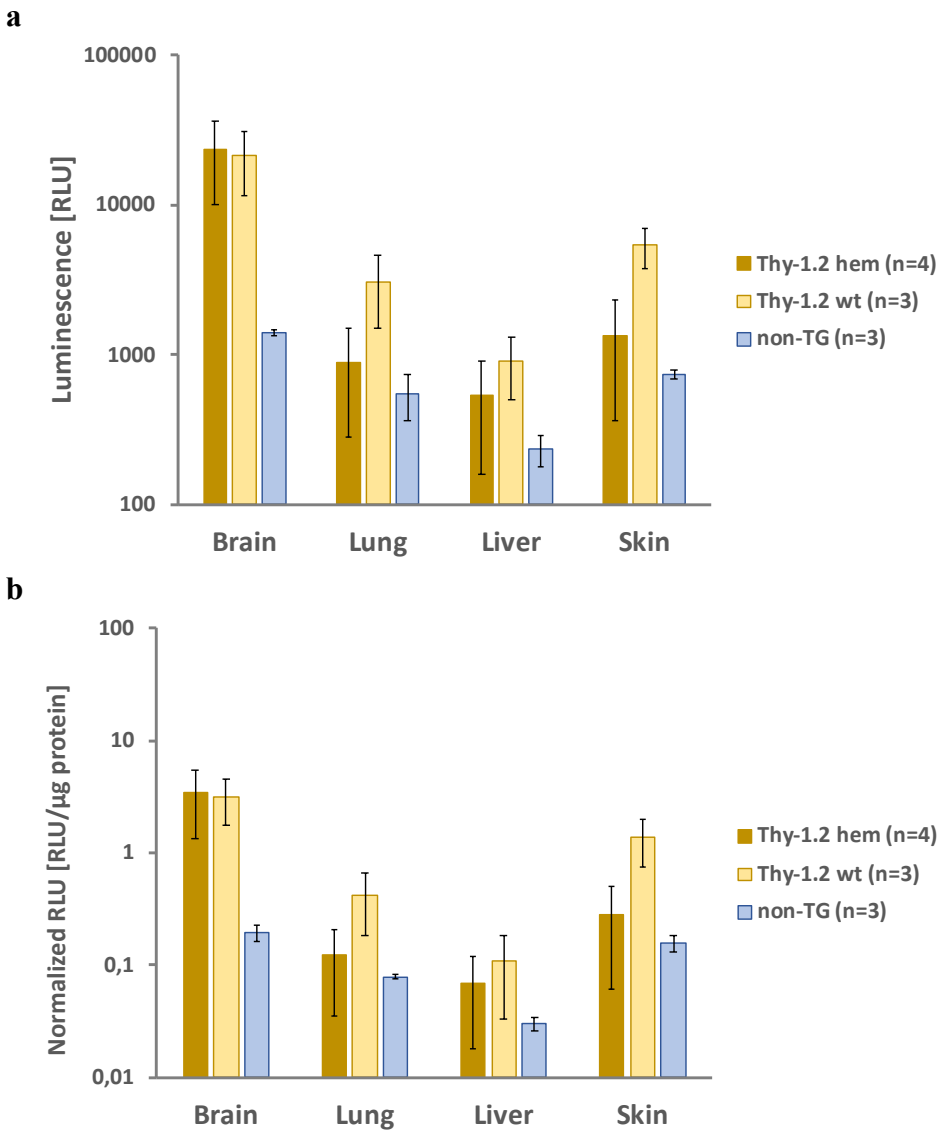


Figure 21. Transgenic Thy-1.2-Luc mice experiments. Luminescence of different tissues of transgenic Thy-1.2-Luc mouse strains was measured per well (10 µl). Logarithmic presentation: **a.** absolute values, **b.** normalized RLU. Luciferase activity was normalized according to the amount of protein per sample (500 µl). Dark yellow columns represent Thy-1.2 hem, yellow columns represent Thy-1.2 wt and blue columns represent non-TG. n = number of animals. Results are expressed as means ± SD.

In figure 22 and 23, an overview of all examined Tg Thy-1.2-Luc mice can be seen in detail. Figure 22 represents luminescence measured individually for each animal in one graph, including the average values of three non-transgenic mice as a background. Figure 23 shows normalized RLUs. Tg Thy-1.2-Luc mice which do not correspond to the respective genotype are marked as outliers with a red asterisk. Missing columns are marked with a red pound because protein amount could not be determined since the signal was out of the measure capacity. A detailed description of the obtained results and correlation of *ex vivo* luciferase assay with *in vivo* bioluminescence imaging and genotype will be discussed in 7.3.2.

The obtained values of examined Tg Thy-1.2-Luc mice can be seen separately for each mouse in detail in figure 25 (appendix).

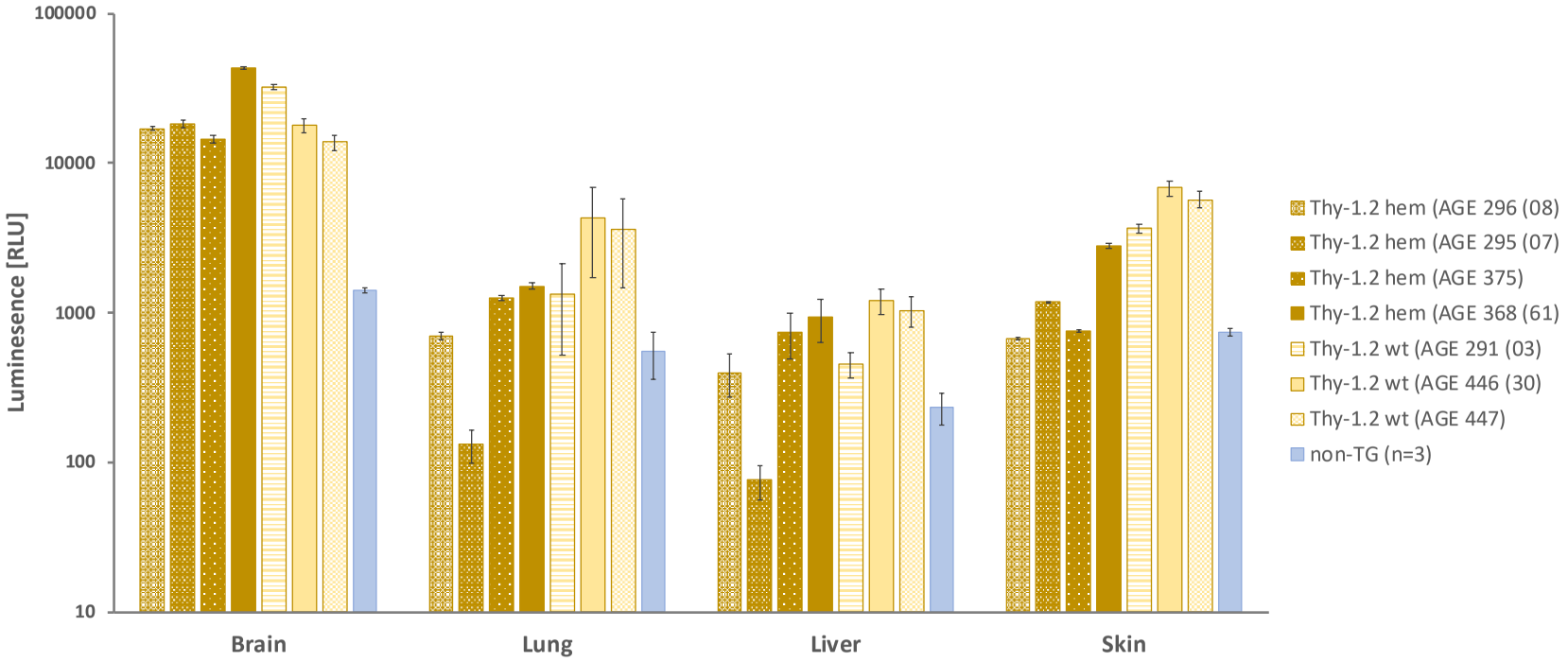


Figure 22. Overview of transgenic Thy-1.2-Luc mice experiments. Luminescence of different tissues measured from transgenic Thy-1.2 mouse strains, more detailed presentation. Non-TG values are expressed as mean ± SD (n=3).

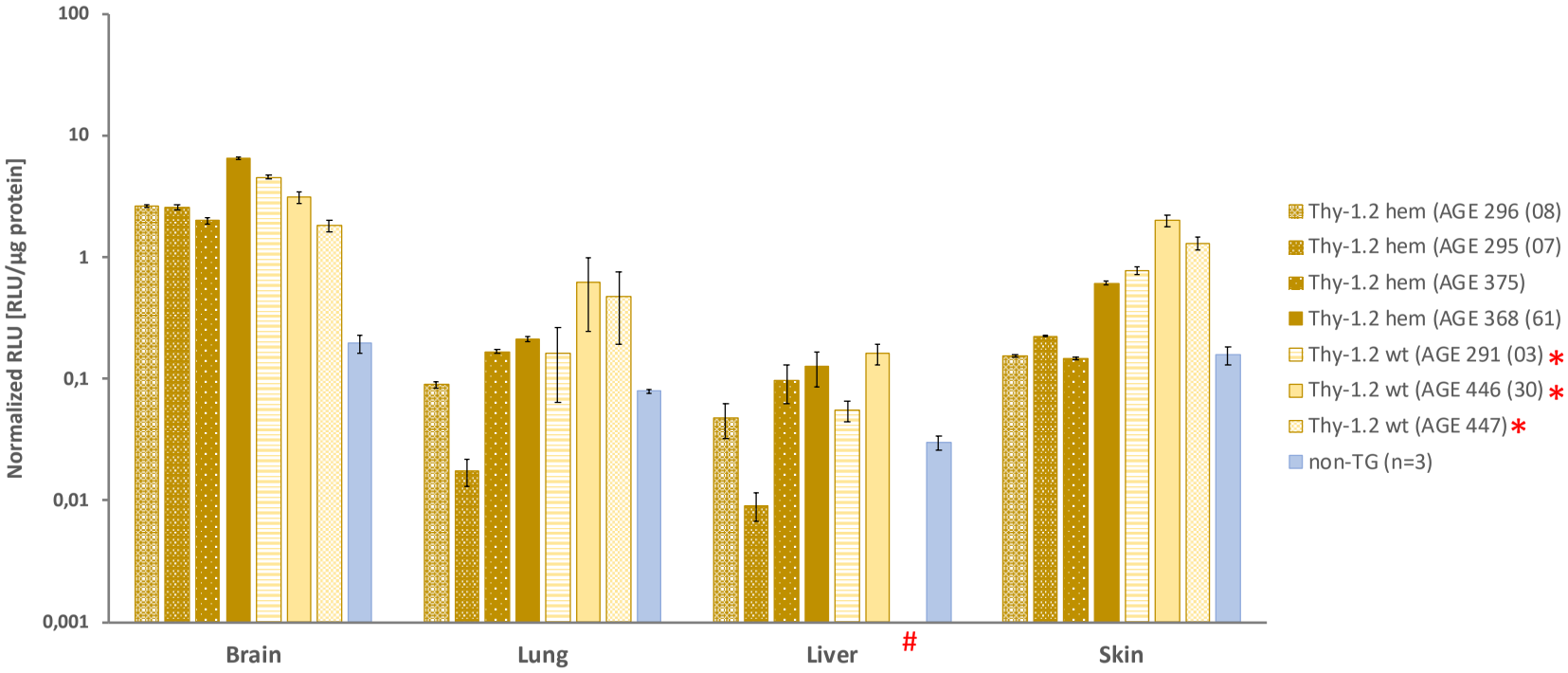


Figure 23. Overview of transgenic Thy-1.2-Luc mice experiments. Normalized luminescence of different tissues measured from transgenic Thy-1.2 mouse strains, more detailed presentation. Luciferase activity was normalized according the protein amount per sample (500 µl). #: Protein amount could not be determined since signal was out of measure capacity. Outliers marked with a red asterisk (*). Non-TG values are expressed as mean ± SD (n=3)

7.3 Correlation of *ex vivo* luciferase expression with *in vivo* bioluminescence imaging

As a last step we wanted to compare the results of our *ex vivo* assays with the results obtained from *in vivo* studies. The laboratory mice used for this work were genotypically characterized by PCR, in general, and phenotypically characterized before with 2D BLI by another member of the MMCT laboratory. The detailed list of used mice and their genotypic and phenotypic status can be seen in table 5 and table 6. In terms of non-TG mice, the status was not assessed since these mice were clearly negative. The remaining mice were phenotypically determined negative with a signal intensity lower than log6 and positive with a signal of log6 and log7, respectively.

7.3.1 Luciferase expression in Tg Sp-C-Luc mice

Firstly, there are no data available from 2D BLI for the black SP-C hem AGE 309 (51) and the white SP-C wt AGE 456 (40). However, for that SP-C hem mouse, our *ex vivo* assay shows high luciferase activity in skin, medium high activity in brain and liver and low activity in lung (Figure 20). This result is comprehensible since this Tg Sp-C-Luc mouse was genotypic examined as a hem mouse. Luciferase activity in mentioned SP-C wt mouse is relatively high in brain, lung and liver, but medium high in skin.

The negative phenotypic status of SP-C hem AGE 313 (46) correlates clearly with our homogenization assay. The low measured luciferase activity is comparable to the measured signal of non-transgenic mice in all tested tissues. Compared to genotypic status, this mouse is labeled as outlier. Furthermore, both SP-C hem AGE 316 (39) and AGE 312 mice show very high luminescence values for skin, but, compared to the signal of non-transgenic mice, low values for lung in AGE 316 and high in AGE 312, respectively. The phenotypic status of log6 of these both SP-C hem mice confirm our results.

One of the SP-C wh mouse, SP-C wh AGE 342 (60), shows distinct low luciferase activity in all tissues, although this mouse was genetically characterized as a wh and its phenotypic status was measured positive with log6. This may imply a general fault in homogenization process for this mouse. Thus, SP-C wh AGE 342 (60) is labeled as outlier. The remaining SP-C wh mice (AGE 364 (78) and AGE 362 (76)), which have a negative phenotypic status,

have comparable RLU values of luciferase activity in every tested tissue, except of SP-C wh AGE 416 (15) with a high activity in skin. This confirms the positive phenotypic status of log6 for this wh mouse.

The both SP-C wt mice show each low (AGE 457 (41) to medium high (AGE 469 (24) luciferase activity in brain, lung, liver and skin, compared to the measured signal of the non-TG mice. This correlates with the phenotypic status lower than log6. Relating to genotypic status, SP-C wt (AGE 456 (40) and (AGE 469 (24) are labeled as outliers, because of the relative high signal.

Table 5. Tg Sp-C-Luc mice in transgenic study. Mice used for application of optimized homogenization protocol and their genotypic and phenotypic status. #: phenotypic status not determined; **Ph log6** and **Ph log7**: phenotype positive, **Ph -**: phenotype lower than log6 and accordingly negative; **(b)**: black mouse, **(w)**: white mouse

Tg Sp-C-Luc mice used for transgenic study				
Mouse Strain	***Mouse ID***	***Genotype***	***Phenotype***	***ex vivo Assay compared to Genotype***
SP-C	AGE 309 (51)	hem	# (b)	as expected
SP-C	AGE 313 (46)	hem	Ph – (b)	outlier (↓ low signal)
SP-C	AGE 316 (39)	hem	Ph log6 (b)	as expected
SP-C	AGE 312	hem	Ph log6 (b)	as expected
SP-C	AGE 364 (78)	wh	Ph – (b)	as expected
SP-C	AGE 342 (60)	wh	Ph log6 (b)	outlier (↓ low signal)
SP-C	AGE 416 (15)	wh	Ph log6 (b)	as expected
SP-C	AGE 362 (76)	wh	Ph – (b)	as expected
SP-C	AGE 456 (40)	wt	# (w)	outlier (↑ high signal)
SP-C	AGE 469 (24)	wt	Ph – (w)	outlier (↑ high signal)
SP-C	AGE 457 (41)	wt	Ph – (w)	as expected

7.3.2 Luciferase expression in Tg Thy-1.2-Luc mice

In terms of Tg Thy-1.2-Luc can be said, that in all examined mice a high luciferase activity was measured in brain (Figure 23). The highest luminescence can be found in brain tissue of Thy-1.2 AGE 368 (61), which correlates with the highest, for this work, measured positive

phenotypic status of log7. Interestingly, both Thy-1.2 wt mice (AGE 446 (30) and AGE 447) with a negative phenotypic status (Table 6) show the highest luciferase activity in skin of all examined Tg Thy-1.2-Luc mice, in addition to the high signal in brain tissue. For Thy-1.2 wt (AGE 291 (03) we measured high luciferase activity in brain and skin, this correlates with the positive phenotypic status of log6. Compared to genotypic status, all three wt mice are labeled as outliers. A possible explanation for the high signals in all three Thy-1.2 wt mice could be a spillover of enzyme. The activity of luciferase enzyme in Thy-1.2 wt mice was measured in the same experiments as Thy-1.2 hem or SP-C hem mice. According to the pattern of the plate, the homogenate of wt mice were always pipet first, then the homogenate of hem mice. However, an overlap of signals during the measurement cannot be excepted completely.

Table 6. Thy-1.2-Luc mice in transgenic study. Mice used for application of optimized homogenization protocol and their genotypic and phenotypic status. **Ph log6** and **Ph log7**: phenotype positive, **Ph -**: phenotype lower than log6 and accordingly negative; **(b)**: black mouse, **(w)**: white mouse

Tg Thy-1.2-Luc mice used for transgenic study				
Mouse Strain	***Mouse ID***	***Genotype***	***Phenotype***	***ex vivo Assay compared to Genotype***
Thy-1.2	AGE 296 (08)	hem	Ph log6 (w)	as expected
Thy-1.2	AGE 295 (07)	hem	Ph log6 (w)	as expected
Thy-1.2	AGE 375	hem	Ph log6 (w)	as expected
Thy-1.2	AGE 368 (61)	hem	Ph log7 (w)	as expected
Thy-1.2	AGE 291 (03)	wt	Ph log6 (w)	outlier (↑ high signal)
Thy-1.2	AGE 446 (30)	wt	Ph – (w)	outlier (↑ high signal)
Thy-1.2	AGE 447	wt	Ph – (w)	outlier (↑ high signal)

8 Conclusion

In present study we mainly investigated the *ex vivo* activity of Firefly luciferase in tissues of the transgenic mouse strains Tg Sp-C-Luc and Tg Thy-1.2-Luc. For this, we firstly developed and optimized a homogenization protocol for specific mouse tissues. We compared our obtained *ex vivo* results with the respective genotype and phenotypic status.

11 transgenic Sp-C-Luc mice were investigated, of which 8 had a positive and 3 a negative genotype. One SP-C hem mouse (AGE 313 (46), one SP-C wh mouse (AGE 342 (60) and two SP-C wt mice (AGE 456 (40) and (AGE 469 (24) showed other results than expected from genotypic status. For this, these mice were labeled as outliers.

7 transgenic Thy-1.2-Luc mice were examined, of which 4 were genotypically positive and 3 were genotypically negative. All 4 Thy-1.2 hem mice confirmed their genotype. The 3 Thy-1.2 wt mice showed high signals and were labeled as outliers. Only Thy-1.2 wt (AGE 291 (03) showed a high signal for phenotypic status, as well.

Generally speaking, a correlation between our *ex vivo* assays and observations of *in vivo* studies is visible. In some cases, the results of our homogenization assay correspond clearly with the phenotypic status. On the other hand, analyses of certain mice, like SP-C wh AGE 342 (60), show different results as expected from the phenotypic characterization. A similar observation of outliers did a colleague of the MMCT in her study. She imaged mice of the same transgenic strains using 2D BLI, in order to determine their phenotypic status.[72]

A possible reason for the variability in present study could be found in the homogenization process, although the homogenization protocol has been optimized successfully by using different parameters. As known, it is important to work as fast as possible and under cool conditions. Besides to prechill the used buffer and microcentrifuge tubes and other equipment that was used, the most important approach is actually to cool during homogenization. For this work, we used the Precellys 24 homogenizer device of another laboratory from the department. This Precellys 24 homogenizer did not come with a cooling device to protect the luciferase enzyme from possible heat degradation during homogenization. At least it is better to move all equipment in a cold room and to carry out all operations at 0-4°C.

Apart from technical requirements as a possible reason for unexpected results in our study, a general variance between individuals may play a role. Although the chosen Precellys lysing kit CKMix is suitable for soft and hard tissue homogenization[69], lung tissue is tougher compared to brain or liver, thus the rate of completely homogenized tissue can be reduced. The extent of luciferase release and, accordingly, its activation can vary. Further experiments with more tissue samples should be done to obtain more significant results.

As Colin et al.[60] reported, haemoglobin may cover the detection of luciferase activity. For further studies, it would be interesting to investigate, how a remove of haemoglobin would

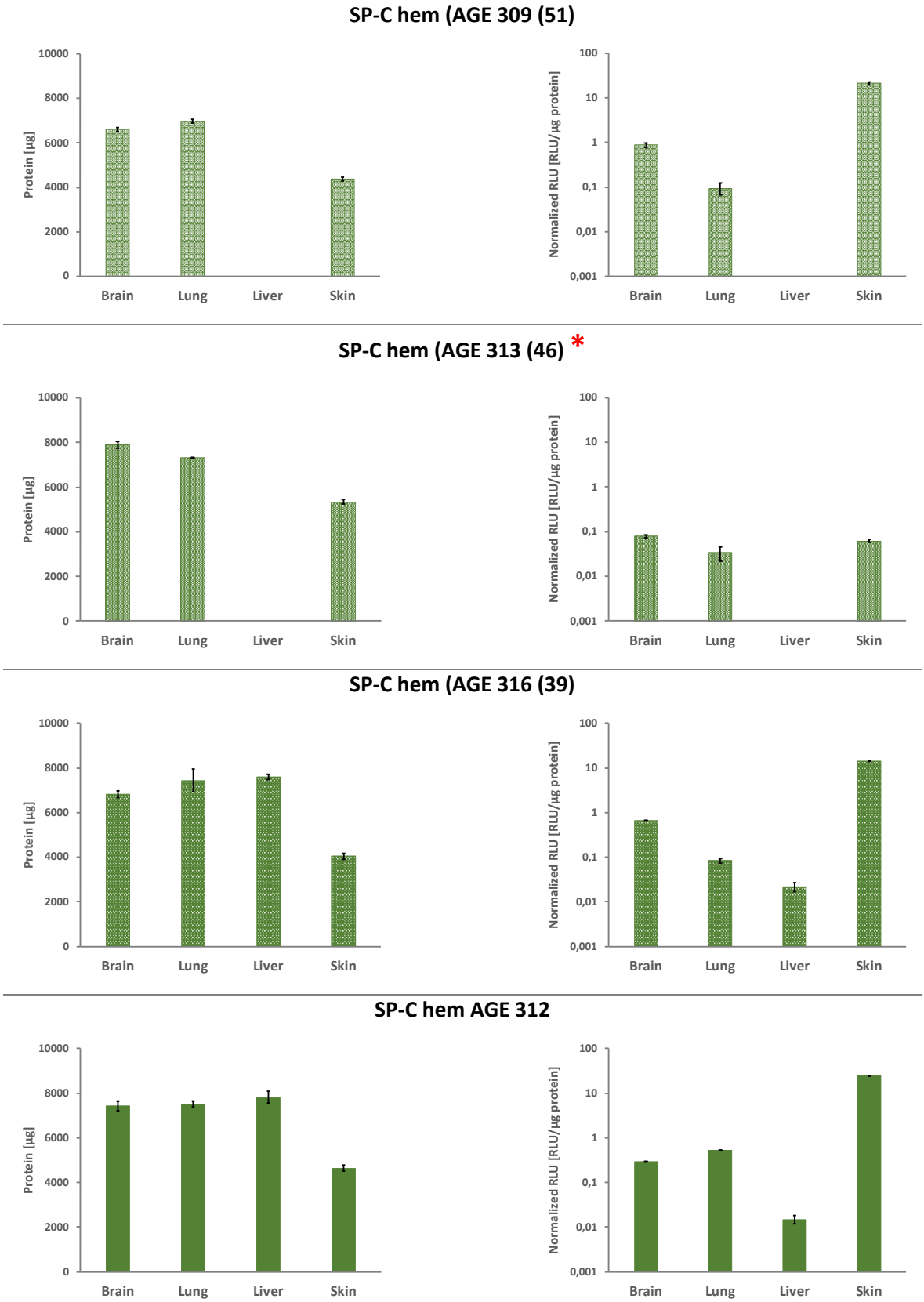
work for our homogenization assay and if this enhance the measurement of luciferase activity.

9 Appendix

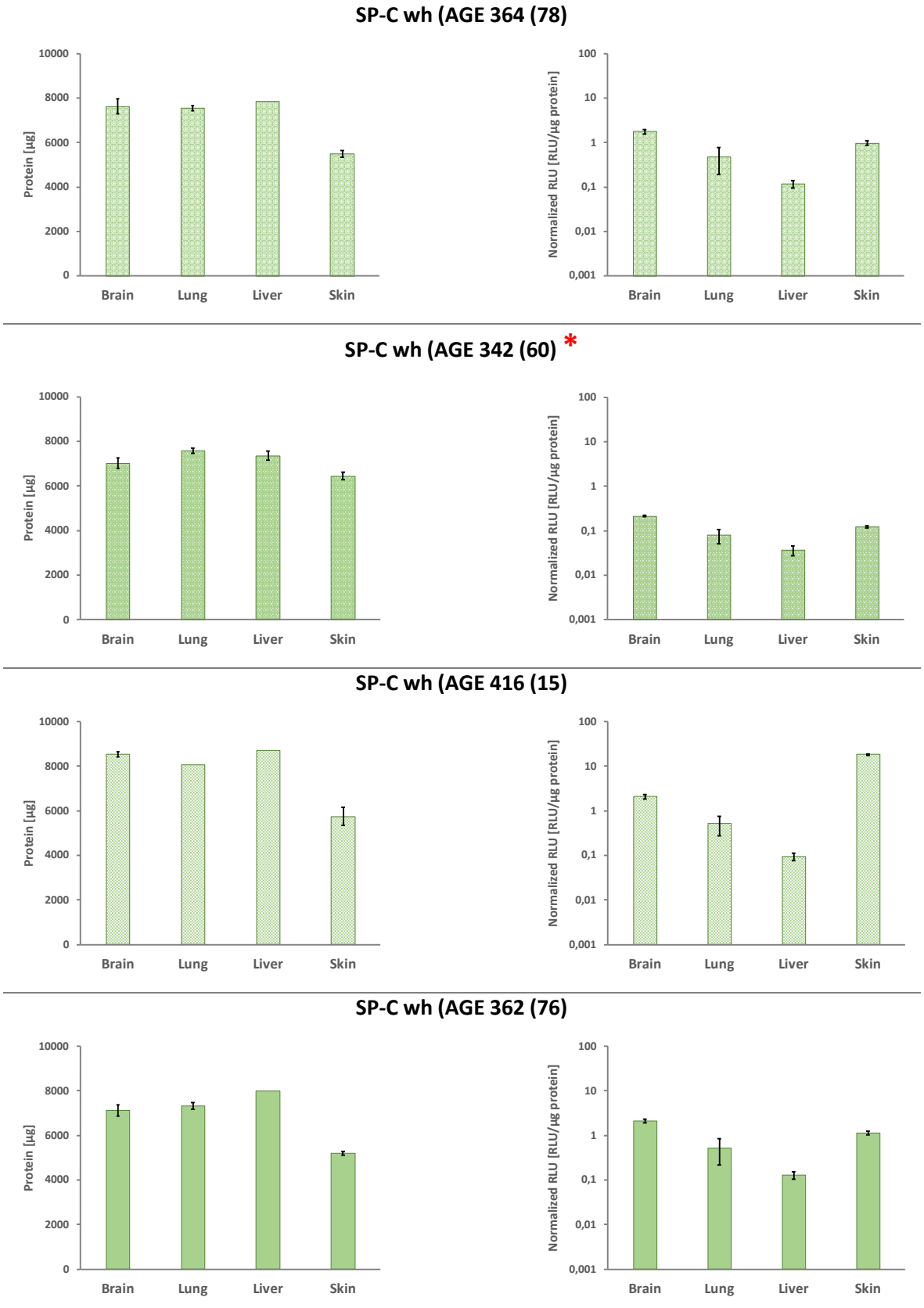
9.1 Transgenic mice with SP-C promoter driven luciferase - Graphs

The following graphs are a detailed presentation of the obtained results of Firefly assay based luciferase expression for Tg Sp-C-Luc mice.

a


SP-C hem (AGE 309 (51)
Protein [µg]
10000
8000
6000
4000
2000
0
Brain
Lung
Liver
Skin
Normalized RLU [RLU/µg protein]
100
10
1
0,1
0,01
0,001
SP-C hem (AGE 313 (46) *
SP-C hem (AGE 316 (39)
SP-C hem AGE 312

b


SP-C wh (AGE 364 (78)
Protein [µg]
10000
8000
6000
4000
2000
0
Brain
Lung
Liver
Skin
Normalized RLU [RLU/µg protein]
100
10
1
0,1
0,01
0,001
SP-C wh (AGE 342 (60) *
SP-C wh (AGE 416 (15)
SP-C wh (AGE 362 (76)

c

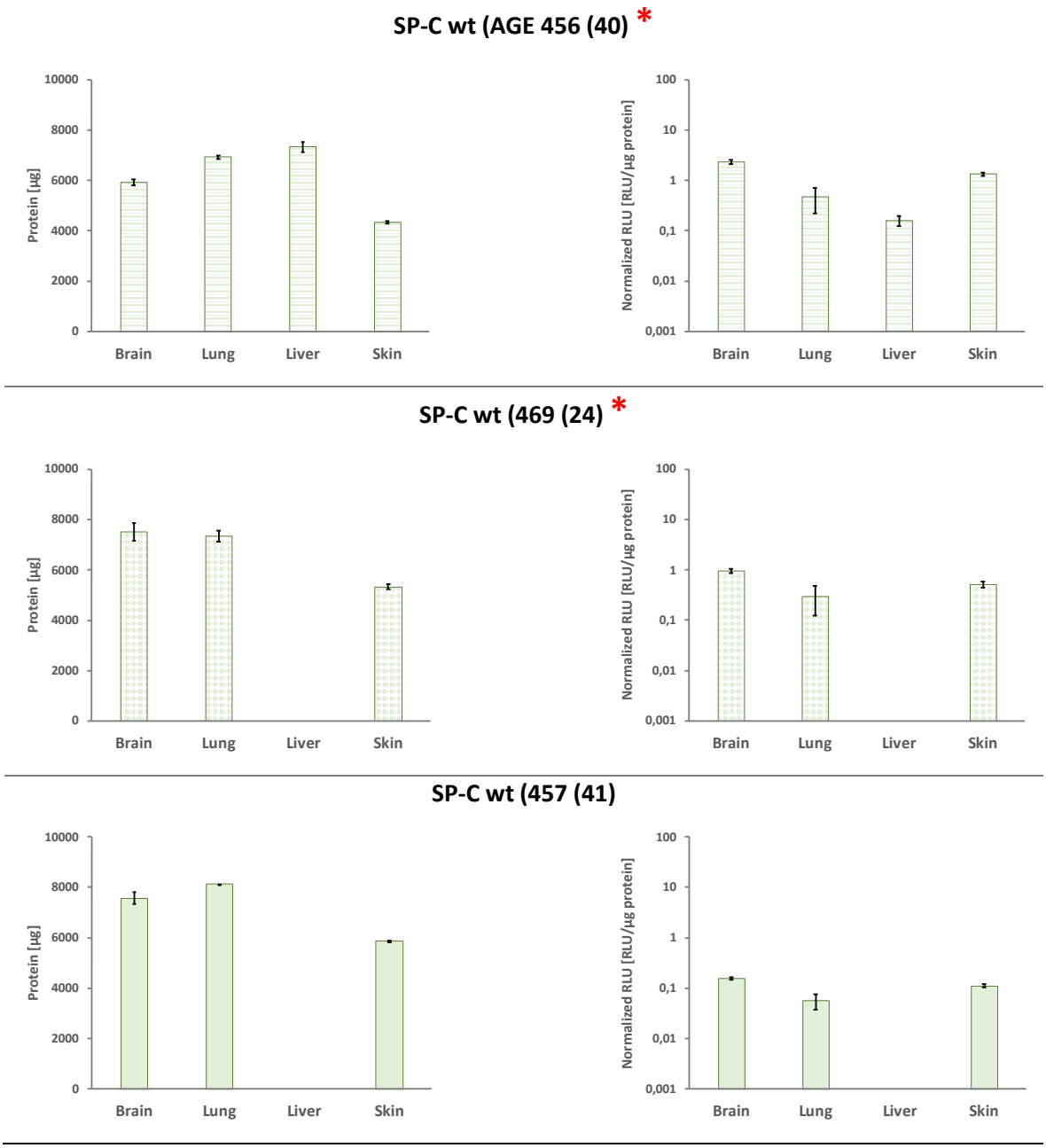
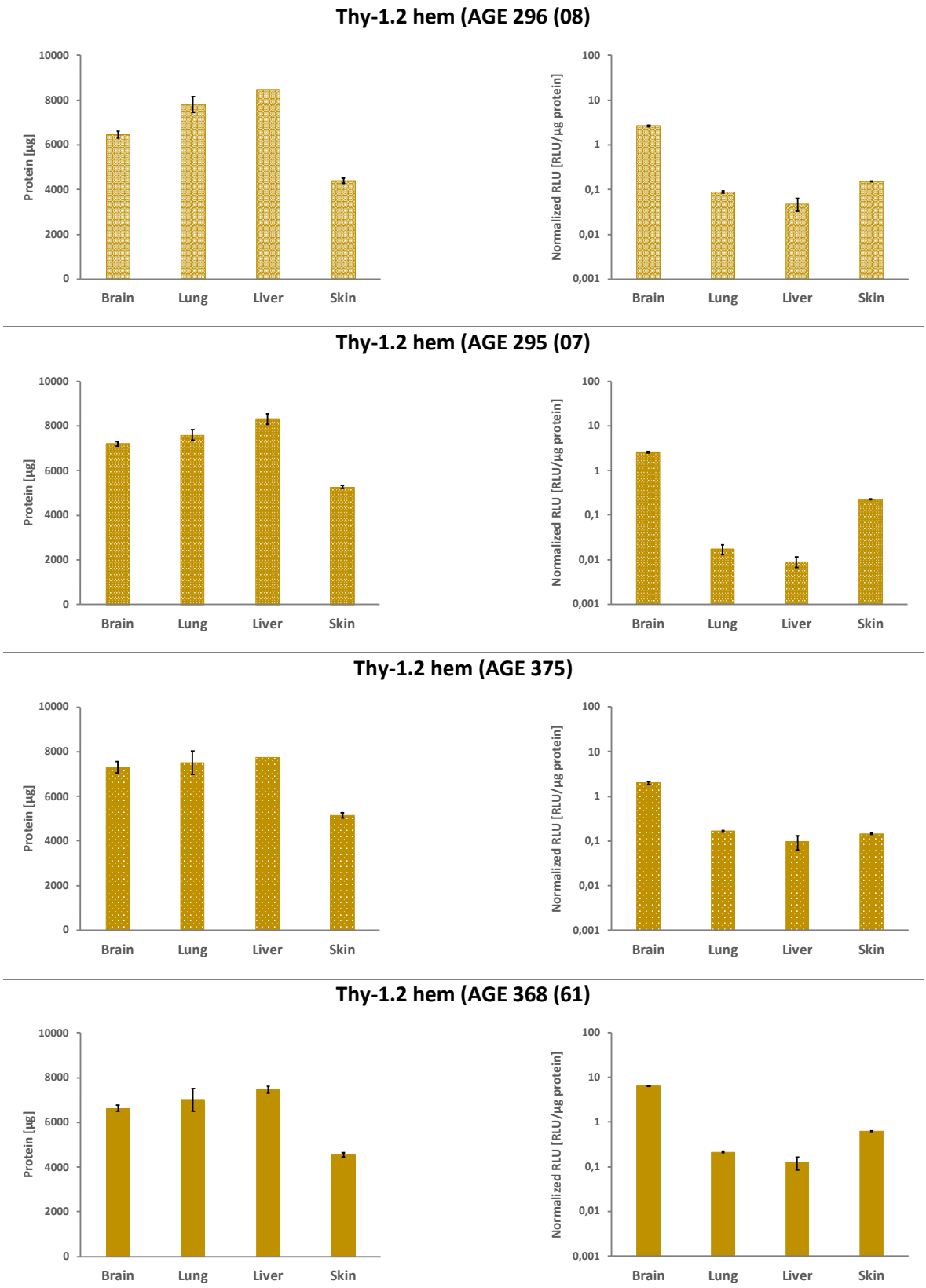


Figure 24. Transgenic Sp-C-Luc mice experiments. Protein amount per sample (500 µl) of all examined Tg Sp-C mice (**a**. SP-C hem, **b**. SP-C wh, **c**. SP-C wt). Luciferase activity was normalized according to the amount of protein per sample (500 µl). The absence of columns means the values were outside the measure capacity of the device. The absence of SD means insufficient measured values (only one measured value was available). Outliers are marked with a red asterisk (*).

9.2 Transgenic mice with Thy-1.2 promoter driven luciferase - Graphs

The following graphs are a detailed presentation of the obtained results of Firefly assay based luciferase expression for Tg Thy-1.2-Luc mice.

a


Thy-1.2 hem (AGE 296 (08)
Protein [µg]
10000
8000
6000
4000
2000
0
Brain
Lung
Liver
Skin
Normalized RLU [RLU/µg protein]
100
10
1
0,1
0,01
0,001
Thy-1.2 hem (AGE 295 (07)
Thy-1.2 hem (AGE 375)
Thy-1.2 hem (AGE 368 (61)

b

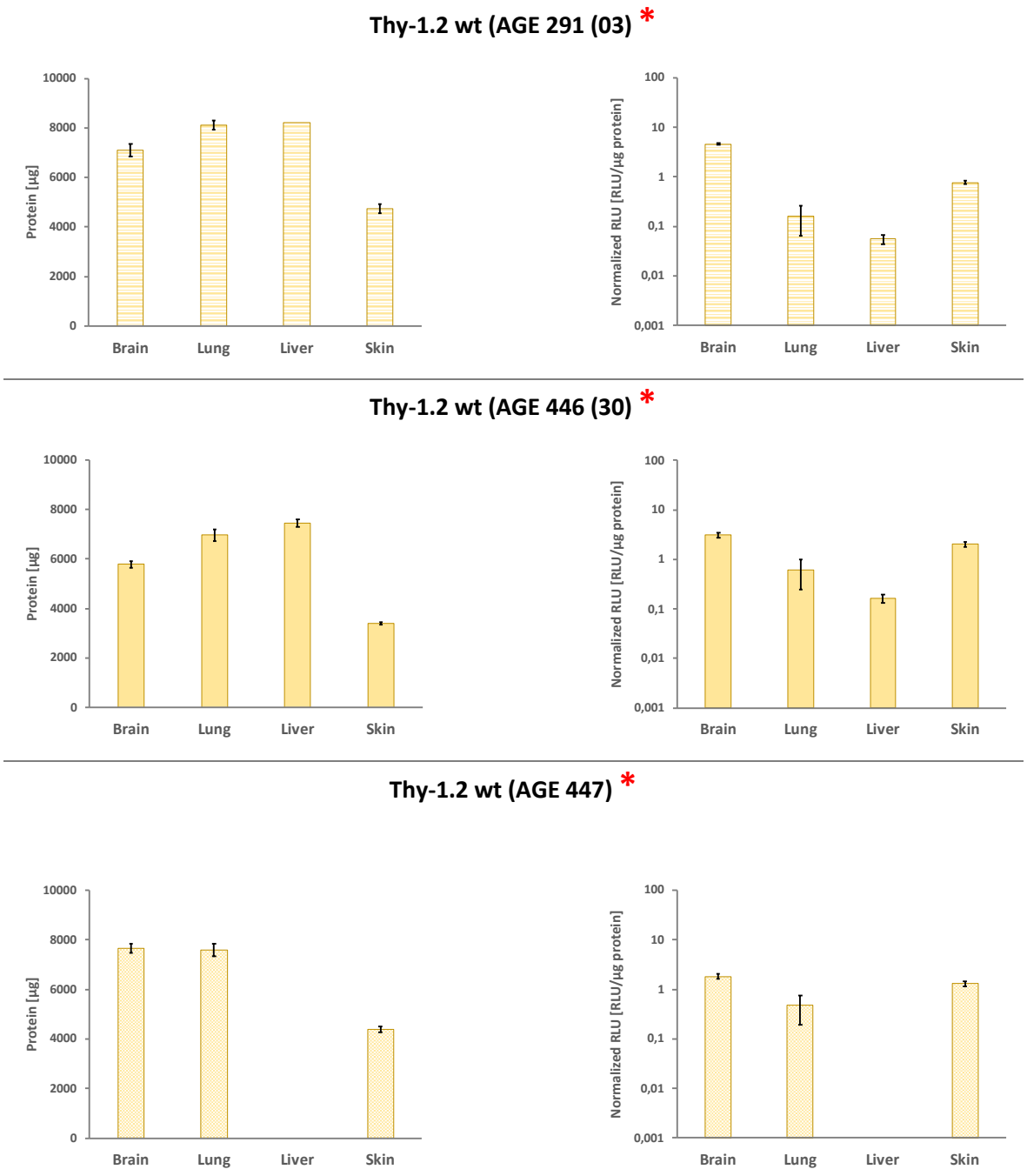


Figure 25. Transgenic Thy-1.2-Luc mice experiments. Protein amount per sample (500 µl) of all examined Tg Thy-1.2-Luc mice (**a**. Thy-1.2 hem, **b**. Thy-1.2 wt). Luciferase activity was normalized according to the amount of protein per sample (500 µl). The absence of columns means the values were outside the measure capacity of the device. The absence of SD means insufficient measured values (only one measured value was available). Outliers are marked with a red asterisk (*).

9.3 Application of optimized homogenization protocol for luciferase based transgenic mice – standard curves

The following standard curves were generated and used during application of optimized homogenization protocol.

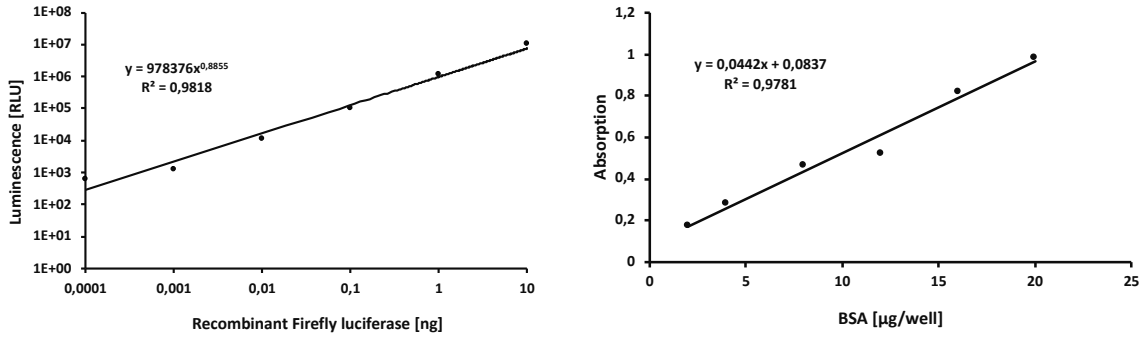


Figure 26. Recombinant Firefly luciferase and BSA standard curve. Examined mice: **SP-C wt (AGE 456 (40)** and **SP-C hem (AGE 309 (51)**

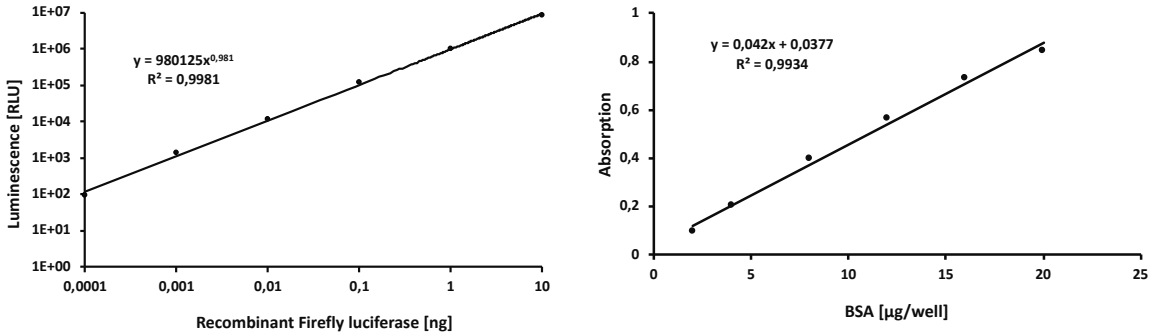


Figure 27. Recombinant Firefly luciferase and BSA standard curve. Examined mice: **SP-C wt (AGE 469 (24)** and **SP-C hem (AGE 313 (46)**

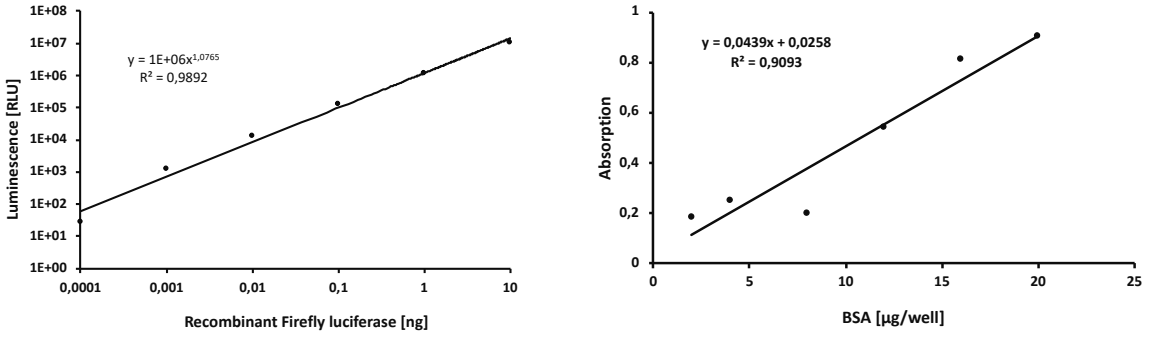


Figure 28. Recombinant Firefly luciferase and BSA standard curve. Examined mice: **SP-C wh (AGE 364 (78)** and **SP-C wh (AGE 342 (60)**

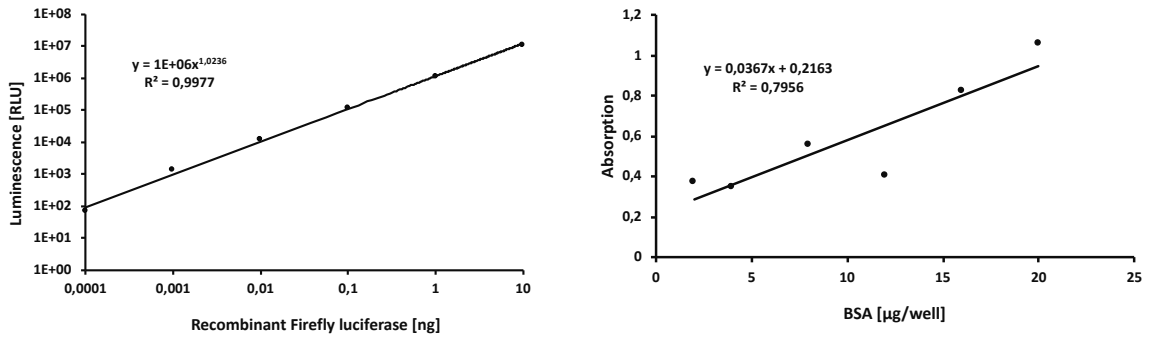


Figure 29. Recombinant Firefly luciferase and BSA standard curve. Examined mice: **SP-C wh (AGE 416 (15)** and **SP-C wt (AGE 457 (41)**

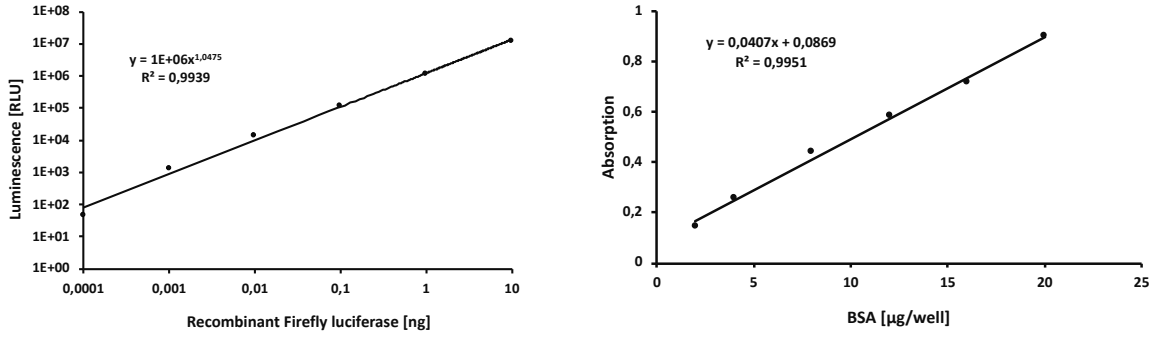


Figure 30. Recombinant Firefly luciferase and BSA standard curve. Examined mice: **non-TG (MCT 83)** and **non-TG (MCT 84)**

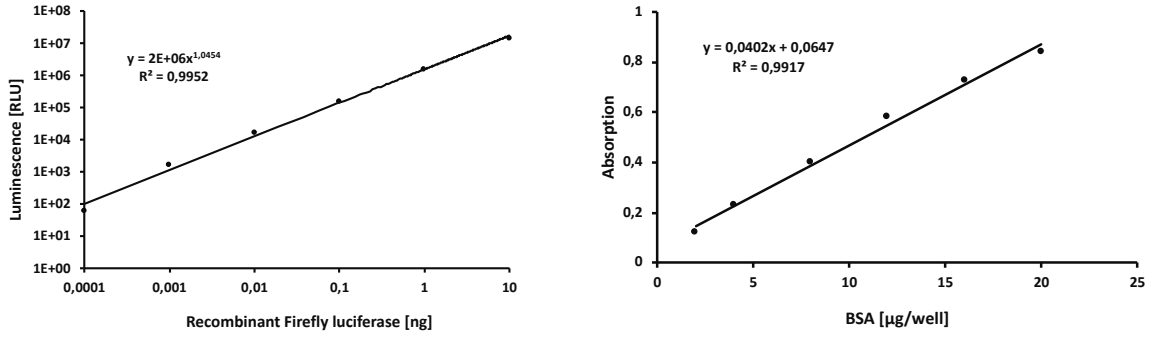


Figure 31. Recombinant Firefly luciferase and BSA standard curve. Examined mice: **Thy-1.2 wt (AGE 291 (03)**, **Thy-1.2 hem (AGE 296 (08)** and **Thy-1.2 hem (AGE 295 (07)**

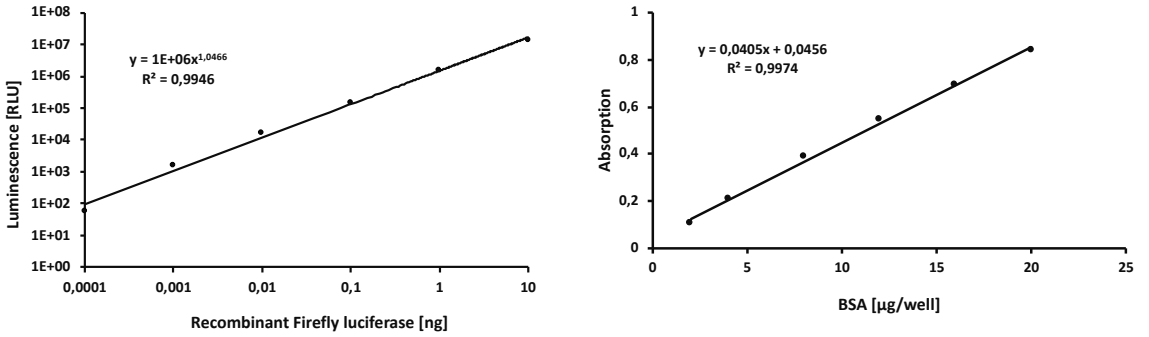


Figure 32. Recombinant Firefly luciferase and BSA standard curve. Examined mice: **non-TG MCT 77 (05)** and **non-TG MCT 78 (06)**

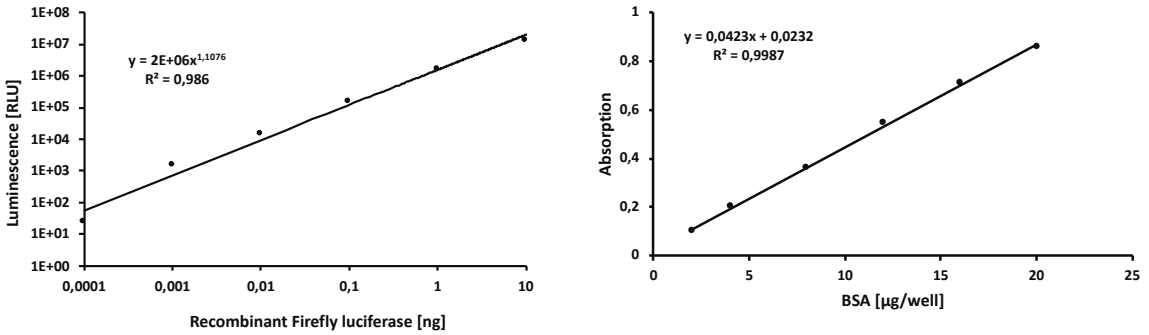


Figure 33. Recombinant Firefly luciferase and BSA standard curve. Examined mice: **Thy-1.2 wt (AGE 446 (30)**, **Thy-1.2 hem (AGE 368 (61)** and **SP-C hem (AGE 316 (39)**

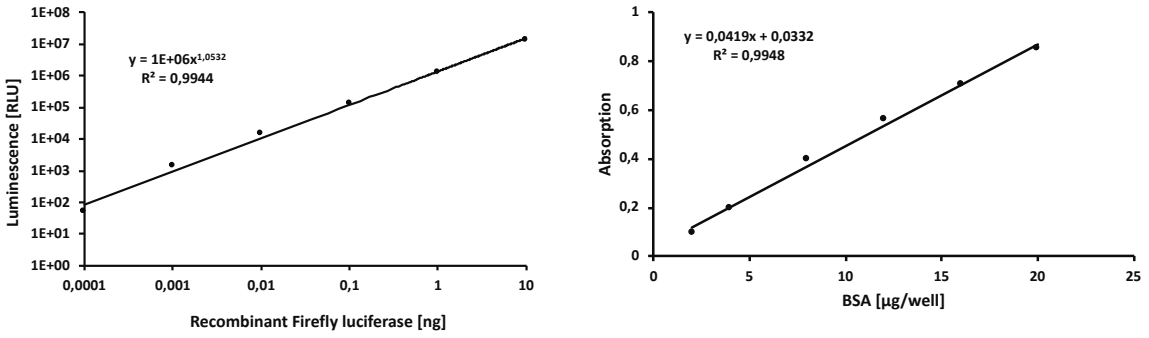


Figure 34. Recombinant Firefly luciferase and BSA standard curve. Examined mice: **Thy-1.2 wt (AGE 447)**, **Thy-1.2 hem (AGE 375)** and **SP-C hem (AGE 312)**

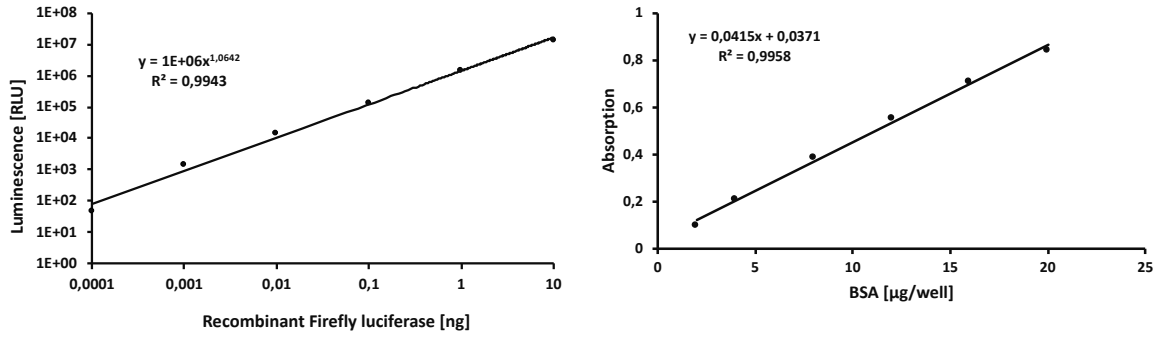


Figure 35. Recombinant Firefly luciferase and BSA standard curve. Examined mice: **SP-C wh (AGE 362 (76)** and **non-TG (MCT 79 (07)**

9.4 Standard operating procedures

SOP – Recombinant Firefly luciferase assay standard curve

Materials:

- Stock C (100 ng/µl) of recombinant Firefly luciferase (rFFluc)
- Cell Culture Lysis Reagent 5X (CCLR)
- Bovine Serum Albumin (BSA 2 mg/ml)
- MQ-water
- White 96-wellplate, flat bottom

Preparation for rFFluc standard curve:

1. At least 2 h before measurement: thaw appropriate number of frozen LBL-aliquots stored in -80°C in LBL-box

(*Note*: 100 µl injection volume/well + 2 ml for priming the injector)

→ do not thaw LBL more than two times

2. Thaw CCLR 5X for 20 min. and appropriate amount of BSA
3. Thaw dilution no. 1 at 4°C 20 min. prior to usage

or prepare new dilutions of no.1 out of stock C and aliquot them, store at -80°C

4. Switch ON plate reader
5. Prepare necessary amount of CCLR 1X (BSA 1mg/ml):
    - mix BSA with MQ-water to obtain a BSA concentration of 1,25 mg/ml
    - prepare CCLR 1X (BSA 1 mg/ml) by mixing 1 volume of CCLR 5X with 4 volumes of BSA (1,25 mg/ml)
6. Prepare rFFluc standard curve dilutions (pipet into wells of a white plate) and store at 4°C until measurement

Dilution ID	Volume from stock C (100 ng/µl)	CCLR 1X (BSA 1mg/ml)	Final volume	Concentration	Volume to be used for rFFluc assay	Amount of enzyme in 10 µl	measured yes/no
1	2 µl	18 µl	50 µl	10 ng/µl	10 µl	100 ng	n
1:10 dilution series							
2	5 µl from 1	45 µl	50 µl	1 ng/µl	10 µl	10 ng	y
3	5 µl from 2	45 µl	50 µl	0,1 ng/µl	10 µl	1 ng	y
4	5 µl from 3	45 µl	50 µl	0,01 ng/µl	10 µl	0,1 ng	y
5	5 µl from 4	45 µl	50 µl	0,001 ng/µl	10 µl	0,01 ng	y
6	5 µl from 5	45 µl	50 µl	0,1 pg/µl	10 µl	0,001 ng	y
7	5 µl from 6	45 µl	50 µl	0,01 pg/µl	10 µl	0,1 pg	y
8	-	50 µl	50 µl	0	10 µl	0	y

SOP – Organ homogenization

Materials:

- Tweezers
- Cell Culture Lysis Reagent 5X (CCLR)
- Bovine Serum Albumin (BSA 2 mg/ml)
- Preceyllys Lysing Kit tubes (CKMix, 1.4 / 2.8 mm, 2 ml)

Method:

1. Thaw required organs after taking out of -80°C or get fresh organs directly from animal house

Note: all steps should be done under cool conditions

2. Prepare necessary amount of CCLR 1X (BSA 1mg/ml):
    - mix BSA with MQ-water to obtain a BSA concentration of 1,25 mg/ml
    - prepare CCLR 1X (BSA 1 mg/ml) by mixing 1 volume of CCLR 5X with 4 volumes of BSA (1,25 mg/ml)
3. Weigh organs and transfer them in new microcentrifuge tubes
4. Cut organs on the cooling plate by using a scalpel (use aluminum foil as padding)
5. Transfer approx. 200mg of tissue in lysing kit
6. Add 500 µl of CCLR 1X (BSA) and weigh full kit tubes (for balance)
7. Homogenize with Precellys 24 device (Lab of Dr. Haslberger, Ernährungswissenschaften)
8. Set up following adjustments:
    - For brain: 6500-2x15-15
    - For lung, liver and skin: 6500-2x10-15
9. Transfer tissue homogenate to new microcentrifuge tubes
10. Centrifuge with Micro Star centrifuge: 13,3 RMP, 4°C, 10 min.
11. Use clear supernatant for further measurements (e.g. luminescence or absorption)

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