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Uptake kinetics and biodistribution of ^{14}C -D-luciferin—a radiolabeled substrate for the firefly luciferase catalyzed bioluminescence reaction: impact on bioluminescence based reporter gene imaging

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Abstract

Purpose—Firefly luciferase catalyzes the oxidative decarboxylation of D-luciferin to oxyluciferin in the presence of cofactors, producing bioluminescence. This reaction is used in optical bioluminescence-based molecular imaging approaches to detect the expression of the firefly luciferase reporter gene. Biokinetics and distribution of the substrate most likely have a significant impact on levels of light signal and therefore need to be investigated.

Methods—Benzene ring ^{14}C (U)-labeled D-luciferin was utilized. Cell uptake and efflux assays, murine biodistribution, autoradiography and CCD-camera based optical bioluminescence imaging were carried out to examine the in vitro and in vivo characteristics of the tracer in cell culture and in living mice respectively.

Results—Radiolabeled and unlabeled D-luciferin revealed comparable levels of light emission when incubated with equivalent amounts of the firefly luciferase enzyme. Cell uptake assays in pCMV-luciferase-transfected cells showed slow trapping of the tracer and relatively low uptake values (up to 22.9-fold higher in firefly luciferase gene-transfected vs. nontransfected cells,

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$p=0.0002$). Biodistribution studies in living mice after tail-vein injection of ^{14}C -D-luciferin demonstrated inhomogeneous tracer distribution with early predominant high radioactivity levels in kidneys (10.6% injected dose [ID]/g) and liver (11.9% ID/g), followed at later time points by the bladder (up to 81.3% ID/g) and small intestine (6.5% ID/g), reflecting the elimination routes of the tracer. Kinetics and uptake levels profoundly differed when using alternate injection routes (intravenous versus intraperitoneal). No clear trapping of ^{14}C -D-luciferin in firefly luciferase-expressing tissues could be observed in vivo.

Conclusions—The data obtained with ^{14}C -D-luciferin provide insights into the dynamics of D-luciferin cell uptake, intracellular accumulation, and efflux. Results of the biodistribution and autoradiographic studies should be useful for optimizing and adapting optical imaging protocols to specific experimental settings when utilizing the firefly luciferase and D-luciferin system.

Keywords

Optical imaging; Luciferase; D-Luciferin; Reporter Genes; Biodistribution

Introduction

Optical imaging techniques, either fluorescence- or bioluminescence-based (e.g., green fluorescence protein [GFP]-based fluorescence microscopy, luciferase-based benchtop luminometry), have been in use for a long time for in vitro applications in molecular and cellular biology [1]. An extension of this concept toward noninvasive in vivo imaging with light photons represents an interesting approach for collecting relevant biological information from small living subjects. In molecular imaging, visualization of reporter gene expression is used for noninvasive monitoring of gene expression in living subjects in various experimental settings [2]. Optical imaging allows for a relatively low-cost alternative for studying reporter gene expression in small animal models compared to other strategies such as positron emission tomography (PET) [3].

One key advantage of optical bioluminescence imaging is that it can be used to detect very low levels of signal because the light emitted from a sample in the dark chamber of a charged coupled device (CCD) camera is—in contrast to fluorescence imaging—virtually background-free. It is quick and easy to perform and allows rapid testing of biological hypotheses in living experimental small animal models.

Luciferases consist of a wide range of enzymes that catalyze the oxidation of substrate luciferins to yield nonreactive oxyluciferins and the release of photons of light that may be detected, collected, and quantified externally. Some luciferins require the presence of a cofactor to undergo oxidation, such as Ca^{2+} or adenosine triphosphate (ATP). Other systems like the sea pansy *Renilla* luciferase which uses a different substrate (coelenterazine) and is not ATP dependent as well as the *Gaussia* luciferase system have been recently validated for applications in living subjects [4, 5].

The most common luciferase–luciferin system used in molecular imaging is that derived from the firefly *Photinus pyralis* [6]. The firefly luciferase gene (*fluc*) encodes for firefly luciferase (FL), an enzyme that oxidizes its substrate D-luciferin in the presence of ATP,

magnesium, and oxygen to result in light emission (bioluminescence, Fig. 1a). The system has several favorable characteristics: It does not need external light excitation, the fast rate of enzyme turnover in the presence of substrate D-luciferin allows for real-time measurements, and the relationship between the enzyme concentration and the peak height of emitted light in vitro is linear up to seven to eight orders of magnitude. These properties potentially allow for sensitive, quantitative non-invasive imaging of fluc expression in living subjects [7].

In vitro transgene expression assays were utilizing the fact that light emission is proportional to the concentration of FL when substrate is provided in excess. However, for in vivo experiments aimed to image fluc expression in various organs after systemic administration of D-luciferin, a concern for fluc-based gene imaging is whether local substrate delivery/distribution can affect luminescence intensity.

In this work, we report cell uptake characteristics of a fully functional, ^{14}C -labeled D-luciferin substrate in native and fluc-expressing cells in cell culture. We examine biodistribution patterns of ^{14}C -labeled D-luciferin in mice using different injection routes (intravenous/intraperitoneal) of the tracer. The in vitro and in vivo biodistribution data are substantiated by autoradiographic studies. Finally, biodistribution data in mice with adenoviral-mediated fluc expression are discussed in context of the cell culture results and efforts to work toward a multimodality optical/ radiotracer-based reporter probe system.

Materials and methods

Production of ^{14}C -D-luciferin

Benzene ^{14}C (U) luciferin was obtained from Moravek Biochemicals (<http://www.moravek.com>) with a specific activity of 2.11 GBq/mmol and a radiochemical purity of 97.0%. The tracer was synthesized by this company following the pathway described in detail in [8]. Condensation of *p*-anisidine [^{14}C] with carbamoyl thiocarbonyl thioacetic acid was the critical step in this synthesis. In the end product, D-luciferin [benzene ring- ^{14}C (U)]-, approximately one carbon atom of the benzene ring per molecule was randomly replaced by ^{14}C , but no foreign atom or charge was introduced into the molecule (Fig. 1b).

Verification of the functionality of ^{14}C -D-luciferin in buffer solution

To a buffer solution with fixed amounts of ATP and MgSO_4 and rising amounts of FL concentrations (from 64 to 400 ng/ μl), a fixed amount of ^{14}C -labeled or unlabeled D-luciferin solution (125 ng) was added, and the bioluminescence signal was measured using a Luminometer (Turner 20/20, Turner, Sunnyvale, CA, USA).

The bioluminescence signal of labeled and unlabeled D-luciferin, measured in relative light units per minute (RLU/min), were then compared in samples with same amounts of FL. The buffer ingredients used in this experiment were the following: 20 mM tricine, 2.5 mM MgSO_4 , 0.1 mM ethylenediamine tetraacetic acid, 33.3 mM dithiothreitol, 530 μM ATP, and 270 μM coenzyme A (CoA)-Li salt. The buffer was prepared at room temperature,

CoA–lithium salt and potassium Luc were added to the solution after the pH was adjusted to 7.8.

Verification of the functionality of ^{14}C -D-luciferin in cell culture

To test the functionality of ^{14}C -D-luciferin in cell culture, 2.5×10^5 293T cells were plated in 12-well plates in modified Eagle's medium (MEM) containing 10% fetal bovine serum (FBS). pCMV-luciferase plasmid was built by cloning fluc in the pcDNA3.1(+) backbone (Invitrogen USA, <http://www.invitrogen.com>), utilizing the *NheI*–*XhoI* restriction enzyme site. Transient transfections were performed 24 h later (~80% confluency) by using Superfect transfection reagent. Each transfection mix contained the pCMV-luciferase plasmid (2 μg CMV-Luc per row). Three hours after transfection, the cell medium was added, and the cells were incubated for 24 h. The medium was carefully removed, and phosphate-buffered saline (PBS) containing 1 μg ^{14}C -D-luciferin (~7.4 kBq) or a corresponding amount of unlabeled D-luciferin was added to each well. Light emission of wells with ^{14}C -D-luciferin was compared to light emission of wells with D-luciferin.

The bioluminescence was measured in a cooled CCD camera (Xenogen IVIS; Xenogen). Photons emitted from cells were collected and integrated for a period of 2 min. Images were obtained by using LIVING IMAGE software (Xenogen) and IGOR image analysis software (Wave-Metrics, Lake Oswego, OR, USA). For quantification of measured light, regions of interest were drawn over the specific wells and the maximum photons per second per square centimeter per steradian were obtained.

Cell culture and cell transfection protocols

Human embryonic kidney cancer cells 293T (American Type Culture Collection [ATCC], Manassas, VA, USA), used for most of the experiments, were grown in MEM supplemented with 10% FBS and 1% penicillin/streptomycin solution. Cells were plated in 12-well plates.

Transient transfections were performed 24 h later by using Superfect transfection reagent. Each transfection mix contained pCMV-luciferase plasmid (2 μg CMV-Luc per row, unless stated otherwise). Three hours after transfection, the cell medium was added, and the cells were incubated for 24 h before cell uptake tests were carried out.

Cell uptake and cell efflux experiments

Cell uptake experiments were done using native 293T cells and transiently with the CMV-Luc plasmid-transfected 293T cells. In one experiment, stably fluc transfected C6 rat glioma cells were utilized. The C6 cells were obtained from the ATCC (<http://www.atcc.org>). The stably fluc-transfected C6 rat glioma cells were created by utilizing the previously introduced pcDNA3.1(+)-fluc plasmid and selecting with the G418 (Neomycin) antibiotic marker.

Transfections were carried out as described above. Two micrograms of CMV-Luc plasmid per row in a 12-well plate was used, unless stated otherwise.

Cell uptake experiments were done in 12-well plates 24 h after CMV-Luc transfection. During the tracer uptake time, cells were kept either in cell media containing FBS or in PBS without serum.

^{14}C -D-Luciferin (3.7 kBq) was added to each 12-well plate. In the experiments in which mass levels of carrier D-luciferin was added, 1,500 μg unlabeled D-luciferin per well was used.

Cells were incubated at 37°C for different uptake times. At the end of each incubation period, radioactivity in the medium was measured. The wells were washed with cold PBS, the cells were harvested using a cell scraper, and the radioactivity in the cell pellet was determined. Triplicate samples were performed for all uptake studies. The same wells were also used to determine total protein content. Data are expressed as the net accumulation of probe in $[\text{dpm cells}/\text{total count} (\text{dpm cells} + \text{dpm supernatant})/\mu\text{g total protein}] \pm \text{standard error}$ or in $(\text{dpm cells}/\text{total count}) \times 100 \pm \text{standard error}$.

For the efflux experiment, 293T Luc cells and native 293T cells were incubated for 1 h with 3.7 kBq ^{14}C -D-luciferin per well in cell media and FBS. Then, the cells were washed two times with PBS, and nonradioactive media were added. At different time points, 100 μl of the supernatant was taken, cells were washed twice with PBS, and the cells were lysed. The cell media, supernatant sample, and the cell lysate sample were measured in a Beckmann LS 9000 liquid scintillation counter with cytosint (ICN, Costa Mesa, CA, USA) scintillation fluid. Corrections for background activity and efficiency (96.1% for ^{14}C) based on calibrated standards (Beckmann) also were performed to obtain disintegrations per minute (dpm). Data are expressed as $[\text{dpm cells}/\text{total count} (\text{dpm cells} + \text{dpm supernatant})] \times 100\% \pm \text{standard error}$.

Murine biodistribution studies

^{14}C -D-Luciferin percent injected dose per gram (%ID/g) was quantified by biodistribution studies. The experiments were carried out in nude mice without fluc expression.

Mice were coinjected via the tail vein with 18.5 kBq ^{14}C -D-luciferin and 3 mg unlabeled D-luciferin. Mice were killed, and organs were immediately removed. The organs were weighed and counted for [^{14}C] radioactivity. For ^{14}C tissue counting, small samples of the tissue were dissolved with hyamine and heated at 500°C for ~ 12 h before counting with cytosint scintillation fluid. Corrections for background activity and efficiency (96.1% for ^{14}C) based on calibrated standards (Beckmann) also were performed to obtain disintegrations per minute. Corrections for the efficiency of tissue sample counting were performed by sending random tissue samples (PTRL, Richmond, CA, USA) for counting by a combustion method.

Two mice per time point and three tissue samples of each tissue type were measured. Radioactivity determinations were normalized by the weight of the tissue and amount of radioactivity injected to obtain %ID/g.

Identical experiments were done after intraperitoneal injection of unlabeled and ^{14}C -labeled D-luciferin and after tail-vein injection of 18.5 kBq ^{14}C -D-luciferin without coinjection of mass amounts of the carrier.

Digital whole-body autoradiography

The ^{14}C -D-luciferin injection procedure described in the biodistribution studies above was repeated using two mice per time point. After killing, each animal was frozen in liquid nitrogen in preparation for digital whole-body autoradiography (DWBA) using a 45- μm slice thickness [9]. The exposure time of the plates was 24–72 h. All digital plates were scanned on a Fuji BAS 5000 Phosphor-imager at a resolution of $\sim 100\ \mu\text{m}$. DWBA was not quantified to calculate the absolute uptake value in %ID/g but performed to correlate the relative accumulation of the tracer in different organs as compared with the biodistribution studies.

In vivo CMV-Luc transfection and optical imaging of mice

Experiments were done in nude mice at least 72 h after injection of 1×10^9 plaque-forming units Adenovirus (Ad-CMV-luciferase) via tail vein. Control mice were injected with the control virus (Ad-CMV- βGal) with the same titer. Mice were injected with 18.5 kBq ^{14}C -D-luciferin and 3 mg of carrier D-luciferin (one mouse of each group was only injected with ^{14}C -D-luciferin without mass amounts of unlabeled D-luciferin). D-Luciferin was injected via tail vein or intraperitoneally, and uptake differences in %ID/g tissue in luciferase-expressing livers vs. control livers were examined.

Mice were killed immediately after CCD camera imaging of the animals so that light signals from transfected and control mice could also be documented. The bioluminescence signal in mice was measured in a cooled CCD camera, and biodistribution studies were carried out as described previously. Different time points from 1 min to 1 h were chosen, including killing the animals when peak light emission occurred.

Statistical analysis

Statistical analysis was carried out utilizing the unpaired Student's *t* test algorithm and the Microsoft EXCEL software. If *p* values were less than 0.05, findings were deemed significant.

Results

^{14}C -D-Luciferin demonstrates activity preservation both in buffer solution and when exposed to intact cells

^{14}C -labeled and unlabeled D-luciferin reveal no significant differences in light yields ($p=0.36$) when incubated with same amounts of FL: $7,393 \pm 1,068$ vs. $6,706 \pm 1,208$ RLU when utilizing 400 ng FL/ μl (Fig. 2a). These results clearly indicate that ^{14}C -D-luciferin is still a functional substrate for the FL-catalyzed reaction.

To test the functionality of ^{14}C -D-luciferin in cell culture, 293T cells, plated in a 12-well culture plate, were transfected with pCMV-luciferase plasmid (CMV-Luc, 2 μg CMV-Luc

per row). Twenty-four hours later, 1.0 μg ^{14}C -D-luciferin or a corresponding amount of unlabeled D-luciferin was added to each well (cells in PBS). The bioluminescence light emission of wells measured in an optical CCD camera with ^{14}C -D-luciferin was compared to light emission of wells with unlabeled D-luciferin. Up to 173 min after adding the substrate to the FL-expressing 293T cells, we found slightly lower light levels ($p=0.02$) in wells with labeled D-luciferin. At time point 720 min, no significant differences between wells exposed with unlabeled or ^{14}C -labeled D-luciferin are observed ($p=0.2$; Fig. 2b).

FL-expressing cells in FBS-containing media show only minimal enhanced ^{14}C -D-luciferin uptake levels compared to cells not expressing FL

The 293T control cells and transiently transfected cells expressing FL were exposed for up to 70 min to cell media containing FBS with ^{14}C -D-luciferin. Only minimally enhanced ($p=0.2$, not statistical significant) accumulation levels of ^{14}C -derived radioactivity in transfected cells—as compared to nontransfected cells—are observed (see Supplemental Fig. 1 on the website).

Adding mass amounts of cold D-luciferin to the cell media further enhances intracellular ^{14}C -D-luciferin uptake levels

Adding mass amounts of unlabeled D-luciferin (1,500 μg per well in a 12-well plate) additionally to trace amounts (3.7 kBq per well) of ^{14}C -D-luciferin enhances ^{14}C -D-luciferin uptake in cells expressing FL when the cells are kept in cell media containing FBS. The ^{14}C -D-luciferin cell uptake at 15 min is 1.6-fold higher when adding mass amounts of unlabeled D-luciferin ($p=0.04$; Fig. 3). Adding higher activity levels of ^{14}C -D-luciferin alone is not able to further enhance the cell uptake levels. There is also no peak uptake in the first 5 min (data not shown).

The FL-expressing cells kept in serum-free PBS compared to native cells kept in PBS show significantly higher level of intracellular accumulation of ^{14}C -D-luciferin over time

293T cells transiently transfected with CMV-luciferase (2 μg per row) show significantly higher uptake levels in comparison to native 293T cells when exposed to PBS containing ^{14}C -D-luciferin (3.7 kBq per well, 12-well plate). Cells show the highest uptake values (up to 22.9-fold higher in fluc-transfected vs. nontransfected cells, p value= 0.0002) after a 3-h exposure with PBS containing ^{14}C -D-luciferin (Fig. 4a).

CMV-Luc-transfected 293T cells in PBS without FBS show highest intracellular trapping of ^{14}C -D-luciferin after transfection with 10 μg CMV-Luc

293T cells transiently transfected with different amount of CMV-Luc plasmid (from 2 up to 10 μg per row in a 12-well plate) show highest intracellular uptake levels after transfection with 10 μg CMV-Luc per row, when exposed to PBS containing ^{14}C -D-luciferin (3.7 kBq per well; Fig. 4b). Incubation time of the cells with ^{14}C -D-luciferin in PBS was 1 h. The amount of transient intracellular accumulation of ^{14}C -D-luciferin is dependent on the intracellular FL levels. Accumulation of radioactivity in the transfected cells is significantly higher as compared to native 293T cells ($p < 0.005$ after transfection with 10 μg CMV-Luc per row).

Efflux experiments carried out in FL-expressing cells kept in media demonstrates that most of the efflux takes place in the first 30 min after the uptake period

293T Luc cells and native 293T cells were incubated for 1 h with ^{14}C -D-luciferin containing cell media and FBS. Then, the cells were washed two times and kept with fresh medium. At different time points, 100 μl of the supernatant was taken and measured for ^{14}C -D-luciferin. The cell lysate was also measured in the scintillation counter. As displayed in Fig. 5, most of the efflux takes place in the first 30 min after termination of the cell uptake period. However, FL-expressing cells show significantly higher radioactivity levels as compared to native cells up to 2.5 h after start of the efflux experiment ($p < 0.05$).

Biodistribution after tail-vein coinjection of carrier cold D-luciferin added with ^{14}C -D-luciferin in mice without fluc expression

Experiments were carried out in nude mice ($N = 8$) without fluc expression. ^{14}C -D-Luciferin (18.5 kBq) and 3 mg D-luciferin was coinjected via the tail vein. Two mice per time point and three tissue samples of each tissue type were measured for ^{14}C -derived radioactivity. Biodistribution of ^{14}C -D-luciferin after tail-vein injection shows early high uptake values in the kidneys; 15 min after injection, significant uptake was observed in most of the organs with low uptake in the brain, and 30 min after injection, high activity in the gastrointestinal tract was observed. Blood levels and liver activity (approximating blood pool at early time points) peak early but then fall rapidly. The data are detailed in Fig. 6a.

Biodistribution after intraperitoneal injection of carrier cold D-luciferin added with ^{14}C -D-luciferin in mice differ from the results after tail-vein injection

The experiments were carried out in nude mice ($N=8$) without fluc expression. ^{14}C -D-Luciferin (18.5 kBq) and 3 mg D-luciferin were coinjected intraperitoneally. Two mice per time point and three tissue samples of each tissue type were measured for ^{14}C -D-luciferin-derived radioactivity. There are significant ($p < 0.05$) differences in biodistribution after intraperitoneal in comparison to intravenous injection: Blood levels rise slower and do not peak to as high levels but are detectable for a prolonged time after intraperitoneal injection. Kidney activity peaks much later. In general, organ activity does not get as high as after intravenous injection but stays increased for a prolonged time. Brain uptake is low with both routes; highest levels are reached in the first 5 min after injection but not significantly higher than after intraperitoneal injection of the tracer ($p=0.09$). Bowel/intestine shows high activity after (local) intraperitoneal injection. The data are listed in Fig. 6b.

Significant differences of biodistribution patterns after intravenous injection of labeled/or injection of carrier cold D-luciferin added with ^{14}C -D-luciferin are detectable in the kidneys

Differences of the biodistribution patterns were evaluated in nude mice ($N=8$) which were intravenously injected with only labeled/or coinjected with mass levels of carrier cold D-luciferin added to the ^{14}C -D-luciferin (18.5 kBq ^{14}C -D-luciferin and 3 mg unlabeled D-luciferin). The results were evaluated 1 and 5 min after injection of the tracer. The data are plotted in Fig. 7. We detected very high radioactivity levels in the kidneys if no mass level of carrier was coinjected, pointing to a possibly higher renal excretion of the tracer. The fact that kidney activity is much higher after only ^{14}C -D-luciferin in comparison to carrier

and ^{14}C -D-luciferin is highly significant ($p=0.0015$ after 5 min). However, no consistent significant uptake differences in organs other than the kidneys could be detected 5 min after injection of only ^{14}C -D-luciferin or ^{14}C -D-luciferin and carrier D-luciferin. One minute after injection of only labeled or carrier added ^{14}C -D-luciferin, observed radioactivity levels in the blood pool are slightly but not significantly higher in coinjected mice ($p=0.07$).

Autoradiographic studies verified the results obtained in the biodistribution experiments

To further confirm the biodistribution results obtained from the different tissues, autoradiograms of animals ($N=6$) injected with the ^{14}C -D-luciferin in similar conditions were studied (Fig. 8). Autoradiographic studies after tail-vein injection of ^{14}C -D-luciferin demonstrate inhomogeneous tracer distribution in mice with early predominant high radioactivity values in the kidneys and later predominant uptake in the gastrointestinal tract, likely reflecting the second elimination route of the tracer via the hepato-biliary system.

Autoradiograms 1 min after injection of only labeled or carrier-added ^{14}C -D-luciferin were developed on the same plate using the exact same color scale (Supplemental Fig. 2 on the website; $N=4$). Consistent with the biodistribution data of radioactivity levels in the blood pool at this time point, we observed—especially in the liver—slightly higher uptake levels if mass levels of unlabeled D-luciferin are coinjected with ^{14}C -D-luciferin.

Optical imaging and biodistribution data in mice expressing FL demonstrates no trapping of ^{14}C -D-luciferin in FL-expressing tissue in vivo

Experiments were done in nude mice at least 72 h after tail-vein injection of equivalent titers of Ad-pCMV-luciferase (Adenovirus carrying the fluc under the control of the CMV promoter; $N=6$) or a control virus ($N=5$). Mice were injected with 18.5 kBq ^{14}C -D-luciferin and 3 mg D-luciferin (one mouse of each group received only ^{14}C -D-luciferin). D-Luciferin was injected via tail vein or intraperitoneally, and uptake differences in %ID/g tissue in fluc-expressing livers vs. control livers were examined. Mice were killed immediately after CCD camera imaging of the animals so that light signals from transfected and control mice could also be documented and correlated to the well counter results. Experiments utilizing intraperitoneal injections of the tracer could not detect uptake differences in %ID/g in fluc-expressing vs. control livers (data not shown). One major obstacle in this setting was that peak organ activity of ^{14}C -D-luciferin is less high after intraperitoneal injection compared to the intravenous route. This makes it difficult to detect possibly relatively small ^{14}C -D-luciferin uptake differences in FL-expressing vs. control tissue.

Time points from 1 min after intravenous injection of ^{14}C -D-luciferin up to 1 h after injection of the tracer were examined. The uptake differences in the %ID/g levels of transfected vs. not transfected tissue detected after intravenous injection are relatively small, with the biggest differences found 4 min after carrier-added injection of ^{14}C -D-luciferin:

Pooled uptake data in %ID/g of six CMV-Luc-transfected livers vs. five control livers are showing $13.24 \pm 1.1\%$ ID/g in the fluc-transfected livers vs. $9.45 \pm 1.79\%$ ID/g in the livers of the control animals ($p=0.047$; Fig. 9). Average light signals of the livers in six fluc-transfected vs. five control mice were $7.29\text{E}+07 \pm 3.12\text{E}+07$ vs. $3.12\text{E}+05 \pm 1.49\text{E}+05$

($p=0.03$); the bioluminescence signal is represented as photons per second per square centimeter per steradian.

Higher ^{14}C -D-luciferin uptake differences between FL-expressing and nonexpressing tissue could not be detected after longer uptake times, shorter uptake time, or after intraperitoneal injection of the tracer (data not shown).

Discussion

For in vitro luciferase assays, a linear relationship between enzyme concentration and light signal can be ensured by controlled reaction conditions which include provision of the D-luciferin substrate in excess [10]. In vivo luciferase reporter gene imaging in mice implies the assumption that the intensity of light signal reliably reflects the regional transgene expression levels. For a fixed concentration of enzyme, a decrease in local substrate availability will cause a decline in the reaction rate [11]. Therefore, local availability and biodistribution of administered D-luciferin are of concern for optical-based in vivo luciferase reporter gene imaging.

We first verified that ^{14}C -D-luciferin can serve as a reliable substrate for FL. In a previous work, Lee et. al. [12] synthesized radiolabeled D-luciferin by electrophilic radio-iodination of D-luciferin with $\text{Na}^{123/125}\text{I}$. Although the authors stated that iodo-D-luciferin partially retained substrate specificity for FL, the introduction of iodine into the molecule still raises questions whether this modification might have a significant impact on the functional characteristic and biodistribution patterns of the substrate relative to the unlabeled form.

Our tracer was produced utilizing an alternate synthesis pathway [8], and the end product, D-luciferin, [benzene ring- $^{14}\text{C}(\text{U})$]-, approximately one carbon atom per molecule in the benzene ring was randomly replaced by ^{14}C . No foreign atoms or charge were introduced into the molecule to ensure that no significant changes in the functionality of the radiolabeled substrate occur. Our experiments in buffer solution demonstrated as expected no significant functional differences between ^{14}C -labeled D-luciferin and cold D-luciferin. These results support that ^{14}C -D-luciferin can be utilized as an appropriate tracer for the parent D-luciferin. The slightly lower light emission of ^{14}C -D-luciferin observed in cell culture was not consistent over the whole time frame of the experiment. Results of ^{14}C -D-luciferin cell uptake experiments support the previously published results of relatively low permeability of D-luciferin through cell membranes [13]. Intracellular uptake levels of the tracer were very low in both native and FL-expressing cells. Adding mass amounts of cold D-luciferin as carrier to the medium moderately increased intracellular uptake levels. This supports the theory of a diffusion-based uptake of D-luciferin. The serum proteins in the medium, to which trace amounts of ^{14}C -D-luciferin can bind and of which the mass amounts could displace the ^{14}C -D-luciferin, might also contribute to the observed increased intracellular uptake levels in the presence of the carrier.

No significant differences in uptake patterns of the tracer could be detected in native versus fluc-transfected cells, when the cells were kept in media containing FBS. It is interesting to note, however, that significant accumulation of radioactivity was observed in fluc-

transfected cells in culture when the cells were exposed to PBS containing radiolabeled D-luciferin. It can be speculated that FBS-containing media with saturated level of proteins may interfere with the intracellular entry of D-luciferin, so that the minimal intracellular accumulation of the substrate could only be detected in cells kept in protein-free PBS. The polar nature of the converted oxyluciferin [14] might contribute to the observed intracellular accumulation of radioactivity in cells expressing FL.

The results of the ^{14}C -D-luciferin efflux experiments with the fast efflux of the tracer do not support the concept of a prolonged intracellular trapping of D-luciferin or its metabolites in FL-expressing cells.

Biodistribution of ^{14}C -D-luciferin after tail-vein injection of carrier-added ^{14}C -D-luciferin shows early high uptake values in the kidneys indicating early renal excretion of the tracer. Early high activity levels in liver tissue represent blood pool activity. Five and 15 min after injection, significant uptake is present in most of the organs with low uptake in brain tissue.

There are significant differences in biodistribution after intraperitoneal injection in comparison to intravenous injection of carrier-added ^{14}C -D-luciferin: A general advantage when using the intravenous injection route was the low variation of uptake values in %ID/g in tissues of different mice. After intraperitoneal injection, organ uptake is prolonged in comparison to intravenous injections. Brain uptake is low with both routes. Intestinal uptake is high after (local) intraperitoneal injection.

The measured higher activities in the organs at later time points (45 min after injection) in the biodistribution study by Lee et. al. [12] is probably in part due to the accumulation of free iodine and might not reflect to the full extent the tracer levels. The relatively high activity levels in the stomach measured at later time points by Lee et al. are also indicative of free iodine (transported into the lumen of the stomach by the sodium iodide symporter).

Biodistribution data in our study confirm relatively low organ activities of the tracer, which likely influence the light signal. It is critical for the planning of optical imaging experiments to decide the injection route. If early and high uptake values are the key, then intravenous injection should be preferred. Intraperitoneal injections are better suited if prolonged organ uptake is important. Location of the area to be studied can also have an impact on the choice of the injection route. The exact timing of the data acquisition relative to time of injection is also critical.

Comparisons of mice intravenously injected with only labeled or carrier-added ^{14}C -D-luciferin clearly showed much higher kidney activity (approximately sevenfold after 1 min, approximately fourfold after 5 min) in mice which were only injected with labeled tracer. Renal excretion of ^{14}C -D-luciferin, the predominant route of excretion of the tracer, is decreased when adding higher amounts of cold D-luciferin to the injection volume. One minute after injection, a higher blood pool activity in coinjected mice was detectable.

We also examined mice after tail-vein injection of high titers of adenovirus carrying the CMV-Luc gene, which leads to high luc expression levels in the liver (adenoviral infection is mediated by coxsackievirus and adenovirus receptor which are expressed in high density

in liver tissue of mice) [15]. Although we found higher organ activity 4 min after injection of carrier-added ^{14}C -D-luciferin in fluc-transfected livers (with a low statistical significance level), consistent data to prove significant trapping of the tracer in tissue expressing high levels of FL could not be demonstrated in vivo. Significant intracellular accumulation of ^{14}C -D-luciferin could not be observed in FL-expressing cells in vivo. It can be speculated that this might be in part due to the low cell membrane permeability of the tracer, which in combination with protein binding and fast renal excretion leads to a rapid tracer washout.

In summary, we successfully introduced for the first time ^{14}C -D-luciferin as a substrate tracer for the FL-catalyzed bioluminescence reaction. We detailed in vitro cell uptake and efflux characteristics as well as biodistribution patterns of this tracer in living mice. However, we did not observe accumulation of the tracer in luciferase-expressing cells in vivo.

Future studies will benefit from the evaluation of biodistribution patterns of radiolabeled coelenterazine and other substrates of optical imaging probes in efforts to characterize alternate optical imaging systems. In future studies of optical imaging probes, a search for probes in which intracellular trapping of the radiolabeled substrate correlates with light yield would be useful. Such intracellular trapping of a substrate in vivo would possibly allow the development of a dual modality optical/radiotracer reporter system.

Since optical/PET scanners are already in development [16], a combined radiolabeled PET/optical multimodality tracer—with corresponding and correlative trapping of the substrate in light emitting cells—would be a significant step in the emerging scientific field of multimodality molecular imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

FL	firefly luciferase
Fluc	firefly luciferase gene
CMV-Luc	CMV-luciferase plasmid
PBS	phosphate-buffered saline
FBS	fetal bovine serum

Ad-CMV-Luciferase	adenovirus carrying fluc under the control of the cytomegalie virus promoter
293T Luc cells	293T cells expressing the Firefly luciferase gene
%ID/g	percent injected dose/gram

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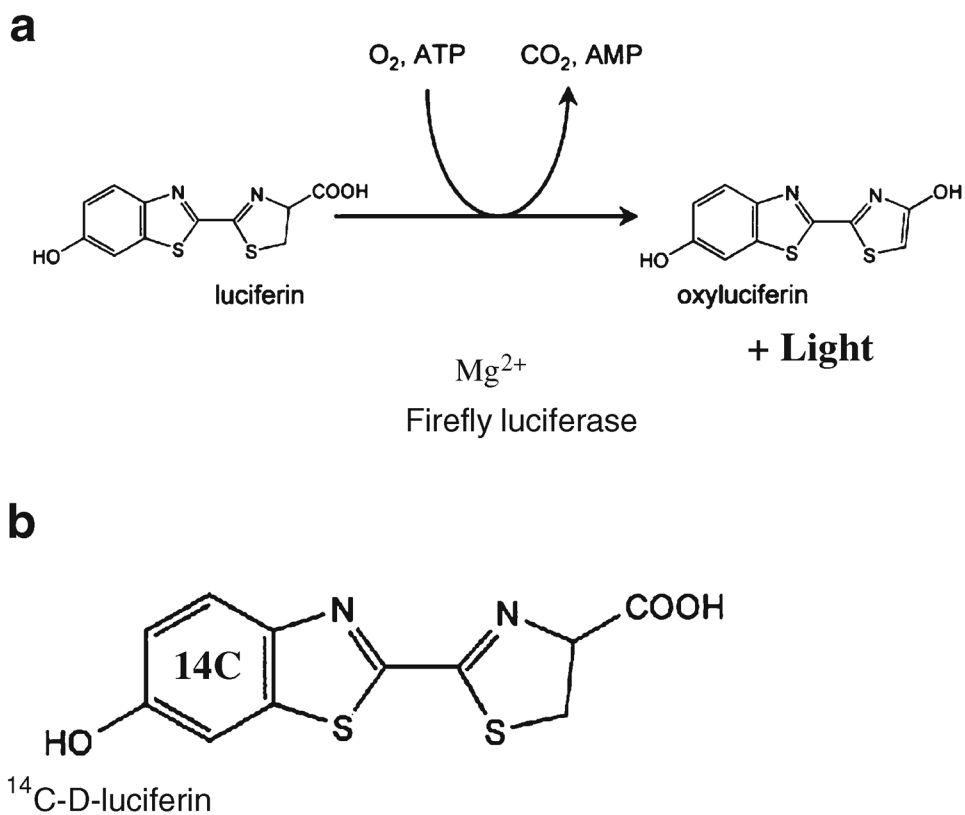
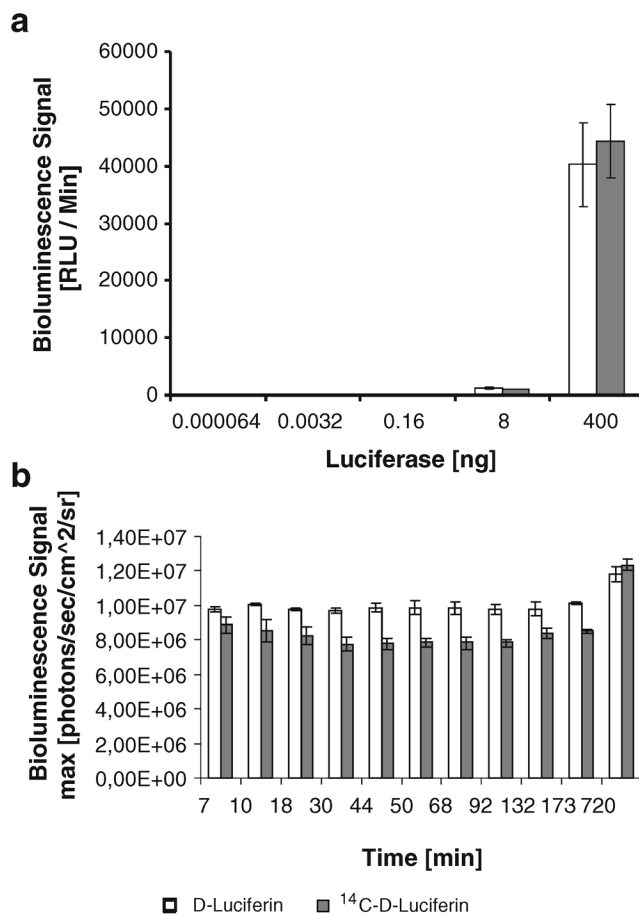


Fig. 1.
a Firefly Luciferase catalyzes the oxidative decarboxylation of Luciferin in the presence of ATP, O_2 , and Mg^{2+} , producing yellow-green light ($\lambda_{max}=560$ nm). **b** Chemical structure of D-luciferin, [benzene ring- ^{14}C (U)]-: Approximately one carbon atom of the benzene ring in this radiotracer is randomly replaced by ^{14}C . The functionality of ^{14}C -D-luciferin was preserved in all our tests

**Fig. 2.**

a To a buffer solution with rising amounts of FL concentration and fixed amounts of ATP and MgSO₄, fixed amounts of ¹⁴C-D-luciferin or D-luciferin were added, and the bioluminescence signal was measured. Light signal was detected by the luminometer from an amount of 8 ng FL on. The detected amount of light signal is displayed on the y-axis and given in relative light units (RLU) per minute. There were no significant differences in the measured light yields when using ¹⁴C-D-luciferin in comparison to unlabeled luciferin ($p=0.36$). **b** To test the functionality of ¹⁴C-D-luciferin in cell culture, 293T cells expressing FL and native cells were investigated. After adding corresponding amounts of unlabeled or ¹⁴C-labeled D-luciferin to the wells, light emission was measured in a CCD camera. Up to 173 min after adding the substrate to the FL-expressing 293T cells, we found slightly lower light levels ($p=0.02$) in wells with labeled D-luciferin, at time point 720 min, no significant differences were observed ($p=0.2$)

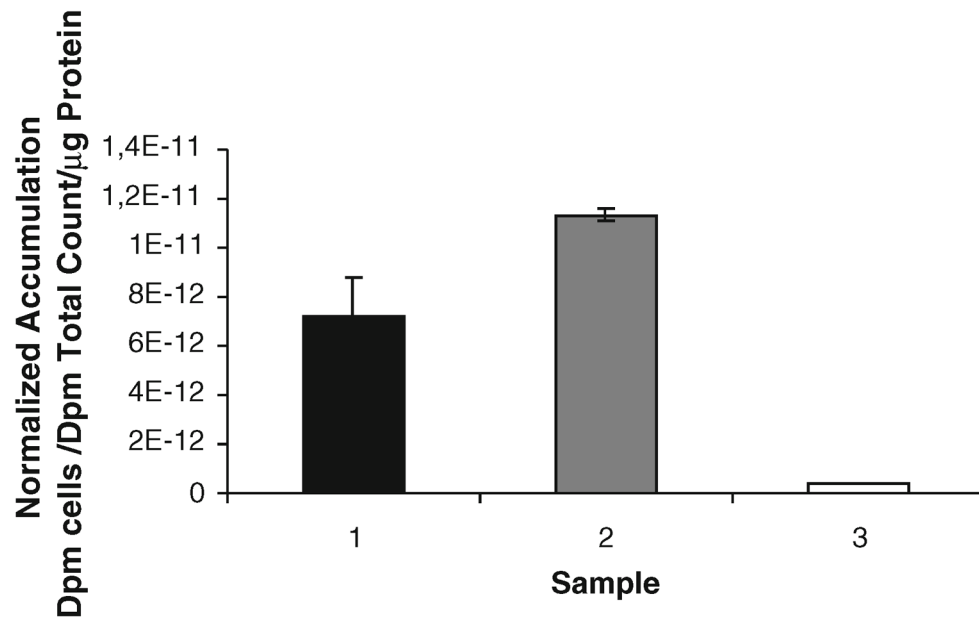


Fig. 3. 293T CMV-Luc cells were exposed for 15 min with ^{14}C -D-luciferin, followed by cell uptake measurements for ^{14}C -derived radioactivity. The cell uptake of ^{14}C -D-luciferin is 1.6 fold higher ($p=0.04$) in the presence of mass amounts of unlabeled D-luciferin during the uptake period. *Sample 1:* 293T-CMV-Luc cells, + ^{14}C -D-luciferin. *Sample 2:* 293T-CMV-Luc cells, + ^{14}C -D-luciferin+mass amounts of carrier D-luciferin. *Sample 3:* No radioactivity added (background control with 293T-CMV-Luc cells)

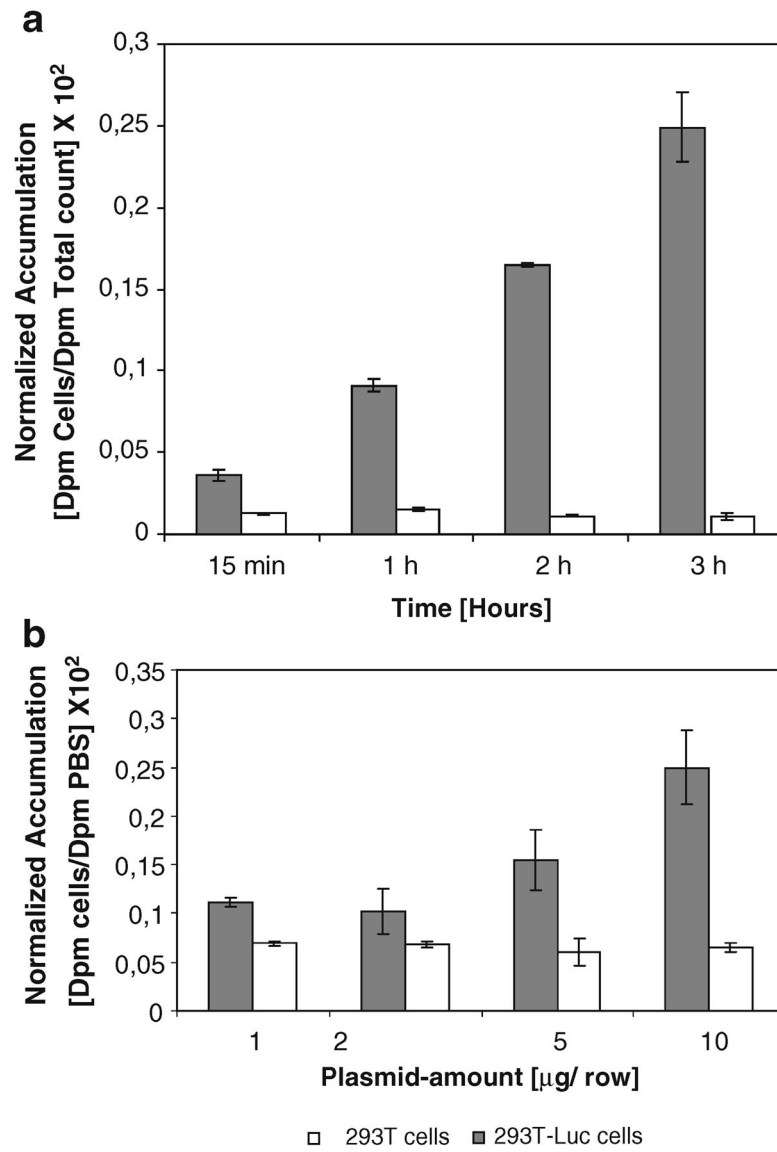


Fig. 4.
a 293T cells transiently transfected with CMV-Luc show significant ($p=0.0002$) higher uptake levels in comparison to native 293T cells, when exposed to PBS containing ¹⁴C-D-luciferin. The transfected cells show highest uptake values (up to 22.9-fold higher in fluc transfected vs. nontransfected cells) after a 3-h exposure with PBS containing the radiotracer. **b** 293T cells transiently transfected with different amount of CMV-Luc plasmid show highest intracellular uptake levels of ¹⁴C-D-luciferin after transfection with 10 μ g per fluc plasmid per row. Cell uptake time was 1 h

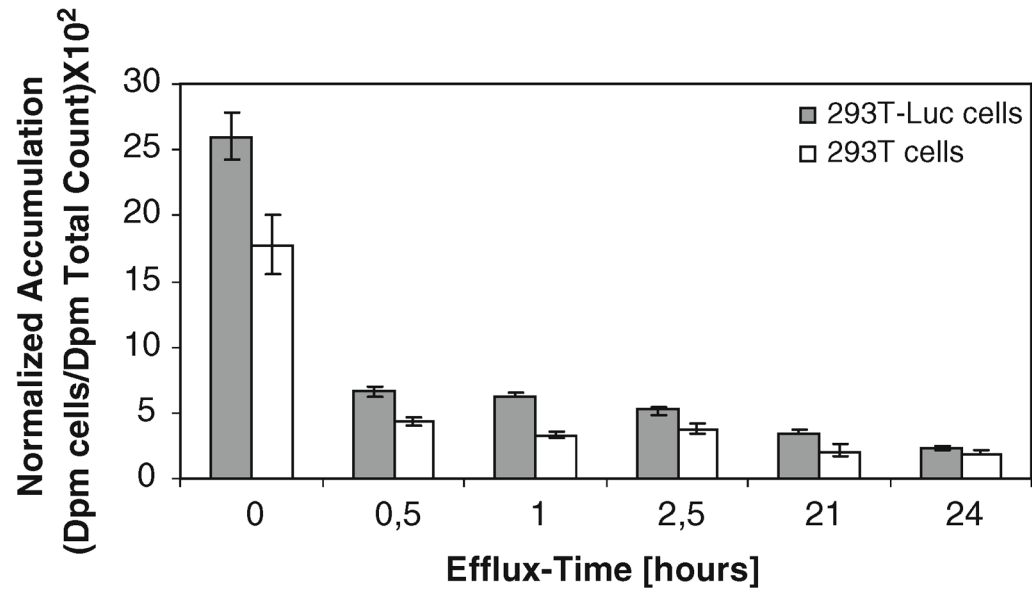


Fig. 5.
293T CMV-Luc cells and native 293T cells were incubated for 1 h with ¹⁴C-D-luciferin containing cell media and FBS. Then, the radioactivity efflux from the cells was measured. Most of the efflux takes place in the first 30 min

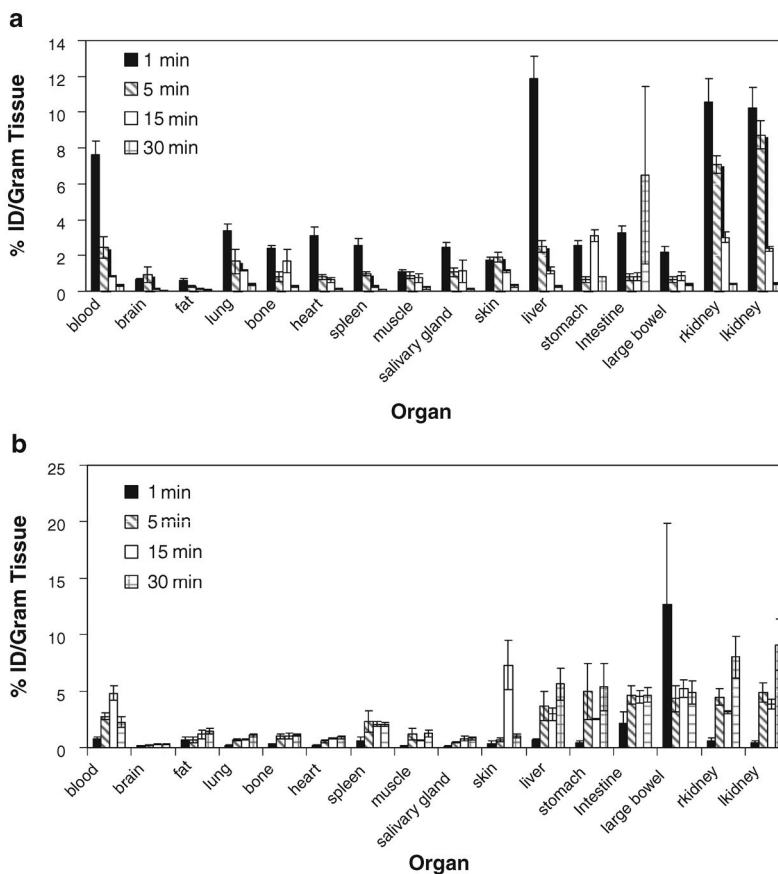


Fig. 6.
a Biodistribution of ¹⁴C-D-luciferin in nude mice without fluc expression after tail-vein injection of 18.5 kBq ¹⁴C-D-luciferin and 3 mg carrier D-luciferin. Note the early high uptake values in the kidneys due to fast renal excretion of the tracer; 15 min after injection, significant uptake in most of the organs is demonstrated with low uptake in the brain. Blood levels and liver activity, which equals blood pool at early time points, peak early but fall rapidly. **b** Biodistribution of ¹⁴C-D-luciferin in nude mice without fluc expression after intraperitoneal injection with the same amounts of tracer and carrier like above. Note the differences in biodistribution after intraperitoneal injection in comparison to intravenous injection: Blood levels rise less high but are longer detectable. Kidney activity peaks later. In general, organ activity is not getting as high as after intravenous injection. Bowel/intestine activities are at early time points high probably due to the injection route

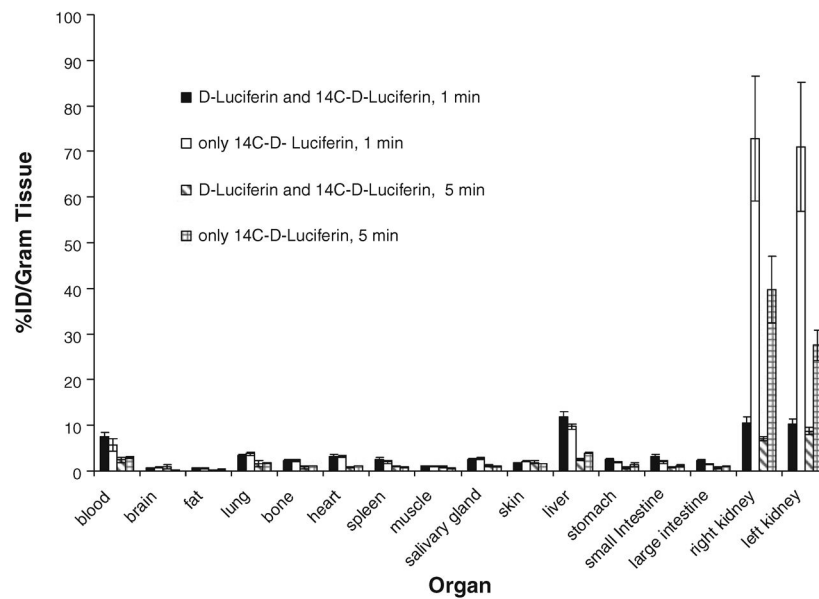


Fig. 7. Differences of the biodistribution patterns were evaluated in nude mice not expressing FL which were intravenously injected with only labeled ^{14}C -D-luciferin or mass levels of carrier added to the ^{14}C -D-luciferin (18.5 kBq ^{14}C -D-luciferin and 3 mg D-luciferin). The results were evaluated 1 and 5 min after injection of the tracer. Kidney activities are significantly higher, if only ^{14}C -D-luciferin is injected, but other organ activities do not differ significantly with our without coinjection of mass levels of carrier substrate

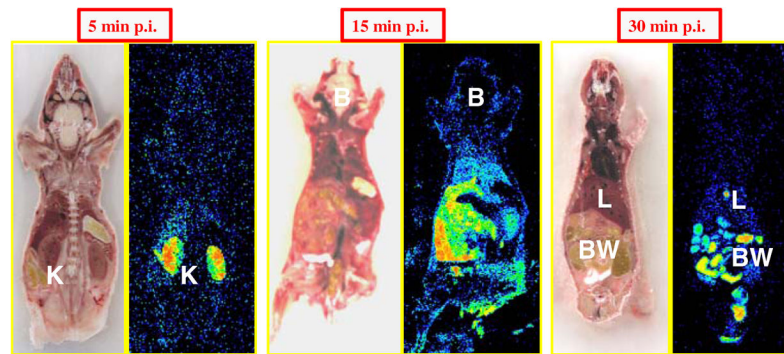


Fig. 8. Autoradiograms ($N=6$ mice) 5, 15, and 30 min after coinjection via the tail vein of 18.5 kBq ^{14}C -D-luciferin and 3 mg carrier D-luciferin. Autoradiographic studies verified results from the biodistribution experiments with early high uptake values in the kidney followed by accumulation in the gut system (K kidney, B brain, L liver, BW bowel)

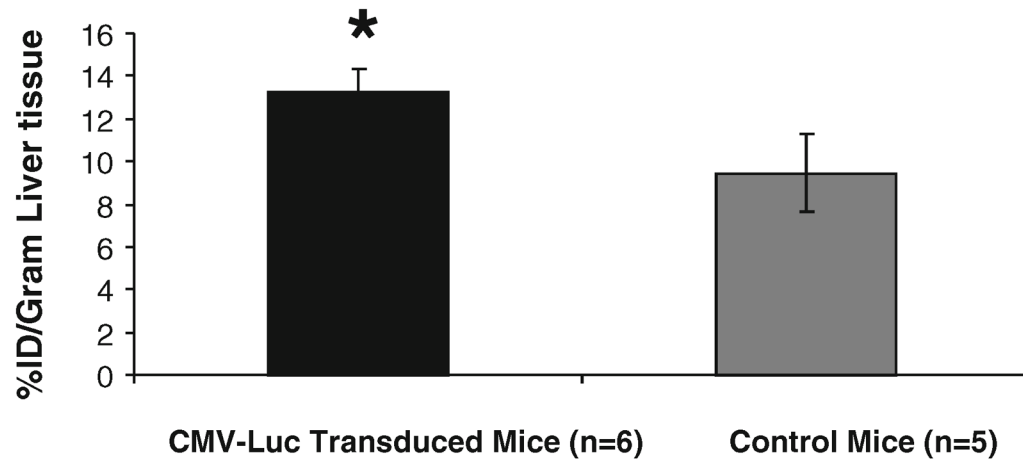


Fig. 9.

Mice were transduced by injecting Ad-CMV-luciferase via the tail vein; the control mice were injected with a control virus without the fluc sequence. Five minutes after tail-vein injection of ¹⁴C-D-luciferin and mass level of carrier D-luciferin, mice were imaged in a CCD camera, and organs were harvested immediately after imaging to measure uptake in %ID/g. In pooled data over six fluc-transfected and five control mice, the difference in ¹⁴C-D-luciferin uptake was only borderline significant ($p=0.0047$). No higher uptake differences between fluc-transfected and control tissue could be detected at earlier or later time points in similar experiments