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Gaussia luciferase reporter assay for monitoring of biological processes in culture and in vivo

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Abstract

Secreted reporters are a useful tool in the monitoring of different biological processes in the conditioned medium of cultured cells as well in the blood and urine of experimental animals. Described here is a protocol for detecting the recently established naturally secreted *Gaussia* luciferase (Gluc) in cultured cells as well as in blood and urine *in vivo*. Further, the assay for detecting the secreted alkaline phosphatase (SEAP), the most commonly used secreted reporter in serum is also presented. The Gluc reporter system has several advantages over the SEAP assay including: a much reduced assay time (1-10 min vs. 1.5 - 2 h); 20,000-fold (*in vitro*) or 1000-fold (*in vivo*) increased sensitivity and a linear range covering over 5 orders of magnitude of cell number. Additionally, the Gluc signal can be detected in urine and the signal can be localized in animals using *in vivo* bioluminescence imaging.

INTRODUCTION

Secreted reporters in the blood or serum have shown to be important tools for the detection, quantitation and non-invasive monitoring of different biological processes in experimental models¹⁻⁸. The most commonly used serum reporter is the secreted alkaline phosphatase or SEAP^{2,3,5-7,9-13}. Normally, alkaline phosphatase is not secreted, however, Berger et al. showed that the placental alkaline phosphatase is expressed and released efficiently into the conditioned medium of mammalian cells that have been engineered to express this reporter⁹. The level of SEAP in the conditioned medium is linearly related to the changes in its intracellular mRNA level as well as cell number^{9,10}. It is also possible to quantitatively detect SEAP in the serum of animals. In 1997, Abruzzese et al., used the SEAP serum level to monitor the induction of gene expression followed by gene transfer to the muscles of adult mice¹⁴. Since then, the SEAP reporter assay has been used as a surrogate serum marker to monitor different *in vivo* processes including tumor growth and drug efficacy³, DNA vaccination¹⁵; promoter and transcription factors activation and inhibition^{2,6,13}; viral gene transfer¹⁶; inflammatory events⁷; viral infection and replication¹⁷; and endoplasmic reticulum stress¹¹.

Recently, we have established a novel secreted reporter from the marine copepod *Gaussia princeps* named *Gaussia* luciferase or Gluc^{1,4,8,18,19}. We have shown that this reporter is

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the HTML version of this article for details).

naturally secreted from mammalian cells in an active form and that the levels of Gluc in the conditioned medium are linear with respect to cell number, growth and proliferation^{1,19}. The Gluc reporter assay has been shown to be a sensitive reporter in monitoring endoplasmic reticulum stress and the secretory pathway^{1,4,18,20}; promoter activity²¹; small interference RNA (siRNA) silencing and micro RNA (miRNA) biogenesis^{22,23}; as well as protein-protein interaction²⁴. *In vivo*, the Gluc reporter assay has been used to localize tumors and to monitor tumor growth and proliferation using bioluminescence imaging^{19,25}. Since Gluc is naturally secreted, we have shown that its level in the blood or urine can be used as a marker to monitor different *in vivo* biological events such as tumor growth and response to therapy, viral infection and replication as well as the viability of circulating cells⁸.

Advantages of using secreted reporter assays

In general, secreted reporters have several advantages over conventional methods for monitoring gene expression such as bioluminescence imaging^{26,27}. In culture, secreted reporters can be quantified in the cell-free conditioned medium avoiding the need for cell lysis and leaving the cells intact and available for confirmation analysis. *In vivo*, secreted reporters can be easily monitored in animals by withdrawing a few microliters of blood or urine and assaying for their activity allowing real-time monitoring of *in vivo* processes. Although *in vivo* bioluminescence imaging has been successfully used in different fields²⁸, including immunology²⁹, oncology³⁰, virology³¹, and neuroscience³², this method requires expensive instrumentation (cooled CCD camera). In addition, this imaging technique is time-consuming, involves frequent anesthesia and repeated systemic substrate injections and is limited by photon absorption by tissues as a function of depth²⁷. These issues make it difficult to monitor relatively large cohorts of animals at small time intervals.

Comparison of reporter assays

The SEAP reporter assay is a traditional serum marker for monitoring *in vivo* processes^{2,3,5-7,9-13}. The availability of a wide range of SEAP substrates including fluorescent and chemiluminescent reagents allows it to be quantified quickly and sensitively^{7,9-11,14}. SEAP activity is not affected by serum¹² and is heat stable therefore interference by endogenous alkaline phosphatase can be eliminated by heating the samples at 65 °C¹⁰. On the other hand, beside it being naturally secreted, Gluc is over 1000-fold more sensitive than the commonly used luciferases from the American firefly or sea pansy *Renilla reniformis*¹⁹ and over 20,000-fold more sensitive than SEAP in cultured mammalian cells¹. Additionally Gluc is very stable in culture medium (half-life around 6 days)⁸, therefore samples can be stored at 4 °C for several days without a significant change in Gluc reporter activity. *In vivo*, the Gluc reporter assay has several advantages over SEAP which are presented in Table 1.

Limitations of secreted reporter assays

In vivo, the SEAP reporter is assayed in serum which requires that the samples are diluted and heat deactivated to inhibit endogenous alkaline phosphatase activity before measurement using a plate reader. The linear range of the SEAP reporter assay is limited which can depend on the efficiency of cell transduction and the amount of SEAP produced by the cells. On the other hand, the Gluc reporter reaction using the coelenterazine substrate has flash kinetics¹⁹, therefore, a luminometer with a built-in injector is needed to perform the assay. Since Gluc is very stable in the conditioned medium, a negative untreated cells control should always be included to which the results are normalized. However, this is not a problem *in vivo*, since the Gluc half-life in circulation is very short. The Gluc reporter assay is very sensitive making it prone to pipetting errors. Using a larger assay volume (10 - 50 µl) can circumvent this problem but care should be taken not to saturate the assay.

Experimental Design

Plasmid or viral vectors expressing the reporter constructs (Gluc or SEAP)—

Initially, efficient gene transfer to the cells of interest needs to be accomplished. This can be performed using any DNA transfection reagent such as lipofectamine2000¹⁹. Alternatively, and more preferably, gene transfer using viral vectors such as lentiviruses can be used^{8,33,34}. The advantage of lentivirus vectors is that they integrate the reporter transgene into the genome of cells thus daughter cells inherit a copy of the reporter gene resulting in stable expression. This is particularly important when the biological process of interest needs to be monitored over a long period of time. In general, lentivirus vectors transduce many different cell types with high efficiency including tumor cells and human stem cells. Furthermore, constructs that also express a fluorescent protein such as GFP are advantageous as they allow for easy monitoring of transduction efficiency by fluorescent microscopy. One way to express both the reporter (Gluc or SEAP) as well as GFP is to clone both of them in the same expression cassette in a lentivirus vector under the control of the constitutive cytomegalovirus (CMV) promoter separated by an internal ribosomal entry site (IRES) elements¹. A detailed protocol for the preparation of lentivirus vectors is described in Nature Protocols³⁴. On the other hand, one might choose to subclone the Gluc or SEAP reporter into a viral vector which is already used in a reader's own laboratory and will efficiently transduce the specific cell type that they are interested in. For optimization of the Gluc assay presented here, a lentivirus vector expressing both the Gluc and the blue fluorescent protein, cerulean (CFP) separated by an IRES under the control of CMV promoter was used¹. For SEAP assays, a similar lentivirus vector was used which expresses both SEAP and the red-fluorescent protein, mCherry under the control of CMV promoter⁸.

Transduction of cells to express the reporter—Cells of interest are transduced with the viral vector and are propagated in cell-specific growth media. In general, many human tumor cell lines including, Gli36 and U87 cells, as well as the commonly used 293T human fibroblast cells are propagated in DMEM high glucose media supplemented with 10% (vol/vol) Fetal Bovine Serum (FBS) and 1% (vol/vol) penicillin/streptomycin (pen/strep). Transduced cells are plated into triplicate wells of a 24-well plate, at around 20% confluency. An aliquot of the conditioned medium is assayed for the reporter activity (Gluc or SEAP) (5 – 50 µl) at different time points (12, 24, 48, 72 h) to confirm that stable transduction of the viral vector was achieved and that the reporter activity is linear with respect to time. i.e., cell growth and proliferation. This is particularly important when using SEAP as it does not have a wide linear range with respect to cell number^{1,8}. When low signals are observed, possibly due to inefficient gene transfer, the experiment can be scaled up by assaying larger volumes of conditioned medium. Similarly, if the signals obtained are too high, possible for Gluc with high gene transfer, the experiment can be scaled down by diluting the conditioned medium in growth media (10x) before assaying for Gluc activity.

Choice of cell lines—For optimization of the Gluc and SEAP assays, we used Gli36 human glioma cells which infect very well (>90%) with lentivirus vectors⁸. The doubling time for Gli36 cells is around 24 h which leads to doubling of the reporter level over this period. *In vivo*, these cells form tumors rapidly which grow exponentially over time^{8,19,35}. On the other hand, the transfection efficiency of Gli36 cells with reagents such as Lipofectamine2000 is very low (<20%). When a viral vector is not available and transient gene transfer is needed, cell lines that transfect well (e.g., 293T cells; >60%) should be used¹. The disadvantage of using transient transfection is that the gene is lost during cell division and therefore biological processes can be monitored only over a short period of time (few days).

Secreted reporter assays in culture—The biological process of interest should be monitored in culture *in vitro* first before moving to a more complex *in vivo* system. For instance

when monitoring a novel tumor therapeutic drug, the cells expressing the reporter should be plated into 24-well plates at 50-80% confluency and treated with the drug of interest. It is important to include negative controls of growth media alone as well as cells cultured with the diluent of the drug (vehicle) at the same concentration used in the experimental wells. It is always ideal to include a positive control (if available) which could be a drug known to kill the cells in the same mechanism as the drug of interest. Aliquots of the conditioned medium are assayed for the reporter activity at different time points. The results should always be normalized to the negative control treated with the vehicle. This protocol can be followed for any biological process such as promoter activation, endoplasmic reticulum stress, etc.

If a lentivirus vector is unavailable and transfection reagent is to be used for the reporter gene transfer, it is important to normalize for the transfection efficiency between different wells. If the reporter plasmid also expresses a fluorescent protein such as GFP, it can be used for normalization using fluorescence microscopy followed by cell counting or FACS analysis³⁶. Alternatively, and more quantitatively, co-transfection with another bioluminescent reporter which uses a different substrate such as firefly luciferase (Fluc) can be performed. In this case, the cell lysates are analyzed at the end of the experiment for Fluc activity and the Gluc or SEAP reporter values are normalized to the Fluc values¹.

Monitoring of *in vivo* processes—In general, athymic nude mice are used for tumor models and *in vivo* bioluminescence imaging. In our hands, Gluc can be expressed for at-least 3 weeks in non-athymic wild-type mice without the signal being compromised due to the presence of neutralizing antibodies. For experiments which require longer monitoring of the Gluc signal in wild-type mice, one might want to initially test whether neutralizing antibodies affect the Gluc signal throughout the duration of the experiment. One way of achieving this is to inject (i.v.) filtered conditioned medium from Gluc-expressing cells. The Gluc half-life in circulation is then monitored by collecting blood samples every 5 min for 1 h and measuring Gluc activity as described in Box 1⁸. These injections are repeated 2x/day over 7 days period. Then, these injections are repeated once a week (for the duration of the experiment) followed by Gluc half-life measurement as above. In cases where antibodies have no effect on Gluc signal, the half-life of Gluc in circulation should remain the same (around 20 min).

The *in vivo* experiment is carried out in a similar manner to the *in vitro* assay described above. Mice are anesthetized with an intra-peritoneal (i.p.) injection of ketamine/xylazine mixture which provides a 100 mg kg⁻¹ body weight dose of ketamine and a 10 mg kg⁻¹ dose of xylazine. Normally, for a 25 g mouse, 110 µl of anesthesia mixture (see reagent set-up) is used. Initially, cells-expressing the reporter are implanted in the location of interest. For tumor monitoring, the easiest model is to inject the tumor cells subcutaneously¹⁹. In this case, approximately 1 million cells (in 50 µl PBS) are mixed with 50 µl of Matrigel (ice cold) and immediately implanted using an insulin syringe. A more complicated model would be to inject the cells in a biologically relevant environment such as the brain for Gliomas⁸. Two controls are generally essential for the monitoring of *in vivo* processes: (1) Blood or urine from mice which are not implanted with cells (background) and when evaluating the effects of drugs on tumorigenesis (2) blood or urine from mice which are treated with the vehicle (the drug diluent; negative control). The time point at which to withdraw blood or urine will depend on the biological process of interest. For tumor monitoring or circulating cells (such as stem cells and T lymphocytes) viability and proliferation, blood or urine can be withdrawn every 3 days over the course of a month and aliquots of whole blood/urine or serum are assayed for Gluc or SEAP activity, respectively. As an example, when monitoring the effect of a novel chemotherapeutic drug, tumor cells-expressing the reporter are implanted subcutaneously. Blood or urine (5 µl) is withdrawn every 3 days and assayed for the reporter activity. Following tumor formation (around 2 weeks depending on the cell type used), different doses of the drug of interest are injected into the animal (intravenous (i.v.), i.p. or intra-tumoral, depending on the drug).

Around 5 animals/drug dose should be used to obtain statistical significance. Blood, urine or serum samples are monitored for the reporter activity as described above for at least 1 month. If using Gluc as a blood or urine marker, the signal can be localized in the animal using *in vivo* bioluminescence imaging once a week. A similar protocol can be followed for monitoring of other biological processes except that the time point at which blood, urine or serum is assayed for the reporter activity can be different and needs to be optimized for each process. For instance, endoplasmic reticulum stress is normally immediate after the treatment with thapsigargin, a known inducer of this type of stress¹¹, therefore, blood, urine or serum samples will be assayed every 1 h for the first 8-12 h post-treatment, and then again after 24 h.

***In vivo* Gluc bioluminescence imaging**—Coelenterazine is normally injected i.v. at a dose of 4 mg kg⁻¹ body weight. For a 25 g mouse, 20 µl of 5 mg ml⁻¹ coelenterazine is mixed with 130 µl of ice cold PBS and injected i.v. immediately before imaging is performed. Injection through the tail-vein is normally tricky, however, intra-ocular injections are normally much easier to perform and one might choose to inject the substrate this way. If this procedure is followed, one might observe some photons around the eye area which can be due to the way the injection was performed however this is not usually detrimental to the experiment or the mice.

The SEAP assay—There are many kits available which measures the alkaline phosphatase activity. One kit which is used most is the Great EscAPe SEAP assay (Clontech). Another kit is the Phospha-Light SEAP reporter gene assay system (Applied Biosystems). Both of these kits basically use the same protocol. A home-made colorimetric SEAP assay system can also be prepared, albeit it will be much less sensitive (100-1000-fold lower) than these kits (<http://home.ccr.cancer.gov/lco/ColorimetricSEAP.htm>).

MATERIALS

Reagents

- A Lentivirus vector expressing Gluc, codon-optimized for mammalian gene expression (Targeting Systems, El Cajon CA, cat. no. LP-07).

CRITICAL store at -80 °C.

CAUTION Although lentivirus vectors are safe, generally, a permit is required to work with this vector. Use under the hood in BL2 facility.

- *Gaussia* luciferase-expressing plasmid, pCMV-Gluc (Nanolight, Pinetop AZ, cat. No. 202).

CRITICAL store at -20 °C.

- • Lipofectamine 2000 (Invitrogen, Carlsbad CA, cat. no. 11668) or other transfection reagents.

CRITICAL store at 4 °C

- Polybrene (Sigma-Aldrich, St. Louis MO, cat. no. 107689).
- Coelenterazine free base, the *Gaussia* luciferase substrate (Nanolight, Pinetop AZ, cat. no. 303 NF-VTZ-FB). <Critical> prepare a solution of 5 mg/mL in acidified methanol, aliquot and store at -80 °C; or GAR-2 reagent (Targeting Systems, El Cajon CA, cat. no. GAR2-0090A)
- Hydrochloric acid (Sigma-Aldrich, St. Louis MO)

CAUTION Harmful. Use under a ventilated hood.

- Methanol (Sigma-Aldrich, St. Louis MO)
- Sodium Chloride (NaCl; Sigma-Aldrich, St. Louis MO)
- Ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, St. Louis MO)
- pSEAP2 control vector (Clontech, Mountain View CA, cat. no. 631717).

CRITICAL store at -20°C .

- 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)-tricyclo[3.3.1.1.3,7]-decan}-4-yl) phenyl phosphate (CSPD) chemiluminescent substrate (Clontech, Mountain View CA, cat. no. 631725). Alternatively, Great EscAPe SEAP Reporter System 3 which contains the SEAP reporter plasmid and all necessary reagents to detect SEAP in the media or in serum (Clontech, Mountain View CA, cat. no. 631706)
- Standard DNA cloning reagents
- Standard media for mammalian cell culture, Dulbecco's Modified Eagle Medium or DMEM (Invitrogen, Carlsbad CA, cat. no. 11995),
- Penicillin/streptomycin (Invitrogen, Carlsbad CA, cat. no. 15140),
- Fetal bovine serum (Invitrogen, Carlsbad CA, cat. no. 16000)
- Trypsin-EDTA (Invitrogen, Carlsbad CA, cat. no. 25-052-CI)
- Phosphate buffer saline or PBS (Invitrogen, Carlsbad CA, cat. no. 14080-055)
- Ketamine (Bedford Lab. Bedford OH, cat. no. KTVA04).

CAUTION Harmful

- Xylazine (AnaSed Injection; LLOYD Lab., Shenandoah IA, cat. no. 139-236).
CAUTION Harmful
- 0.9% (wt/vol) sterile NaCl (Abbot Lab., Abbot Park IL, cat. no. NDC 0074-1966-07)
- Matrigel (BD Bioscience, San Jose, CA, cat. no. 354230).
- CRITICAL store at -20°C . thaw slowly on ice before use.
- Mice, preferably nude athymic especially for Gluc and *in vivo* bioluminescence imaging (NCI-Frederick, Frederick MD, Strain 01B75).

CAUTION All animal studies should be carried out under institutional and national guidelines

Equipment (add city and state for companies)

- Coulter counter (Beckman Coulter, Fullerton CA) or hemacytometer (Advanced Microscope, Mill Creek WA, Cat. No. AMD-N01)
- Microcentrifuge (Eppendorf, Westbury NY)
- Fluorescence microscope to monitor DNA transduction efficiency (Zeiss LSM 510; Thornwood NY)
- A luminometer to monitor both the *Gaussia* luciferase and SEAP activity (EG&G Berthold Microlumat; Oak Ridge TN).
- Optional: a cooled couple-charged device (CCD) camera for *in vivo* bioluminescence imaging of Gluc (Xenogen, Alameda CA).

Reagent Setup

Lentivirus vectors—Vectors are usually stored at -80°C and they are stable for 2 years. Vectors should be thawed immediately before cell infection. Avoid freezing and thawing since this will decrease the vector infection efficiency.

CAUTION Although lentivirus vectors are safe, generally, a permit is required to work with this vector. Work under the hood

Coelenterazine—Coelenterazine is not soluble in aqueous solution. First, it should be dissolved in acidified methanol (add 1 drop of concentrated HCl to 10 ml of methanol) to a concentration of 5 mg ml^{-1} . Aliquots of $100\text{ }\mu\text{l}$ are stored at -80°C which is stable for >1 year. An aliquot which is used often can be stored at -20°C .

Critical Coelenterazine is prone to auto-oxidation resulting in a decrease in signal over time. To stabilize this substrate, dilute it to a working concentration in DMEM and incubate for 30 min in the dark at $20 - 25^{\circ}\text{C}$ before use. PBS with 5mM NaCl , pH 7.2 could be used as an alternative diluent for coelenterazine to give higher light output and more stability of the substrate.

Anesthesia—Prepare a mixture of ketamine and xylazine in saline solution by mixing 2 parts of ketamine (50 mg ml^{-1}) with 1 part of xylazine (20 mg ml^{-1}) and 1 part of 0.9% (wt/vol) NaCl. This mixture is stable at $20 - 25^{\circ}\text{C}$ indefinitely.

CAUTION Harmful. Always wear gloves

Polybrene—Dilute polybrene in sterile water to a final concentration of 8 mg ml^{-1} and store at 4°C for a maximum of 12 months

Coelenterazine. Prepare a solution of 5 mg ml^{-1} coelenterazine in acidified methanol, aliquot and store at -80°C for maximum of 2 years.

PROCEDURE

Transduction of cells to express the reporter (Gluc or SEAP) Timing 48-72 h

1| Plate cells of interest such as tumor cells in a well of a 6-well plate in 2 ml of growth media (DMEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) Pen/Strep) at 70-80% confluency and incubate for 24 h in an incubator at 37°C , 5% CO_2 .

2| Wash the cells with 3 ml of PBS

3| Transduce the cells with the appropriate construct. This can be done using option (A) Using a lentivirus construct or Option (B) Using a plasmid construct.

Option (A) Using a lentivirus construct

(i) Mix the lentivirus vector [multiplicity of infection (MOI) of 10; e.g., use 10 million transducing units (normally $100\text{ }\mu\text{l}$ of non-concentrated lentivirus vector is sufficient) for 1 million cells] with 1 ml of growth media containing polybrene ($8\text{ }\mu\text{g ml}^{-1}$) and add directly to the cells and incubate for 24 h.

CAUTION Although lentivirus vectors are safe, generally, a permit is required to work with this vector. Work under the hood in BL2 facility.

Option (B) Using a plasmid construct

(i) Using transfection reagents such as Lipofectamine2000, in tube 1, mix 100 μ l of growth media with 1 μ g of the plasmid expressing the reporter (Gluc or SEAP) and GFP. In tube 2, mix 100 μ l growth media with 3 μ l of Lipofectamine2000. Mix tube 1 and 2 and incubate for 45 min at 20 – 25 °C. Increase the volume of the mix to 1 ml with growth media. Remove the media from the cells and add the mix and incubate at 37 °C in a 5% CO₂ incubator for 4 h.

4| Remove the media and wash the cells with 2 ml of PBS. Remove the PBS and add 2 ml of fresh culture medium and incubate the cells for a further 24 h.

5| Determine the transduction/transfection efficiency by counting the number of GFP positive cells using fluorescence microscopy in 3 different fields (10x) normalized to the GFP negative cells in the same fields. When the Gluc reporter plasmid or lentivirus vector does not contain an eGFP expression cassette, transduction efficiency can be estimated by measuring 15 μ l aliquots of the conditioned medium for Gluc or SEAP activity using the protocols described in Box 1 and Box 2, respectively.

PAUSE POINT When using viral vectors that stably integrate into the host genome such as lentiviruses, the cells may be grown and stored in liquid nitrogen indefinitely for later use.

Optimization of the linearity of the reporter assay with respect to time and cell number Timing 48-72 h

6| Plate different amounts of cells (ranging from 10,000 to 100,000 cells) expressing the reporter construct in 0.5 ml of growth media into 24 well plates.

7| At different time points (12, 24, 48 and 72 h), remove 10 - 50 μ l of the conditioned medium and transfer it into a clean white or black 96-well plate and assay for Gluc or SEAP reporter as described in Box 1 or Box 2, respectively.

CRITICAL STEP It is important not to use clear plates since chemiluminescence signals can transfer from one well to the next leading to cross-contamination of signals.

Pause Point Aliquots of the conditioned medium may be stored at –20 °C or –80 °C and are stable for >1 month and can be assayed together at the last time point of the experiment.

8| Analyze the data by plotting the signal obtained as relative light unit (RLU) with respect to cell number or time (Fig. 1)^{1,4,18}.

?TROUBLESHOOTING

Reporter activity assay to monitor biological processes in culture Timing 48-96 h

9| Plate cells expressing the reporter constructs at 50% confluency in 0.5 ml in 21 wells of a 24 well plate and incubate for 24 h.

10| Wash the cells with PBS as described in Step 4

11| Prepare 5 different concentrations of the drug of interest in fresh media. Treat triplicate wells of cells with 0.5 ml of each drug concentration. Prepare 3 negative control wells by adding fresh media only and 3 vehicle control wells by adding media containing the drug diluent.

CRITICAL STEP Always include as a negative control triplicate wells of cells treated with the drug diluent (vehicle) at the same concentration and volume of media used in the experimental wells.

12| At different time points, remove 15 μ l of the conditioned medium and assay for Gluc or SEAP reporter activity as described in Box 1 or Box 2, respectively.

PAUSE POINT Aliquots of the conditioned medium may be stored at -20°C or -80°C and are stable for >1 month and can be assayed together at the last time point of the experiment.

13| Analyze the data by first normalizing the signals obtained from the drug-treated wells to the negative control at the different time points and plotting the % RLU in which the negative control is set to 100%¹.

CRITICAL STEP It is ideal if the results are validated with a well-established technique such as Western blotting for protein analysis or real-time PCR for mRNA analysis for Gluc or SEAP as well as other genes that are expected to be up- or down-regulated in response to the drug treatment¹.

?TROUBLE SHOOTING

Monitoring of Biological Processes *In Vivo* Timing 2 – 30 d

14| Transduce the cells of interest with the SEAP or Gluc reporter as in step 3.

15| After 7 days of culture (or longer depending on the cell of interest), remove the medium and wash the cells with PBS as described in Step 4. Add 4 ml of trypsin and incubate the cells for 1 min. Neutralize the trypsin by addition of 20 ml of growth media.

16| Count the cells using a coulter counter or a hemacytometer.

17| Pellet the cells by centrifugation for 5 min at 1500 g.

18| Resuspend the cells in PBS adjusting the concentration of the cells to $20,000\ \mu\text{l}^{-1}$.

19| Anaesthetize the mice by i.p. injection of a ketamine/xylazine mixture which gives a ketamine dose of $100\ \text{mg kg}^{-1}$ and xylazine dose of $10\ \text{mg kg}^{-1}$ body weight.

CAUTION All animal studies should be carried out under institutional and national guidelines.

20| Implant the cells expressing the reporter in the location of interest or systemically (i.v.) depending on the biological process⁸. For subcutaneous tumor model, mix 50 μl of ice cold Matrigel with 50 μl of cells (1 million cells total) and implant them using an insulin syringe.

21| Immediately before (time zero) and at different time points after cell implantation (once every 3 days for tumor growth), withdraw 5-10 μl of blood by simple tail-vein cut or collect a similar volume of urine and assay for the Gluc or SEAP reporter activity^{8,12} as described in Box 3 and 4, respectively.

22| When tumor is formed, generally 1 week when using Gli36 tumor cell line, divide the mice into groups (generally 5 mice/group) and inject i.p. or i.v. each group with either the vehicle (negative control) or with different doses of the drug of interest. The dose as well as the route of injection will depend on the drug used.

CRITICAL STEP It is essential to assay blood, urine or serum samples from mice which have either have not been implanted with any cells as a background control and from mice bearing reporter-expressing cells and treated with the vehicle as a negative control.

23| At different time points after treatment (once every 3 days for tumor therapy), withdraw 5-10 μl of blood by making a small incision in the tail of mice (no anesthesia required) or similar volume of urine and assay for Gluc or SEAP reporter activity as described in Box 3 and Box 4, respectively.

PAUSE POINT Blood or urine aliquots (for Gluc) or serum (for SEAP) may be stored at -20°C or -80°C (stable for 1 month) and assayed for the reporter activity at a later time or together with samples collected at different time points.

24| Analyze the data by plotting the relative light units (RLU) on the y-axis with respect to time on the x-axis^{8,12}.

?TROUBLESHOOTING>

***In Vivo* Gluc Bioluminescence Imaging. Timing 30 min**

25| Anesthetize the mice as described in Step 19.

26| Immediately before use, for each 25 g mouse, mix 20 μ l of coelenterazine (5 mg ml^{-1}) prepared in acidified methanol as described in the REAGENT SET-UP section with 130 μ l cold PBS and inject immediately into the tail vein of mice or intra-ocularly.

CAUTION A small precipitate/cloudy solution might form during injection which normally does not interfere with imaging. However, if coelenterazine diluted in PBS is incubated for a long period of time, a larger precipitate will form which might lead to blood-clots and death of the animal.

27| After coelenterazine injection, acquire photon counts over a 1-5 min period using a cooled CCD camera with no illumination¹⁹.

CRITICAL STEP Since *Gaussia* luciferase reaction is fast with flash kinetics, it is important to image the animals immediately after coelenterazine injection. It is also essential to keep the time between each injection and the imaging constant. Normally, imaging is carried out 1 min after coelenterazine injection.

28| Take a light image of the animal in the chamber using dim polychromatic illumination¹⁹.

29| Process the images using an imaging program such as ImageJ (NIH; <http://rsb.info.nih.gov/ij/>) or other programs available from the supplier of the CCD camera.

30| Define the regions of interest around the tumor using an automatic intensity contour procedure to identify bioluminescence signals with intensities significantly greater than the background¹⁹.

31| Calculate the sum of the photon counts in these regions as a measurement of Gluc activity.

32| Fuse the bioluminescence images with the corresponding white light surface images in a transparent pseudocolor overlay to permit correlation of areas of bioluminescent activity with anatomy¹⁹.

33| Analyze the data by plotting the photons/sec at the y-axis with respect to time on the x-axis^{8,19}.

?TROUBLESHOOTING

Box 1

***In vitro* Gluc reporter assay Timing 30 min**

1| Prepare a solution of 20 μ M coelenterazine by diluting it in PBS supplemented with 5 mM NaCl, pH 7.2.

CRITICAL STEP Incubate the coelenterazine mixture for 30 min at room temperature (18 to 25 °C) in the dark by wrapping the tube in aluminum foil to stabilize it.

2| Using a pipette, transfer 10 – 50 μ l aliquot of the conditioned media to a well of a white or black 96-well plate.

CRITICAL STEP it is important to use white or black plates not clear plates as the bioluminescence signal can transfer from one well to the next leading to cross-contamination.

3| Measure the Gluc activity using a plate luminometer which is set to inject 50 μ l of 20 μ M coelenterazine and to acquire photon counts for 10 sec.

4| Analyze the data by plotting the relative light units (RLU; y-axis) with respect to either the cell number or time (x-axis). In the case that one is performing a dose-response curve, data can be plotted as % Gluc expression in which all data are compared to the control well which is set to 100%.

?TROUBLESHOOTING

End of Box 1

Box 2

In vitro SEAP reporter assay Timing 1.5 h

1| Using the Great EscAPe SEAP assay kit (Clontech), mix 15 μ l of cell free conditioned medium with 45 μ l of 1x dilution buffer

1| Incubate the mixture for 30 min at 65 °C.

2| Cool the sample to room temperature

3| Add 60 μ l of assay buffer containing L-homoarginine and incubate for 5 min at room temperature.

4| Add 60 μ l of the chemiluminescent enhancer containing 1.25 mM CSPD substrate to the sample and incubate for 15 min at room temperature.

5| Transfer the reaction mixture to a clean white or black 96-well plate.

CRITICAL STEP it is important to use white or black plates not clear plates as chemiluminescence signals can transfer from one well to the next leading to cross-contamination.

6| Measure the chemiluminescence signal using a luminometer by acquiring photon counts for 5-10 sec.

7| Analyze the data by plotting the RLU with respect to either cell number or time. In the case that one is performing a dose-response curve, data can be plotted as % SEAP expression in which all data are compared to the control well which is set to 100%.

?TROUBLESHOOTING

End of Box 2.

Box 3

In vivo Gluc Blood and Urine Reporter Assay Timing 30 min

1| Withdraw 5 μ l of blood by making a small incision in the tail of mice using a razor blade (no anesthesia required) or collect 5 μ l of urine

2| In the case of blood, immediately mix the sample with 1 μ l of 50 mM EDTA (as an anticoagulant)

- 3| Prepare a solution of 100 μ M coelenterazine by diluting it in PBS supplemented with 5 mM NaCl, pH 7.2.

CRITICAL STEP Incubate the coelenterazine mixture for 30 min at room temperature (18 to 25 °C) in the dark by wrapping the tube in aluminum foil to stabilize it.

- 4| Transfer 5 μ l of blood or urine sample to a well of 96-well plate (white or black).

CRITICAL STEP It is important to use white or black plates not clear plates as the bioluminescence signal can transfer from one well to the next leading to cross-contamination.

- 5| Measure the Gluc activity using a plate luminometer which is set to inject 100 μ l of 100 μ M coelenterazine and to acquire photon counts for 10 sec.

- 6| Analyze the data by plotting the relative light units (RLU; y-axis) with respect to time (x-axis). In the case that one is performing a drug treatment *in vivo*, data can be plotted as % Photons in which all data are compared to the control group of animals which is set to 100%.

?TROUBLESHOOTING

End of Box 3.

Box 4

In vivo Serum SEAP Reporter Assay Timing 1.5 h

- 1| Withdraw 10 μ l of blood by making a small incision in the tail of mice using a razor blade (no anesthesia required)
- 2| Immediately centrifuge the samples for 5 min at 1500x g and transfer the supernatant (serum) into new eppendorf tubes.
- 3| Mix 5 μ l of the serum with 15 μ l of 1x dilution buffer
- 4| Incubate the mixture for 30 min at 65 °C.
- 5| Cool down the sample to room temperature (18 – 25 °C)
- 6| Add 20 μ l of assay buffer containing L-homoarginine and incubate for 5 min at room temperature.
- 7| Add 20 μ l of chemiluminescent enhancer containing 1.25 mM CSPD substrate and incubate for 15 min at room temperature.
- 8| Transfer the reaction mixture to a clean white or black 96-well plate and measure the chemiluminescence activity using a luminometer by acquiring photon counts for 5-10 s.

?TROUBLESHOOTING

End of Box 4.

TIMING

Steps 1-5, Transduction of cells to express the reporter (Gluc or SEAP) 48-72 h

Steps 6-8, Optimization of the linearity of the reporter assay with respect to time and cell number 48-72 h

Steps 9-13, Reporter activity assay to monitor biological processes in culture 48-96 h

Steps 14-24, Monitoring of Biological Processes *In Vivo* 2 – 30 d

Steps 25-33, *In Vivo* Gluc Bioluminescence Imaging. 30 min

Box 1 In vitro Gluc reporter assay 30 min

Box 2| In vitro SEAP reporter assay 1.5 h

Box 3| *In vivo* Gluc Blood and Urine Reporter Assay 30 min

Box 4| *In vivo* Serum SEAP Reporter Assay 1.5 h

?TROUBLESHOOTING

Troubleshooting advice can be found in table 2.

ANTICIPATED RESULTS

In vitro detection of Gluc and SEAP reporter activity

A linear curve with respect to cell number should be observed in cultured cells with both the Gluc and SEAP reporter assays (Fig. 1a). Further, a linear curve with respect to time as an index of cell growth and proliferation is expected with both reporters. Figure 1b shows the increase of Gluc expression with respect to time. A similar increase in SEAP expression would be expected (data not shown).

In vivo monitoring of tumor growth and proliferation using Gluc and SEAP reporter assays

For both the SEAP and Gluc reporter, a linear curve is expected with respect to the number of cells expressing the reporter (Fig. 2a). In the case of Gluc, similar linearity with respect to cell number is obtained using the urine assay (Fig. 2a) as well as an increase in photons with *in vivo* bioluminescence imaging (Fig. 2b). The level of Gluc in the blood should increase over time indicating the growth of the tumor (Fig. 2c). Upon treatment of tumors with drugs or viruses, the levels of Gluc in the blood should decrease correlating with the efficiency of drug or virus treatment (Fig. 2c).

Monitoring the Gluc and SEAP reporter activity of of circulating cells

Depending on the injected cell type, the results might vary. Initially, expect a high blood level of the reporter upon cell implantation. One-to-two d after implantation, the reporter activity should decrease signaling the death of some cells. At later time points, the signal is either stable indicating that some of the injected cells are still viable but not proliferating (Fig. 2d); the signal increases indicating that the cells which survived the injection are proliferating; or the signal decreases to background levels indicating that all of the implanted cells are dead. The expected results from the last 2 scenarios would be similar to the results presented in Figure 2c.

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Figure 1. *In vitro* detection of secreted reporters

Different numbers of Gli36 human glioma cells expressing SEAP or Gluc were plated in wells of a 96-well plate and the conditioned medium was assayed for Gluc and SEAP after 24 h of culture (a). Cells (20,000) expressing Gluc were plated into 96-well plates and Gluc activity in the medium was assayed at different time points of culture (b). Results presented are mean $\pm$ s.d. (n = 5 with the experiment repeated 3x). RLU, relative light units. Figure adapted from Badr et al., 2007¹.

Figure 2. Blood monitoring of *in vivo* processes

Different numbers of Gli36 human glioma cells expressing both Gluc and SEAP were implanted subcutaneously into 5 athymic nude mice. After 48 h, 5 μ l of blood and urine was assayed for Gluc and 5 μ l of serum assayed for SEAP activity (a). Data presented as mean $\pm$ s.d. (n=5 with experiment repeated 3x). Gluc signal was localized in the animals using a CCD camera and *in vivo* bioluminescence imaging (b). Gli36 cells (1×10^6) expressing the Gluc reporter were implanted subcutaneously into nude mice (n=10). On d 10 after implantation, tumors were injected with either PBS (control, n=5) or an HSV oncolytic vector known to kill tumors (n=5). Gluc reporter activity was monitored in the blood at different time points (c). C17.2 neuroprecursor cells (1×10^6) expressing the Gluc reporter were injected i.v. into 5 nude mice and Gluc activity in the blood was monitored over time. Data presented in c-d are from one representative mouse from each group with the experiment repeated 3x. RLU, relative light units. Figure modified from Wurdinger et al., 2008³⁷.

Table 1

Comparison of Gluc and SEAP assay *in vivo*.

	Gluc reporter assay	SEAP reporter assay
Time to complete the assay	Minutes	hours
Sensitivity	1000-fold more sensitive	less sensitive than Gluc
Dynamic range to cell number	>5 orders of magnitudes	<3 orders of magnitudes
Assay in whole blood	Yes	No, requires sample processing
Half-life	20 min	3 hr leading to accumulation
Detected in urine	Yes, cleared by kidneys	No
Localized with in vivo bioluminescence imaging	Yes	No

Table 2

Troubleshooting table

Step	Problem	Possible reason	Solution
8 and 13; Box 1, step 4 and Box 2, step 7	Signal does not increase with respect to time or cell number. Signal reaches a plateau	A high number of cells are plated or a large volume of conditioned medium is assayed	When performing the linearity of the reporter assay with respect to time and cell number, the signal might reach a plateau at the highest concentration of cells or at the later time points (especially for SEAP). In this case, the protocol needs to be scaled down either by assaying a smaller volume of the conditioned medium and/or plating lower amount of cells. However, it is not recommended to assay less than 10 μ l of conditioned medium as pipetting errors may be higher especially with the Gluc assay
24 and 33	Dose-response with respect to drug concentration is not obtained	Too little or too much drug is added per well	Higher or lower doses of drug may be needed. In the case of no response, use higher concentration of each drug/well. In the case of high response even with the lowest drug concentration, use less drug/well.
24; Box 3, step 6; Box 4, step 8	No reporter activity is observed in blood/serum	Not enough cells are implanted or transduction efficiency is low	More cells-expressing the reporter should be implanted. In the case of tumor and other cells that are known to proliferate <i>in vivo</i> , one may choose to wait 1 – 2 weeks to obtain higher reporter signal before starting the drug treatment.
33	No Gluc activity is observed during bioluminescence imaging	Not enough cells are concentrated in one location of the animal	Implant more cells. In the case of using cells which proliferate <i>in vivo</i> , wait 2 weeks before the next imaging session.
33	Signal obtained from tumors is saturated	Large tumor size	Decrease the imaging time (10 s – 1 min) in order to stay within the linear range of bioluminescence imaging.
33, Box 4, step 8	In the case of cells which are known to proliferate <i>in vivo</i> (e.g., tumors) SEAP activity does not increase over time	The number of cells <i>in vivo</i> is above the linear range of the reporter assay (especially for SEAP). System reached saturation	Dilute the serum sample in the assay buffer (20-fold dilution rather than 4-fold) by mixing 2 μ l of serum with 38 μ l of assay buffer and proceed with the SEAP assay