Gaussia luciferase blood level as an index of cell growth and proliferation

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Method Article

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Abstract

Introduction

Luciferase-mediated bioluminescence is widely used as a reporting tool for monitoring various biological processes in vitro and in vivo. Firefly[1], Renilla[2], and Gaussia[3,4] luciferases are currently used as reporters for monitoring of numerous processes in different fields[5], including immunology[6] oncology[7], virology[8], and neuroscience[9]. After systemic substrate injection, a cooled charge-coupled device \(CCD) camera can be used for the localization of the luciferase photon signals in vivo. In addition, the naturally secreted and non-ATP dependent Gaussia luciferase \(Gluc) expression levels can be easily quantified in cell-free conditioned medium by adding its substrate coelenterazine and measuring emitted photons using a luminometer. Since Gaussia luciferase is naturally secreted from mammalian cells in culture[4], it is secreted into the blood of animals harboring cells expressing this reporter enzyme. Further, expression levels via a constitutive promoter is proportional to cell number irrespective of location of cells in the body, while levels under a physiologic responsive promoter should reflect the physiologic state of the tissue in which the cells were located. Here, we present the protocol for measuring the level of the naturally secreted Gaussia luciferase in few microliters of blood as a quantitative index of the number of cells expressing it. The level of Gluc blood in the blood can be used to monitor tumor growth and response to therapy, as well as the survival and proliferation of circulating cells such as stem cells and T-lymphocytes in vivo. The Gluc blood assay allows a convenient and quantitative assessment of in vivo luciferase reporter activity and should be a significant aid in the monitoring of biological processes in experimental animals.

Reagents

A Gaussia luciferase-expressing plasmid which can be obtained from Nanolight \(Pinetop, Az) or Targeting Systems \(El Cajon, CA). A Lentivirus vector expressing Gluc which can be obtained from Targeting Systems \(El Cajon, CA). Note: It would be helpful to obtain these constructs also expressing a fluorescent protein such as GFP so one can monitor transduction efficiency by fluorescent microscopy. Coelenterazine, the Gaussia luciferase substrate, can be obtained from Nanolight \(Pinetop, Az) or Targeting Systems \(El Cajon, CA). Fetal bovine serum \(FBS), penicillin and streptomycin mix, Dulbecco's modified Eagle's medium \(DMEM) and OPTIMEM, 10x phosphate buffere saline \(PBS) can be obtained from Cellgro by Mediatech Inc. \(Herndon, VA). Tissue culture plates, can be obtained from Fisher Scientific \(Pittsburg, PA)

Equipment

DNA transduction efficiency is monitored using a confocal fluorescence microscope Zeiss LSM 510 \(Jena, Germany). Gaussia luciferase activity is measured using a luminometer \(EG&G Berthold

Microlumat). In vivo bioluminescene imaging is carried out using a cooled couple-charged device \(CCD) camera \(Xenogen, Alamada, CA).

Procedure

1) Gluc vector preparation. If one has a viral vector system which will efficiently transduced the specific cell type that they are interested in, one can subclone the Gluc cDNA into this vector using standard cloning protocols. In general, lentivirus vectors transduce many different cell types with high efficiency. The Gaussia luciferase cDNA, codon optimized for mammalian gene expression[4], and a fluorescent protein such as GFP, separated by an internal ribosomal entry site \(IRES) elements are cloned into a lentivirus vector under the control of the strong constitutive cytomegalovirus \(CMV) promoter to produce LV-Gluc-GFP as described[3,10]. 2) LV-Gluc-GFP transduction of cells of interest. Plate one million cells in a 2 cm^2 well and 24 hrs later, add lentivirus vector so you have a multiplicity of infection \(MOI) of 10 - 50 in the presence of 8 µg/ml polybrene. After 5 h, wash the cells with PBS and add fresh culture medium and incubate the cells for 48 hrs. Alternatively, the cells of interest can be transfected by the plasmid expressing Gluc using transfection reagents such as lipofectamine \(Invitrogen, Carlsbad, CA), though less efficient and transient. Next, determine the transduction/transfection efficiency by analysis of the CFP expression using fluorescence microscopy. In order to determine the Gluc expression, harvest 5 - 20 µl aliquots of the conditioned medium into a clean white or black 96-well plate, and measure the Gluc activity using a plate luminometer which was set to inject 50 µl 20 µM coelenterazine in DMEM and to acquire photon counts for 10 sec. If lentivirus vector was used to transduce the cells, the gene will integrate within the genome and therefore the cells become stably expressing the reporter, therefore, one may choose to grow and freeze the cells for later use. 3) Measurement of Gluc activity from cells-expressing it. Plate different amount of cells in a different well of a 96-well plate in triplicate. 24 hrs later, aliquot 10 µl of conditioned medium into a black or white plate and measure the Gluc activiy after injecting 50 µl 20 µM coelenterazine and acquire the signal for 10 sec using a luminometer. To monitor cell growth and proliferation, plate one to five thousand cells in a well of 96-well plate in triplicate and the next day, start taking 10 µl aliquots at different time points and measure the Gluc activity as above. Note: Coelenterazine is known to auto-oxidizes. To stabilize this substrate, it should be diluted in DMEM and stored at R.T in the dark for 30 min before use. PBS with 5M NaCl could be used as an alternative diluent for coelenterazine to give higher light output and more stability of the substrate. 4) In vivo injection of Gluc-expressing cells. Anaesthetize the mice by i.p. injection of ketamine \(100 mg/kg) and xylazine \(5mg/kg). Inject Gluc-expressing cells or Gluc-expression vectors at the location of interest or systemically \(i.v.) and measure Gluc activity in the blood at several time-points before and after injection \(see below). 5) Gluc blood monitoring. Blood samples are withdrawn by making a small incision in the tail of mice \(no anesthesia required) or by retro-ocular withdrawal \(anesthesia required). Typically 5 µl blood is withdrawn using a p20 pipette and immediately added to 1 µl 20 mM EDTA and stored at 4ºC until all samples are collected \(up to 5 days). Gluc activity is measured using a plate luminometer which is set to inject 100 µl 100 µM coelenterazine \(stabilized by incubated for 30 min at R.T.) in DMEM to 5 µl of blood samples and to acquire photon counts for 10 sec. As a background control, blood from mice

injected with non-Gluc expressing cells is measured. 6) CCD camera imaging of Gluc signal. Mice are anesthetized as above and i.v. injected via tail-vein with 150 µl coelenterazine \(4 mg/kg body weight, around 100 µg/mouse) and photon counts are acquired immediately over 1-5 min using a cooled CCD camera with no illuminationas described4. A light image of the animal is taken in the chamber using dim polychromatic illumination. Following data acquisition, post-processing and visualization is performed using CMIR-Image \(a program developed by the Center for Molecular Imaging Research using image display and analysis suite developed in IDL \(Research Systems Inc., Boulder, CO) or other programs available from the company from which the CCD camera was purchased. Regions of interest are defined using an automatic intensity contour procedure to identify bioluminescence signals with intensities significantly greater than the background. The mean, standard deviation, and sum of the photon counts in these regions are calculated as a measurement of Gluc activity. For visualization purposes, bioluminescence images are fused with the corresponding white light surface images in a transparent pseudocolor overlay, permitting correlation of areas of bioluminescent activity with anatomy. Note: Coelenterazine is not soluble in aqueous solution. First, coelenterazine should be dissolved in acidified methanol \(add a drop of concentration HCl to 10 ml of methanol) to a concentration of 5 g/l. Immediately before injection, mix 20 µl of coelenterazine with 130 µl PBS and i.v. inject it immediately. A small precipitate/cloudy solution might form during injection which normally does not interfere with imaging. However, if coelenterazine in PBS was incubated at R.T., a larger precipitate will form which might lead to blood-clot. Retro-ocular injections are normally easier to be done and one might choose to inject the substrate this way, rather than tail-vein injection. 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 and this is normally fine. In vivo studies. All animal studies should be performed with relevant institutional guidelines and regulations.

Timing

10-20 min

Anticipated Results

In vitro detection of Gluc-expressing cells. Expect a linear curve of the Gluc signal with respect to cell number as well as with respect to time as an index of cell proliferation. For typical results, see Badr et al., \(2007)[3]. Monitoring of Gluc activity in vivo. Expect a linear curve between different amount of implanted cells and the Gluc signal obtained by either the blood assay or in vivo bioluminescence imaging using the CCD camera \(Fig. 1a & b). Also, expect an increase in the Gluc signal with respect to time \(Fig. 1c). Monitoring of circulating cells. Depending on the injected cell type, the results might vary. Initially, you would expect a drop in the Gluc signal since some of the injected cells are going to die. Later, the signal would either: remains the same indicating that the injected cells are still viable but not proliferating \(Fig. 1d); the signal would increase indicating the cells are proliferating; the signal would decrease to background level indicating all cells are dead.

References

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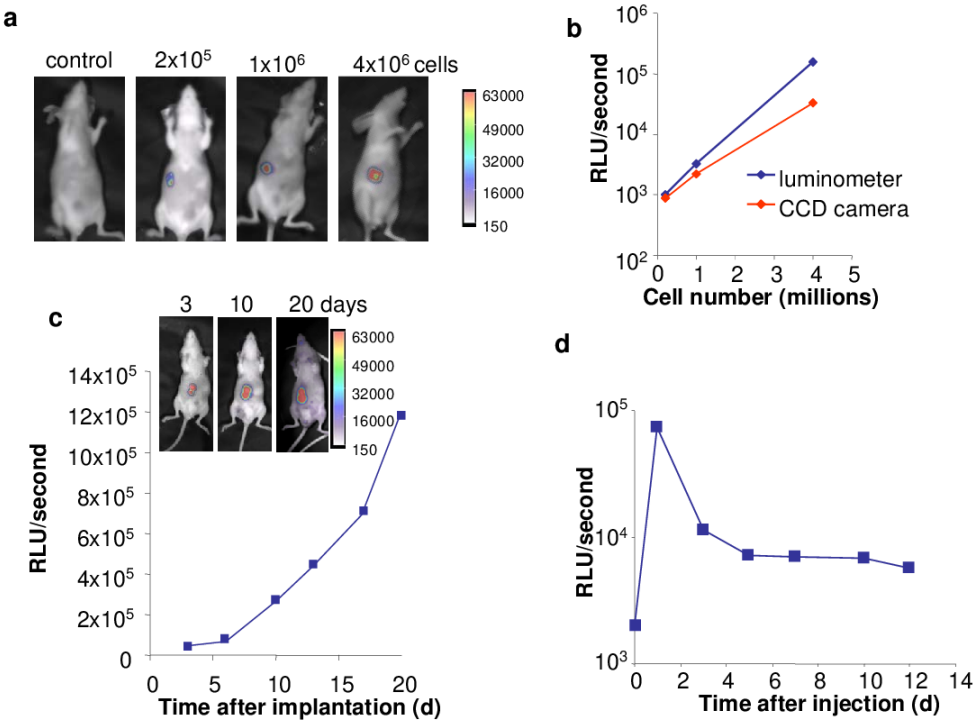
Figures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2x10^5
1x10^6
4x10^6 cells
63000
49000
32000
16000
150
b
RLU/second
luminometer
CCD camera
Cell number (millions)
c
3
10
20 days
RLU/second
Time after implantation (d)
d
RLU/second
Time after injection (d)

Figure 1

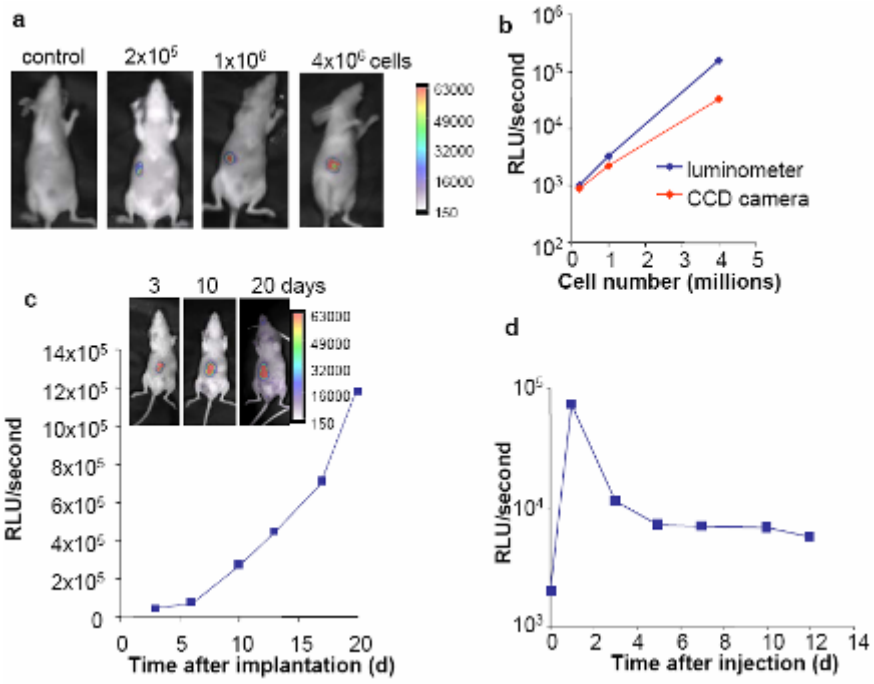

Figure 2

Figure 1