Bioluminescence Imaging using NightOWL LB 981 NC 100

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Abstract

Reporter genes are widely used for monitoring gene expression in *in vitro* and *in vivo* assays. Interest for firefly luciferase gene in *in vivo* bioluminescence imaging is increasing. This technology enables non-invasive visualization of gene expression in intact animals. Cells expressing luciferase gene under the control of a constitutive promoter were used as a model of *in vivo* proliferation of cancer cells. Intensities of their *in vitro* and *in vivo* bioluminescent signals were first compared and the conditions of *in vivo* bioluminescent reaction measurements were determined. Luciferase gene expression was non-invasively monitored with a cooled charge-coupled device (CCD) camera and quantitative analyses allowed us to follow tumor formation and proliferation in living animals. These bioluminescent models will become useful tools for evaluating cancer treatment efficiencies and the role of receptors in invasion and proliferation.

Introduction

Cancer therapy drug development needs new tools to detect the presence of small numbers of tumor cells in the early course of disease or remaining after therapy. It is also essential to evaluate the processes of cell transformation and tumor growth in the context of intact organ systems of living animals (1). Detection of tumor cells in current animal models are often time consuming and numerous animals have to be sacrificed to determine treatment efficiency. For a rapid detection and in order to avoid sacrificing numerous animals, we developed a non-invasive *in vivo* model using bioluminescent cell

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lines. Firefly luciferase gene has been widely used as a reporter gene in both *in vitro* and *in vivo* assays and to tag tumor cells for growth and therapy monitoring in animal models of human disease (2-7). Tagging of biological processes with reporters, which can be monitored and quantified externally, provides a powerful tool for evaluating disease progression and response to therapy (8). In our laboratory, low light imaging has been used for more than 20 years to visualize and quantify bioluminescent and chemiluminescent reactions in order to select cell lines stably expressing bioluminescent reporter genes (9-14). In this study, we used a firefly luciferase pCMV-Luc$^+$-SVNeo reporter gene stably transfected in human colon cancer LS174T cells to follow the growth of xenografts implanted in immunodeficient mice. Non-invasive monitoring and imaging of tumor cells, through their luciferase gene constitutive expression, were performed in living animals using NightOWL LB 981 NC 100 CCD camera from Berthold Technologies. Signals from labeled cells in these whole body images were used to both rapidly localize tumors and quantify cellular proliferation. The high efficiency detection sensitivity of the instrument together with the great bioluminescent cell signal allowed us to detect very early proliferation stages.

Materials and Methods

Materials

Materials for cell culture were obtained from Invitrogen (Cergy-Pontoise, France). Luciferin and geneticin (G418) were purchased from Promega (Charbonnières, France). Tribromo-ethanol (TBE) was purchased from Sigma Chemical (Saint Quentin-Fallavier, France).

Plasmid construction

To obtain pCMV-Luc$^+$-SVNeo plasmid, CMV region was amplified by PCR-cloning and introduced in p(RAR)$_3$-tk-Luc$^+$-SVNeo plasmid (9) previously deleted from its (RAR)$_3$-tk promoter.

Generation of stably transfected reporter cell lines

Human colon carcinoma LS174T cell line (15), originally established from a moderately differentiated human colon adenocarcinoma, was obtained from the American Type Culture Collection (Rockville, MD, USA). The luciferase stably transfected cell line, LSCL$^+$N (LS174T CMV Luc$^+$ Neo), was obtained with the help of the CCD photon counting camera (NightOWL LB 981 NC 100 from Berthold Technologies) as already described for other cell lines (9). Briefly, LS174T cells were transfected with the constitutive luciferase expressing pCMV-Luc$^+$-SVNeo gene and selection of resistant clones by G418 was performed at 1 mg/ml. The most luminescent clone was isolated and called LSCL$^+$N 3.

Cell culture conditions

For strain cultures, cells were grown in RPMI 1640, supplemented with 10% fetal calf serum (FCS), 1% antibiotic (penicillin/streptomycin) and 1 mg/ml G418 in a 5% CO_2 humidified atmosphere at 37°C. These cells were adherent and grew as monolayers. Because phenol red might absorb emitted light, *in vitro* experiments were achieved in phenol red-free medium (test culture medium).

Cell luciferase assay

Dilution series of LSCL$^+$N were seeded in 96-well white opaque tissue culture plates (Becton Dickinson) in 150 µl test culture medium. Experiments were performed in quadruplicate. After 6 hr cell attachment, medium was removed and replaced by 3×10^{-4} M luciferin containing test culture medium. The 96-well plate was then imaged by integrating luminescence signal for 1 min with NightOWL LB 981 NC 100 CCD camera and intact living cell luminescence was measured. The number of photons emitted per well was expressed as a function of cell number in each well. The same plate was rerun three days later to follow bioluminescent signal evolution after cell proliferation.

Animals

Athymic female Swiss nude mice (nu/nu), about 50 days old and weighing 18-20 g, were obtained from Charles-River (L'Arbresle, France). Mice were acclimatized for 1 week before the experiment was started and were housed in self-contained filter-top plastic cages (5 mice/cage) maintained under the following standard conditions: 22±2°C, 45±10% relative humidity, 12 hr light/12 hr dark cycle each day under near-sterile conditions. Mice were given a standard diet (UAR, Epinay-sur-Orge, France) and water *ad libitum*.

All experiments were performed in compliance with the French guidelines for experimental animal studies (Agreement No. B-34-172-27).

In vivo bioluminescence imaging

A subcutaneous xenograft was implanted in the right flank of female Swiss nude mice by injecting 1×10^6 LSCL$^+$N cells in 200 µl serum-free culture medium. Luciferase activity of LSCL$^+$N cells allows tumor visualization and growth quantification by *in vivo* bioluminescence imaging during more than one month. Experiments were achieved with a group of 15 mice, and each mouse was killed by cervical dislocation when tumor volume reached 2,500 to 3,000 mm^3.

To measure luciferase activity, 125 mg/kg body weight of luciferin (sodium salt; Promega) in aqueous solution was injected into the mouse peritoneal cavity (ip) 10 min prior to imaging. The luminescent signal was maximum 10 min after injection and remained stable for 10 min. Mice were sedated by ip injection of 230 mg/kg body weight of TBE. Anesthetic

was dissolved in ethanol (230 mg/ml) and then diluted in physiological serum (1:9). Alternatively, anesthesia was performed using an isoflurane gas anesthesia system from T.E.M. (Bordeaux, France). Mice were first sedated in an anesthesia induction box with 4% isoflurane in air and 1.5% anesthetic in air was then continuously delivered in the dark box *via* a nose cone system.

A gray-scale body-surface reference image was collected using the NightOWL LB 981 NC 100 CCD camera. Photons emitted from luciferase within the animal and transmitted through its tissues were collected and integrated for a 5 min period. A pseudocolor luminescent image from blue (least intense) to red (most intense), representing the special distribution of the detected photons emitted from active luciferase within the animal, was generated using WinLight software (BERTHOLD TECHNOLOGIES). The overlay of the real image and the luminescence representation allowed the localization and measurement of luminescence emitted from xenografts. The signal intensities from manually derived regions of interest (ROI) were obtained and data were expressed as photon flux (counts/s). Background photon flux was defined from a ROI of the same size placed in a non luminescent area nearby the animal and then subtracted from the measured luminescent signal intensity. All light measurements were performed under the same conditions, including camera settings, exposure time, distance from lenses to the animals and ROI size.

Results

In vitro detection limit of LSCL^{+}N cells

To be able to detect a small number of tumor cells in the animal, we engineered a stably transfected reporter cell line constitutively expressing a high level of firefly luciferase. The reporter gene, which is introduced into the target cell DNA as a stable integration, is replicated with cell division and not lost over time. We used human colon carcinoma LS174T cells transfected with the constitutive luciferase expressing pCMV-Luc^{+}-SVNeo gene and called them LSCL^{+}N cells. They were *in vitro* tested for their emitted luminescence using a NightOWL LB 981 CCD camera from BERTHOLD TECHNOLOGIES. Dilution series of LSCL^{+}N cells were achieved in 96-well culture plate. As little as 10 cells were easily detected which demonstrated the high level of firefly luciferase expressed in LSCL^{+}N cells (Fig. 1).

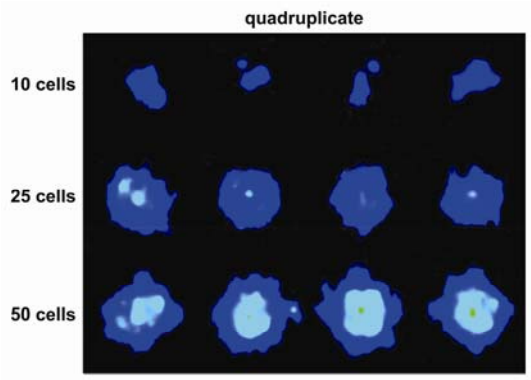

Figure 1: In vitro imaging of luminescent LSCL$^+$N cells. Dilution series of LSCL$^+$N cells were achieved in 96-well cell culture plates (10, 25 and 50 cells per well). Experiments were performed in quadruplicate. After 6 hr cell attachment, intact living cells were imaged by integrating luminescence signal for 1 min using the NightOWL LB 981 CCD camera. A pseudocolor luminescent image from blue (least intense) to green (most intense) is presented. As little as 10 cells were easily detected.

There was a perfect correlation between cell number and luciferase activity measured per well from 10 to 100,000 cells per well (R^2 of 0.9954).

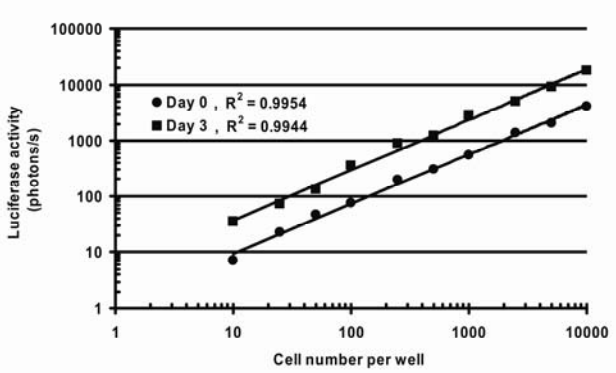

Figure 2: Sensitivity of in vitro LSCL$^+$N cell detection and correlation to cell proliferation. Dilution series of LSCL$^+$N cells were achieved in 96-well cell culture plates over a range of 10 to 100,000 cells per well. Experiments were performed in quadruplicate. After 6 hr cell attachment, intact living cell luminescence was measured by integrating signal for 1 min using the NightOWL LB 981 CCD camera. Total photon counts from each of the quadruplicate wells were determined, background signal was subtracted and means were plotted as a function of cell number (R^2 was 0.9954). Experiment was rerun 3 days later to follow cell proliferation (R^2 was 0.9955).

The same cells were imaged 3 days later to demonstrate that the correlation between cell number and luciferase activity was independent from cell proliferation in each well (R^2 of 0.9944) (Fig. 2). This result allowed us to implant LSCL$^+$N cells in mice to follow their *in vivo* proliferation.

In vivo proliferation of LSCL$^+$N xenografts in nude mice

In a first experiment, LSCL$^+$N cells (10^3, 10^4, 10^5 or 10^6 cells) were incubated with 3×10^{-4} M luciferin and then injected into female Swiss nude mice. Animals were imaged immediately after to measure *in vivo* bioluminescence and compare it with *in vitro* results

obtained above. As little as 10^3 cells could be detected *in vivo* (not shown). A xenograft was obtained when at least 10^6 cells were injected in the animal (Fig. 3A).

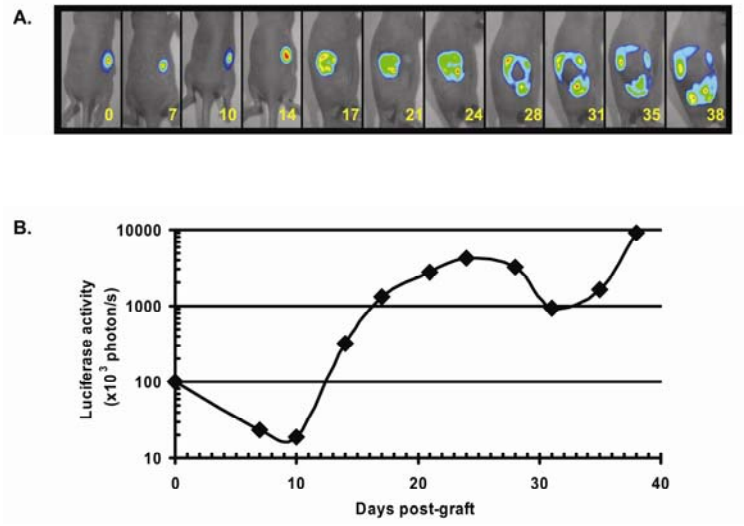

Figure 3: In vivo kinetics of tumor cell growth following subcutaneous injection of tumor cells. $LSCL^+N$ cells were injected into female Swiss nude mice, and animals were imaged twice a week for 38 days. (A) One representative animal injected with 1×10^6 $LSCL^+N$ cells is shown. Luciferin was ip administered and images were obtained by integrating bioluminescent signal for 5 min using the CCD camera. A pseudocolor luminescent image from blue (least intense) to red (most intense) is presented. (B) Kinetics of tumor signals was obtained and quantification data were expressed as photon flux (photons/s) using WinLight software from Berthold Technologies. Cells were detectable from injection day to sacrificing day. From day 0 to day 10 the bioluminescent signal decreased due to cell mortality and the signal then exponentially increased up to day 24 parallely to tumor cell proliferation. At day 28, signal extinction in tumor center indicated necrosis. From this time on, bioluminescent signal was no longer correlated with manually measured tumor volume.

Animals were then observed twice a week for 38 days. *In vivo* bioluminescence imaging was achieved as described in *Materials and Methods* using the CCD camera. Luciferin was ip injected and mice sedated either by ip injection of tribromoethanol or with isoflurane gas anesthesia system. The magnitude of bioluminescence measured *in vivo* varied with time after luciferin injection, as well as with dose, which necessitated that the comparison of the quantitative results took into consideration time after injection. To determine the optimal luciferase activity detection time, time course experiments were performed. Serial pictures were taken at different time intervals following luciferin injection roughly every 2 min. (Fig. 4). Maximal bioluminescent signal was obtained about 10 min after injection and remained stable during ten more minutes.

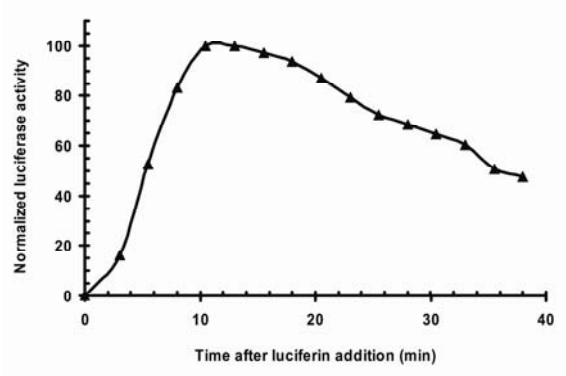

Figure 4: Time course of in vivo light emission from luciferin-treated LSCL$^+$N cells. Data were obtained after subcutaneous implantation of LSCL$^+$N cells in female Swiss nude mice. Mice were anesthetized and luciferin was ip injected. After 2 min imaging with CCD camera, data were extracted using WinLight software (Berthold Technologies) as described under Materials and Methods. The curve represents the normalized light units at each point measured by taking maximum value as 100.

A typical diagram of LSCL$^+$N cell xenograft proliferation in nude mouse followed by bioluminescence signal is reported in Fig. 20B. Similar proliferation patterns were observed in all the grafted animals (15 mice). Cell presence was easily detectable just after they were grafted. After a significant decrease up to day 10, tumor size and bioluminescence increased exponentially until day 24. Xenografts were manually measurable under the animal skin from day 14 only. During exponential proliferation, luciferase activity was well correlated with the manually measured xenograft volume (mm^3) estimated by the formula $d_1 \times d_2 \times d_3/2$, where d_1 is the tumor length, d_2 its width, and d_3 its height (Fig. 5).

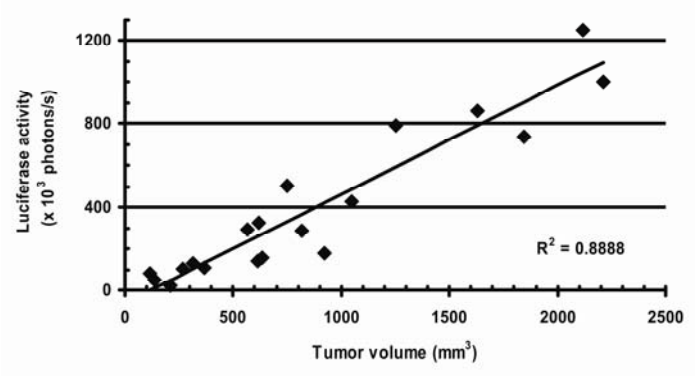

Figure 5: Correlation between luminescent signal and tumor volume during exponential proliferation. Luciferase activity and manually measured xenograft volume were plotted from 5 different mice that gave 19 separate measurements (R^2 was 0.8888). Luciferase activity was determined as described in Materials and Methods and tumor volume (mm^3) was estimated by the formula $d_1 \times d_2 \times d_3/2$, where d_1 is the tumor length, d_2 its width, and d_3 its height.

This result was obtained with 5 different mice giving 19 separate measurements (R^2 was 0.8888). After day 24, bioluminescence in the xenograft center decreased, due to central tumor necrosis most likely induced by insufficient angiogenesis, and there was no longer a correlation between cell signal and tumor volume.

Presence of necrotic LSCL$^+$N cells in xenograft

Loss of bioluminescence signal in the centre of LSCL$^+$N cell xenografts was due to an important necrosis. At day 38, the mouse was sacrificed and xenograft was taken out and cut into four parts named a to d (Fig. 6A). Those parts were placed in a 24-well culture plate to detect and quantify their luciferase activity. Intensity of luminescent signal emitted from these four parts as a function of weight is reported. Anatomo-pathological analysis determined necrosis percentage which was reported on the same graph.

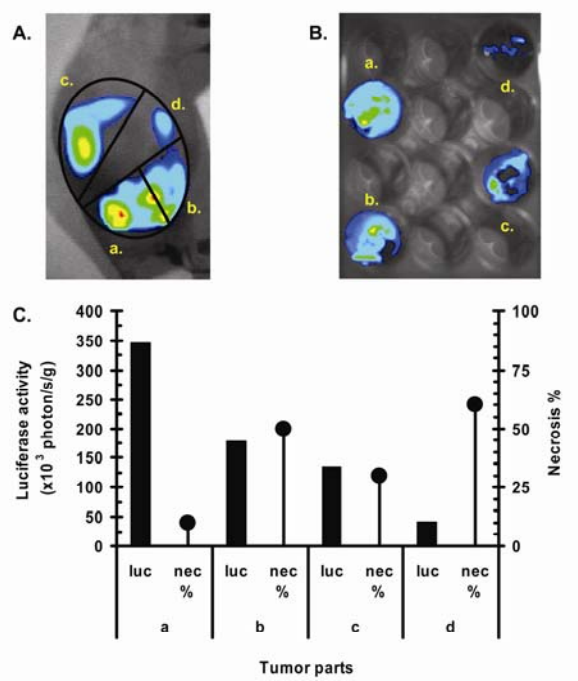

Figure 6: Tumor necrosis measurement at day 38. The representative animal injected with 1×10^6 LSCL$^+$N cells was imaged at day 38 post-injection and then sacrificed. Xenograft was removed and carved into 4 parts named a to d (A). Parts were weighed and then seeded in a 24-wells culture plate. Their luminescence was detected and quantified (B). Luciferase activities of xenograft parts were reported on graph C as a function of their respective weights. Samples were also anatomo-pathologically analyzed to determine their necrosis percentage and values were represented next to luciferase activities, according to the right scale (•).

As expected, part a, which was the most luminescent area, was the least necrotic zone (only 10%). Parts b and c showed 50% and 30% of necrotic cells, respectively, and part d, representing the least luminescent part in the xenograft center, exhibited 60% of necrosis. Although not detectable externally using calipers, tumor cell death was reflected by a decrease in signal intensity. The ability to detect the dynamics of tumor stasis and necrosis *in vivo* should also facilitate studies of angiogenesis inhibitors which are currently being evaluated in preclinical tumor models as well as in clinical trials (17).

Discussion

In this study, bioluminescence-based measurements were performed to evaluate proliferation of luciferase expressing tumor cells. The optical reporter gene that we used in our oncology models is a modified version of that isolated from firefly *P. pyralis*. This reporter is well suited for our study since there is essentially no bioluminescent background in mammals. This contrasts with the use of fluorescent tags with which tissue autofluorescence and photobleaching can be extremely limiting (18). Moreover, fluorescent reporter genes require the use of excitation light, typically of short wavelengths that emit in the green region of the spectrum. These wavelengths do not efficiently penetrate tissues and signals from deep tissue sites may not be accessible. Luciferase enzyme produces light in presence of luciferin substrate, oxygen and ATP (19). *In vitro*, luciferase uses intracellular oxygen and ATP and adding 0.3 µM luciferin, which freely diffuses into living cells, is sufficient to produce a luminescent signal. This signal is approximately 10-fold less intense than a signal after cell lysis but, in intact cells, it is stable for several hours (20). In *in vivo* experiments, emitted light is less intense due to tissue absorption but it can be externally detected and quantified with sensitive light-imaging systems (21). Since luciferin is rapidly eliminated, signal is less stable than in *in vitro* experiments (10 min instead of several hours) (20). Therefore, measurements have to be performed from minute 10 to minute 20 after injection. Furthermore, contrary to other *in vivo* methods, ours does not require sacrificing animals for each measurement.

Signals from labeled cells in whole body images allowed us to both rapidly localize tumors and quantify cellular proliferation. Bioluminescent models could advantageously avoid sacrificing numerous animals. Moreover, bioluminescence tumor imaging takes into account only metabolically active cells and therefore, a decrease in signal intensity occurs as cell die. It allows a more precise evaluation of active tumor masses than by manually measuring its volume which would also include necrotic cells. These new bioluminescent models will be useful tools for evaluating cancer treatment efficiencies.

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