Bioluminescence measurements in mice using a skin window

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Abstract. Studies of bioluminescence in living animals, such as cell-based biosensor applications, require measurement of light at different wavelengths, but accurate light measurement is impeded by absorption by tissues at wavelengths <600 nm. We present a novel approach to this problem—the use of a plastic window in the skin/body wall of mice—that permits measurements of light produced by bioluminescent cells transplanted into the kidney. The cells coexpressed firefly luciferase (FLuc), a vasopressin receptor—*Renilla* luciferase (RLuc) fusion protein, and a GFP^2-β-arrestin2 fusion protein. Following coadministration of two luciferase substrates, native coelenterazine and luciferin, bioluminescence is measured via the window using fiber optics and a photon counter. Light emission from the two different luciferases, FLuc and RLuc, is readily distinguishable using appropriate optical filters. When coelenterazine 400a is administered, bioluminescence resonance energy transfer (BRET) occurs between the RLuc and GFP^2 fusion proteins and is detected by the use of suitable filters. Following intraperitoneal injection of vasopressin, there is a marked increase in BRET. When rapid and accurate measurement of light from internal organs is required, rather than spatial imaging of bioluminescence, the combination of skin/body wall window and fiber optic light measurement will be advantageous. [DOI: 10.1117/1.2795567]

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1 Introduction

Measurements of bioluminescence in experimental animals, particularly mice, play an important role in many areas of biomedical research.[1–8] Mice may be genetically modified to express bioluminescent proteins, and immunodeficient mice can serve as hosts for bioluminescent cells of human or other mammalian origin. One widely used approach for detection and measurement of bioluminescence in mice has been charge-coupled device (CCD) camera imaging.[1–8] However, external recording of bioluminescence from cells or tissues beneath the skin is limited by the poor penetration of light through the skin and other tissues.[4,5,9] Mainly as a result of the presence of hemoglobin in all vascularized organs of the body, light at wavelengths <600 nm is strongly absorbed by tissues. The calculated absorption coefficient of mammalian tissue is 10 cm^{-1} at 400 nm, whereas at 700 nm it is only 0.03 cm^{-1}.[4] Light absorption may cause >99% loss of light from internal organs such as the lung.[4,5,9] Tissue light absorption creates two problems. First, it is more difficult to visualize bioluminescence-producing cells that are located within internal organs of the body than those that are located more superficially. Second, selective loss of light at certain wavelengths interferes with measurements of bioluminescence that require an accurate assessment of the ratio of light at two different wavelengths, such as comeasurement of *Renilla* luciferase (RLuc) and firefly luciferase (FLuc), or comeasurement of a luciferase and an interacting fluorescent protein, which produces light by bioluminescence resonance energy transfer (BRET).[10,11] The strong absorption of light by tissues particularly affects the use of short-wavelength bioluminescent proteins such as RLuc. The combination of RLuc and modified GFP (GFP^2) is very useful, because BRET can occur between these two proteins in the presence of coelenterazine 400a.[10,11] The combination of RLuc fusion proteins and GFP^2 fusion proteins can be used to create cell-based biosensors.[12,13]

For both types of bioluminescence measurements in living animals (BRET and comeasurement of different luciferases), it is necessary to measure light emission accurately and without apparent shifting of the emission wavelength caused by selective loss of light at certain wavelengths. To address these issues, in the present experiments we combined two technologies: transplantation of bioluminescent cells under the capsule of the mouse kidney, together with the use of a plastic window inserted into the skin and body wall adjacent to the kidney. The subcapsular space of the kidney is an excellent site

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for transplantation of both normal and cancer cells, and the abundant vascular supply of the kidney provides the transplant with ready access to molecules circulating in the blood.[14–16] The use of a window inserted into the skin and body wall enables light to be measured from bioluminescent cells in the kidney without interference by skin or other tissues. Windows have been used for a variety of purposes in small animals such as mice, rats, and hamsters. In one form, double-sided transparent chambers have been inserted into the skin to visualize cells or tissue fragments inserted between the two sides of the chamber.[17,18] In another form, windows have been inserted into the skin and body wall to allow visualization of internal organs, including the lung,[19] heart,[20] skeletal muscle,[21] pancreas,[22] mammary gland,[23] and kidney.[24] In this laboratory we earlier developed a simple window formed from polyvinyl film, which allowed visualization of the kidney.[25] However, these windows were not well integrated into the tissues of the host animal and the edges of the window tended to detach from the tissues of the animal after about 10 days. In the present experiments, we devised a completely new form of window for the purpose of allowing light measurements to be made using a fiber optic light guide. The key features of the window are that it is made of lightweight plastic, is optically clear, and has an attached woven polyester collar that enables it to be tightly integrated into the skin and body wall. Its size and shape enable a fiber optic light guide to be temporarily attached to the mouse to enable measurements of light emission. We show that the skin/body wall window allows the accurate measurement of light emission at defined wavelengths from bioluminescent cells transplanted in the kidney.

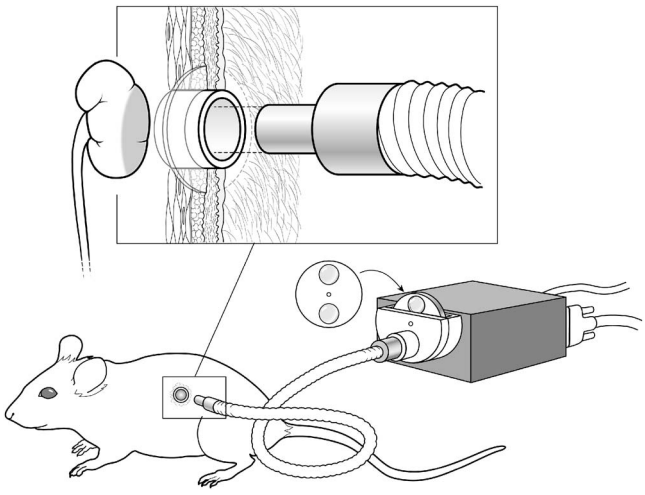

Fig. 1 Diagrammatic representation of a mouse with a skin/body wall window and transplanted bioluminescent cells. The gray shading represents the bioluminescent cells transplanted beneath the capsule of the kidney. A plastic window is fitted in the skin and body wall adjacent to the kidney. A woven polyester collar attached to the window enables the window to be tightly integrated into the tissues of the mouse without loss of integrity of the skin around the edge of the window. When the mouse is anesthetized, the end of a fiber optic light guide may be inserted into the opening of the window. Following injection of luciferase substrates into the peritoneal cavity of the animal, light emitted from the bioluminescent cells is detected by a photon counter. A filter wheel interposed in the light path enables the measurement of light at defined wavelengths. Data from the photon counter are recorded on a computer, which also controls the filter wheel.

2 Materials and Methods

2.1 *Cells Expressing Biosensor Proteins*

Crandell feline kidney (CrFK) cells[26] were transfected with plasmid encoding FLuc (pSV2A/L-AΔ5′),[27] and *neo* and stable transfectants were selected with G418. These cells were then transfected with plasmids pGFP^2-β'-arrestin2 and pV2-vasopressin-receptor-hRLuc (Packard Bioscience, Meriden, Connecticut; hRLuc is a humanized form of RLuc), or a modified version of this plasmid encoding hRLuc mutated at 8 codons, RLuc8.[28] This pair of fusion proteins, V2-vasopressin-receptor-hRLuc/RLuc8 and GFP^2-β-arrestin2, constitute a biosensor for the hormone vasopressin.[12] GFP^2 is a modified form of GFP.[29] Firefly luciferase-expressing cells were transfected with pGFP^2-β-arrestin2 and selected with hygromycin. Stably transfected clones were screened for fluorescence. Selected clones were then transfected with pV2-vasopressin-receptor-hRLuc/RLuc8 and selected with zeocin. Stably transfected clones were screened for luciferase activity. Clones with high luciferase activity were further screened for responsiveness to vasopressin by changes in BRET as described.[12]

2.2 *Construction of Plastic Windows*

Windows were formed from two pieces of polystyrene cut from 10 mm o.d. (8 mm i.d.) tubes (Evergreen Scientific, Los Angeles, California) each 3 mm in height. An annulus of woven polyester (polyethylene terephthalate) material, internal diameter 8 mm, external diameter 14 mm, was sealed between the two pieces using cyanoacrylate glue (see Fig. 1). Circles of transparent polyester film were sealed over the open ends of the tube. Windows were sterilized by exposure to ultraviolet light before use. Light transmission by the assembled windows was measured and was >80% over the range 350 to 1200 nm.

2.3 *Photon Counter and Fiber Optics*

A Hamamatsu H7467 photon counter (Hamamatsu Photonics, Bridgewater, New Jersey) was used in conjunction with a motorized filter wheel (Oriel model 74041, Newport Corporation, Irvine, California). Both photon counter and filter wheel have RS-232 interfaces, allowing computerized measurement of light and control of the position of the wheel. Two of six positions of the filter wheel were occupied by optical filters, either the combination of a 475/50-nm bandpass filter (475/50 BP; Semrock, Rochester, New York) and a 580-nm "3RD Millenium" longpass filter (580 LP; Omega Optical, Brattleboro, Vermont); or a 417/60-nm bandpass filter (417/60 BP; Semrock) and a 500 longpass filter (500 LP; Omega Optical). The filter wheel is connected to a fiber optic light guide (5 × 500 mm Single Flexible Light Guide, Volpi USA, Auburn, New York). The dimensions of the plastic window were designed to allow insertion of one end of the light guide into the window.

2.4 *Surgical Implantation of Biosensor Cells in the Kidney of Immunodeficient Mice and Insertion of Skin/Body Wall Window*

$RAG2^{-/-}, \gamma c^{-/-}$ mice were purchased from Taconic (Germantown, New York). Animals (both males and females) at an age greater than 6 weeks (~25-g body weight) were used in these experiments. Procedures were approved by the institutional animal care committee and were carried out in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Animals were anesthetized with Avertin.[30] An oblique incision (<1 cm) was made in the skin parallel to and adjacent to the long axis of the left kidney. The skin was separated from the body wall to the extent of 1.5 cm longitudinally and 1.0 cm laterally. An incision (<1 cm) was then made in the body wall adjacent to the kidney and the kidney was exteriorized. Biosensor cells (2×10^6) were trypsinized and resuspended in cold culture medium for implantation. The total volume (cells and medium) was 10 to 15 μl. Cells were injected using a blunt 25 gauge needle and glass Hamilton syringe using a transrenal injection, placing the cells beneath the capsule on the side of the kidney adjacent to the body wall. The kidney was returned to the retroperitoneal space. A plastic window was inserted into the incision in the body wall, the base of the window being adjacent to the transplanted cells. Using a continuous suture technique (6-O silk suture), the polyester collar was attached to the outer surface of the body wall. The suture was tightened before being tied off. The skin was sealed around the opening of the window and was placed in close contact with the polyester collar by stretching it around the window and closing the incision with a surgical staple. Postoperative care of animals was as previously described.[15]

2.5 *Bioluminescence Measurements in Mice with Transplanted Cells*

The growth of the transplanted cells was monitored immediately after surgery and at intervals thereafter using epifluorescence (400-nm wavelength illumination, Lightools Research, Encinitas, California). After 15 to 25 days, animals were used in experiments of bioluminescence measurements. Mice were anesthetized using Avertin. Bioluminescence substrates were administered by intraperitoneal injection. D-Luciferin and native coelenterazine were obtained from Biotium, Incorporated, Hayward, California. Coelenterazine 400a (also known as DeepBlue C, a trademark of PerkinElmer) was obtained from Biosynth AG, Staad, Switzerland. Luciferin was dissolved at 30 mg/ml in water for injection. Coelenterazines were dissolved at 1 mM in acidic methanol and were diluted if necessary in saline for injection. A complex of native coelenterazine and 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, Saint Louis, Missouri) was prepared by lyophilization of a mixture of a methanolic solution of coelenterazine and an aqueous solution of cyclodextrin (1:50 molar ratio).[31] For use, the lyophilized complex was resuspended in water at a concentration of 1 mM.

Following injection of luciferase substrate, the anesthetized animal was placed in a light-tight box housing the photon counter and filter wheel. One end of the light guide was placed within the opening of the skin/body wall window (Fig. 1). Measurements were made at 10-s intervals, each measurement typically of 5-s integration time, alternating between the two filters. The software used for data acquisition and control of the filter wheel was written in the Labview programming language (National Instruments, Austin, Texas). Further analysis of the data was performed in Microsoft Excel.

2.6 *In-Vitro Experiments Using Biosensor Cells*

For *in-vitro* measurements, the same photon counter/filter wheel apparatus was used with the light guide inserted into a solid metal block designed to hold a 10×75-mm glass tube. Biosensor cells (100,000 cells) were trypsinized and resuspended in 30 μl of a buffer comprising 160-mM NaCl, 5.4-mM KCl, 1.8-mM $CaCl_2$, 0.8-mM $MgCl_2$, 25-mM HEPES, 5.6-mM glucose, 4-mM glutamine, and pH 7.4. Cells were preincubated in buffer for 15 min. To initiate the bioluminescence reaction, 10-μl coelenterazine 400a was added to give a final concentration of 10 μM. After light acquisition had been started, vasopressin was added (2 μl, giving a final concentration of 25 μM), without interrupting data acquisition. For experiments at 37 °C, the block holding the glass tube was placed on a heating mat.

3 Results

3.1 *A Skin/Body Wall Window Enables Measurement of Light Emission from Bioluminescent Cells Transplanted Into the Kidney*

The aim of the present experiments was to investigate whether a plastic window inserted into the skin and body wall of the mouse could be used to facilitate the measurement of bioluminescence originating in an internal organ. If successful, this method would avoid problems arising from transmission of light through tissues, including the skin, which absorb light strongly at wavelengths <600 nm. To assess whether it was possible to simultaneously measure RLuc and FLuc bioluminescence originating in an internal mouse organ, cells expressing both proteins were transplanted into the kidney of immunodeficient mice and were permitted to establish a vascular supply, as previously described for other cell types.[14–16] Insertion of the window into the skin/body wall and transplantation of bioluminescent cells into the kidney was performed in a single surgical procedure, as described in Materials and Methods in Sec. 2. The size and shape of the window allowed one end of a fiber optic light guide to be inserted within it when the mouse was immobilized under anesthesia. The light guide was used to transmit light to a photon counter (Fig. 1).

The use of a woven polyester collar attached to the plastic window allowed a tight integration of the window into the tissues of the mouse (between the skin and body wall) without loss of integrity of the skin around the window. This was important to prevent infection and fluid loss. Windows remained usable for at least 40 days following surgery. There was no evidence of loss of transparency or of detachment of windows from the surrounding mouse tissues, problems that were encountered with a simple polyvinyl chloride window previously used in this laboratory.[25] Figure 2 shows details of the histological structure of the integration of the polyester collar into the skin. The polyester fibers were found to be united into the tissue between the dermis and the body wall with no evidence of inflammation or foreign body reaction.

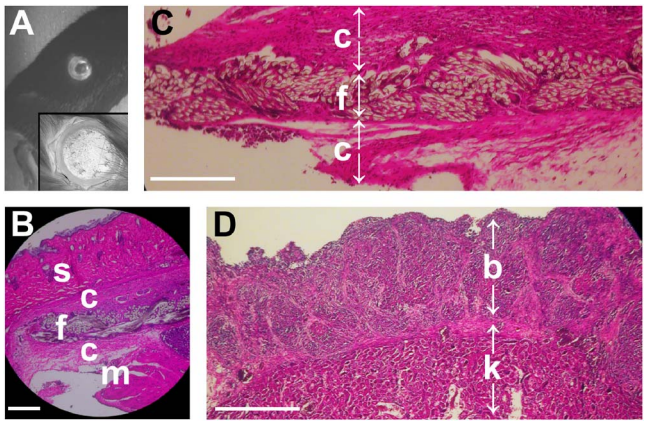

Fig. 2 Appearance and histological structure of skin/body wall window and transplanted cells in the kidney. (a) External appearance of a skin/body wall window at 35 days following surgery; the inset shows a higher-power view of a window in a different animal. (b) Section of skin and body wall close to the window. The woven polyester collar is visible as fibers *f* within connective tissue *c* between the skin *s* and the muscle *m* of the body wall. Hematoxylin and eosin stain. Scale bar =1 mm. (c) Detailed view of polyester fibers *f* showing complete integration into the subdermal connective tissue *c*. Scale bar=1 mm. (d) Bioluminescent cells *b* forming a thick layer beneath the capsule of the mouse kidney *k*. Scale bar=1 mm.

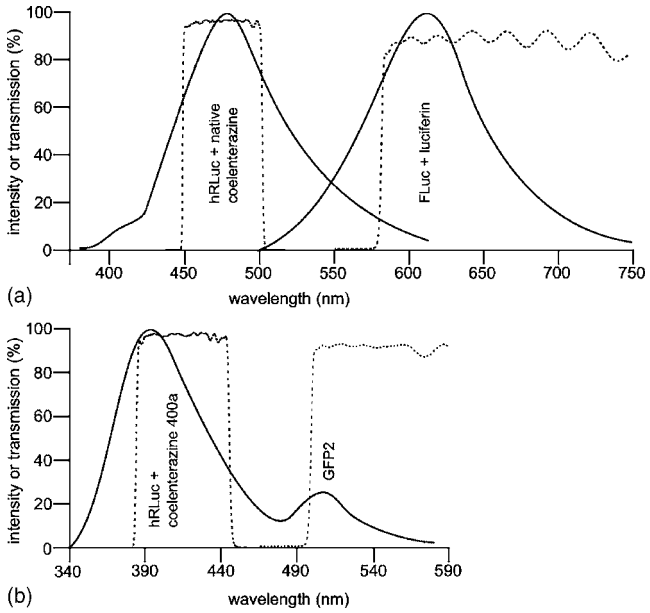

Fig. 3 Transmission characteristics of filters and emission spectra of proteins used in these experiments. Dotted lines indicate transmission characteristics of filters, and solid lines indicate emission spectra of proteins. (a) A 475/50-nm bandpass filter and a 580-nm longpass filter were used for discrimination of bioluminescence of RLuc and FLuc. The spectrum for RLuc (native coelenterazine substrate) is replotted from Ref. 47. The spectrum for FLuc (luciferin substrate) is replotted from Ref. 9. Note that the spectrum shown is the emission of FLuc at 37 °C. The emission peak of FLuc is 612 nm at 37 °C versus 578 nm at 25 °C.[9] (b) A 417/60-nm bandpass filter and a 500-nm longpass filter were used for discrimination of RLuc bioluminescence and light emitted by GFP[2] by bioluminescence resonance energy transfer (BRET) in the presence of coelenterazine 400a. The combined spectrum (RLuc+GFP[2]) is replotted from Ref. 48. Filter transmission characteristics are from data supplied by Semrock and Omega Optical Incorporated.

Connective tissue filled the spaces between the fibers, thus providing a strong bond between the materials of the window and the internal tissues of the mouse. Different forms of polyester fibers have been used in surgical materials for many decades, and the excellent surgical results are consistent with the known biocompatibility of this polymer.[32] The bioluminescent cells formed a thick solid vascularized layer of tissue beneath the capsule of the kidney [Fig. 2(d)].

3.2 *Comeasurement of Light Production by Renilla Luciferase and Firefly Luciferase in Transplanted Bioluminescent Cells*

In these experiments, our aim was to assess the feasibility of simultaneous measurements of bioluminescence of RLuc and FLuc, and also simultaneous measurement of RLuc bioluminescence and GFP[2] fluorescence, which is required for measurement of BRET in cell-based biosensor applications. The characteristics of the optical filters used for these studies are shown in Fig. 3.

In initial experiments, we tested the suitability of the filters for discriminating RLuc and FLuc bioluminescence. We used extracts of cells transfected with either RLuc or FLuc. The photon counter used for light measurements was the same as that later used in animal experiments. When a cell extract containing RLuc was incubated with native coelenterazine as substrate, light emission measured with the 580 LP filter was ~2% of that measured with the 475/50 BP filter. The amount of light detected at >580 nm is slightly less than would be predicted based on the published spectrum of RLuc (Fig. 3). This is probably accounted for by the fact that photomultiplier tubes, such as the one used in the photon counter, are less sensitive to light at longer wavelengths.[33] When a cell extract containing FLuc was incubated with luciferin and ATP, light emission measured with the 475/50 BP filter was ~1% of that measured with the 580 LP filter. This is consistent with the published spectrum of FLuc (Fig. 3).

To assess the suitability of these filters for measurements of light emission at different wavelengths in living animals, cells expressing both RLuc and FLuc were transplanted into the kidneys of a series of immunodeficient mice. Plastic windows were fitted into the skin and body wall adjacent to the transplanted cells, as described earlier. Animals were used for experiments at 20 to 30 days following surgery, when the transplanted cells had become vascularized, as shown in Fig. 2(d). Native coelenterazine, which has been shown to be an optimal *in-vivo* substrate for RLuc, was injected intraperitoneally at a dose previously used in the literature, 2 mg/kg.[34] Light emission rose very rapidly, so that at about 30 s after injection, light detected with the 475/50 BP filter had risen to ~200,000 counts/s [Fig. 4(a)]. Before injection of coelenterazine, light emission was ~10 counts/s. The delay of ~30 s from injection to first measurement is accounted for by the time taken to place the anesthetized mouse into the light-tight box, in which the photon counter and light guide are housed. Light detected with the 580 LP filter was ~3% of the level of that detected with the 475/50 BP filter, and showed the same temporal pattern. We concluded that for RLuc/native

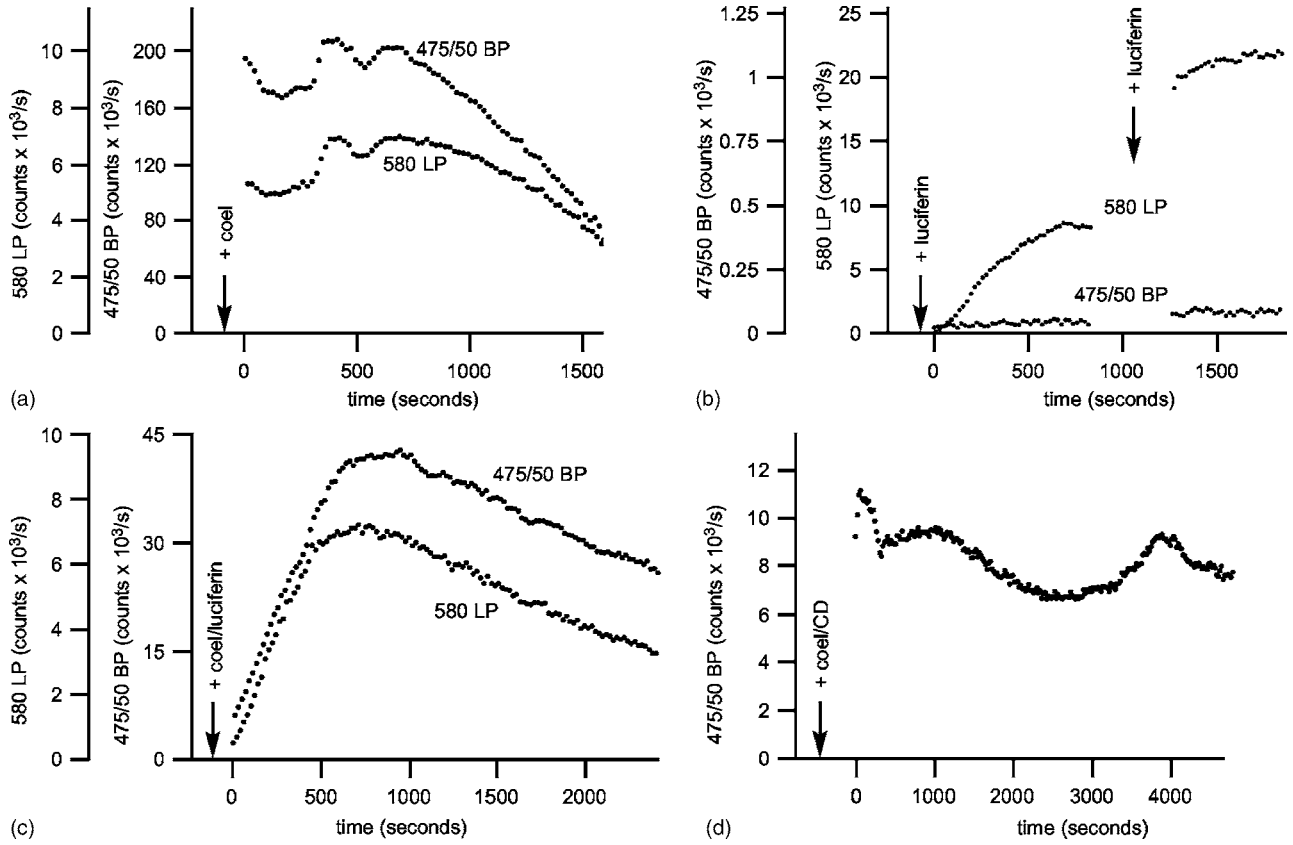

Fig. 4 Discrimination of bioluminescence produced by RLuc and FLuc in transplanted bioluminescent cells. Mice received transplants of RLuc/FLuc-expressing biosensor cells in the kidney and were fitted with skin/body wall windows adjacent to the transplants, as shown in Fig. 1. Experiments were performed at 20 to 30 days following cell transplantation. (a) Native coelenterazine (2 mg/kg) was injected intraperitoneally into a mouse with a bioluminescent cell transplant. Light was measured using 475/50-nm bandpass and 580-nm longpass filters (see Fig. 3) over ~1600 s. (b) Luciferin (150 mg/kg) was injected intraperitoneally into a mouse with a bioluminescent cell transplant. Light was measured using the same pair of filters over ~1000 s. A second dose of luciferin (150 mg/kg) was then injected, and light emission was measured for another ~800 s. (c) A mixture of native coelenterazine (0.05 mg/kg) and luciferin (300 mg/kg) was injected intraperitoneally into a mouse with a bioluminescent cell transplant. Light emission was measured over ~2500 s using the same pair of filters. (d) Coelenterazine/cyclodextrin complex (14-mg coelenterazine/kg) was injected intraperitoneally into a mouse with a bioluminescent cell transplant. Light emission was measured using the 475/50 bandpass filter over ~5000 s.

coelenterazine, there is little difference in light detected with the 580 LP filter when intact animal and *in-vitro* results are compared (~3% versus ~2%). The most likely cause of the slight increase in the intact animal is nonspecific fluorescence of tissues or other components in the light path.[35] We showed that it was not caused by the plastic windows. When an assembled window was placed in the light path *in vitro*, no change in light emission from RLuc was detected with the 580 LP filter.

Luciferin was injected intraperitoneally in a mouse with a kidney transplant of bioluminescent cells. Luciferin was administered at 150 mg/kg, a dose previously used in the literature.[34] In this experiment, light production by FLuc/luciferin in bioluminescent cells in the kidney was less than that from RLuc/coelenterazine (~8,000 counts/s versus ~200,000 counts/s) [Fig. 4(b)]. Injection of a second dose of luciferin produced an increase in light to ~22,000 counts/s. We conclude that the dose of luciferin required to produce a strong level of light emission from FLuc is much greater than the dose of native coelenterazine required for production of light from RLuc (300 mg/kg versus 2 mg/kg). This conclusion is similar to that from previous studies in which RLuc/FLuc bioluminescence in mice was assessed by CCD camera imaging.[34] Even though it is lower than the light emission from RLuc, the light production rate from FLuc following injection of a single dose of luciferin (~10,000 counts/s) would be sufficient for most experiments.

We then performed experiments in animals with transplanted bioluminescent cells in which a small dose of coelenterazine (0.05 mg/kg) was coinjected with a higher dose of luciferin (300 mg/kg) [Fig. 4(c)]. In this case, light detected with the 475/50 BP and 580 LP filters rose at about the same rate over a period of ~750 s and then began a more or less linear decline.

We performed a series of measurements to determine the reproducibility of simultaneous assessments of RLuc and FLuc bioluminescence in living animals. Over a period of 10 days, six separate measurements of bioluminescence from a transplant of biosensor cells were made. On each occasion the combination of 0.05-mg/kg coelenterazine and 300-mg/kg luciferin was injected. Over these six measure-

ments, the RLuc bioluminescence/FLuc bioluminescence ratio was determined to be 10.6±0.9. This result indicates that the RLuc bioluminescence/FLuc bioluminescence ratio can be reproducibly measured from biosensor cells in living animals.

One concern that has been raised regarding the use of coelenterazine is that it is typically dissolved in methanol or ethanol, and that toxicity can arise when animals are repeatedly used for RLuc bioluminescence measurements.[9] We therefore performed experiments in which native coelenterazine was complexed with cyclodextrin.[31] The complex was injected intraperitoneally at a dose of 0.7-mg/kg coelenterazine. Light emission rose very rapidly but never attained the very high levels observed with uncomplexed coelenterazine. However, the cyclodextrin complex had the advantage that light emission was much more stable and remained at >7000 counts/s for ~5000 s [Fig. 4(d)].

3.3 *Bioluminescence Resonance Energy Transfer Measurements in Biosensor Cells in the Kidney*

An important application of skin windows in bioluminescence studies is accurate measurement of the ratio of light emitted at two different wavelengths. This is required for measurement of BRET in cell-based biosensor applications. In the experiment shown in Fig. 5(a), we transplanted cells into the kidney that express two fusion proteins, V2-vasopressin-receptor-RLuc and GFP^2-β-arrestin2. This combination of proteins produces BRET in the presence of coelenterazine 400a, and BRET increases in the presence of vasopressin.[12] We show here that BRET may be observed in the biosensor cells transplanted into the kidney. Following injection of coelenterazine 400a into the animal, the light emission by RLuc rose rapidly; light emission by GFP^2 also rose, giving a relatively stable BRET ratio of ~0.1. Following injection of vasopressin, there was a delay of ~200 s, and then the BRET ratio showed an approximately linear increase over 500 s, stabilizing again at ~0.4.

We compared the *in-vivo* and *in-vitro* responsiveness of the biosensor cells. One factor of interest was the relative response of the cells at room temperature and at 37 °C. Most studies of BRET in cells have been at room temperature (e.g., see Refs. 36 and 37 for cells expressing this vasopressin-responsive biosensor protein pair). In the living animal, the cells function at 37 °C. Figures 5(b) and 5(c) show that when a suspension of biosensor cells was exposed to vasopressin *in vitro*, using the same photon counter apparatus and filters, BRET increases in response to vasopressin were rapid and sustained. The response was greater and more rapid in cells incubated at 37 °C rather than 25 °C. At 25 °C, the BRET ratio rose from 0.016 to 0.030 with a $t_{1/2}$ of 140 s, whereas at 37 °C it rose from 0.016 to 0.034 with a $t_{1/2}$ of 80 s. Therefore, the strong response to vasopressin in biosensor cells in the living animal results at least partially from the higher temperature *in vivo*.

4 Discussion

Monitoring cellular and molecular events within living animals can be performed using various forms of magnetic resonance imaging, positron emission tomography, and optical imaging.[2,3,7] The choice of which technology to use for monitoring cellular and molecular events in living animals depends on the aims of the studies. In some cases, determining the location of the events within the body is critical, whereas precise quantitation of the cellular or molecular event being monitored is not needed. In other cases, spatial information is less important, but precise quantitation of the events being monitored is required. Specifically, it may be desirable to measure the state of activation of biosensor proteins in the animal. Determining the distribution of the proteins in the body might not be needed, because the location has been determined by the experimental conditions, e.g., by transplantation of cells expressing the proteins. In this case, accuracy and rapidity of measurement are important. This is the subject of the present set of experiments.

In biosensor applications, and for many other purposes in intact animals, there is a need for accurate measurement of light at two wavelengths, either from two bioluminescent proteins or the combination of a luciferase and a fluorescent protein that emits light by bioluminescence resonance energy transfer (BRET). However, in intact animals, this presents difficulties because tissues absorb light strongly and the absorption varies with the wavelength of the light.[4,5,9] Tissues also scatter light and fluoresce to various extents.[35] When accurate measurement of light is required in whole animals, any avoidable passage of light through tissues prior to measurement is undesirable.

Several solutions to these problems have been proposed. The first approach is to use superficial (subcutaneous) cells or tissues as the source of light. Using CCD camera imaging, the transmission efficiency of FLuc was found to be 17% for skin and 2% for lung; values were much lower for RLuc.[9] In experiments involving bioluminescence investigations of protein-protein interactions in living animals, CCD camera imaging has been used with subcutaneously injected cells.[38,39] In pioneering experiments on detection of BRET in living animals, subcutaneously injected cells were used in conjunction with CCD camera imaging.[40] The second approach has been to develop methods of detection and measurement that take into account light absorption by tissues.[41] The third approach has been to develop bioluminescent proteins that emit longer wavelength light, which is not as strongly absorbed by tissues.[9] However, most currently available bioluminescent proteins emit light at relatively short wavelengths. In particular, the very useful pair of proteins, RLuc and GFP^2, which can be used for BRET experiments and biosensors, emits light in the range 380 to 550 nm.

An alternative approach is to develop technology that allows accurate measurements of bioluminescence in internal organs of living animals. In the present experiments we used a new technique in which the use of a plastic window in the skin and body wall allows the temporary attachment of the animal to the light-measuring apparatus when required. We make use of the ability of optical fibers to guide light between two locations—the animal and the detector. However, the apparatus is not permanently attached, and therefore animals with bioluminescent cell transplants and windows do not need special housing or treatment, and their behavior is unaffected.

The two salient features of this technology are the use of a skin/body wall window and the transfer of light to a photon counter via a fiber optic light guide. The window was designed to allow the insertion of the light guide into the exter-

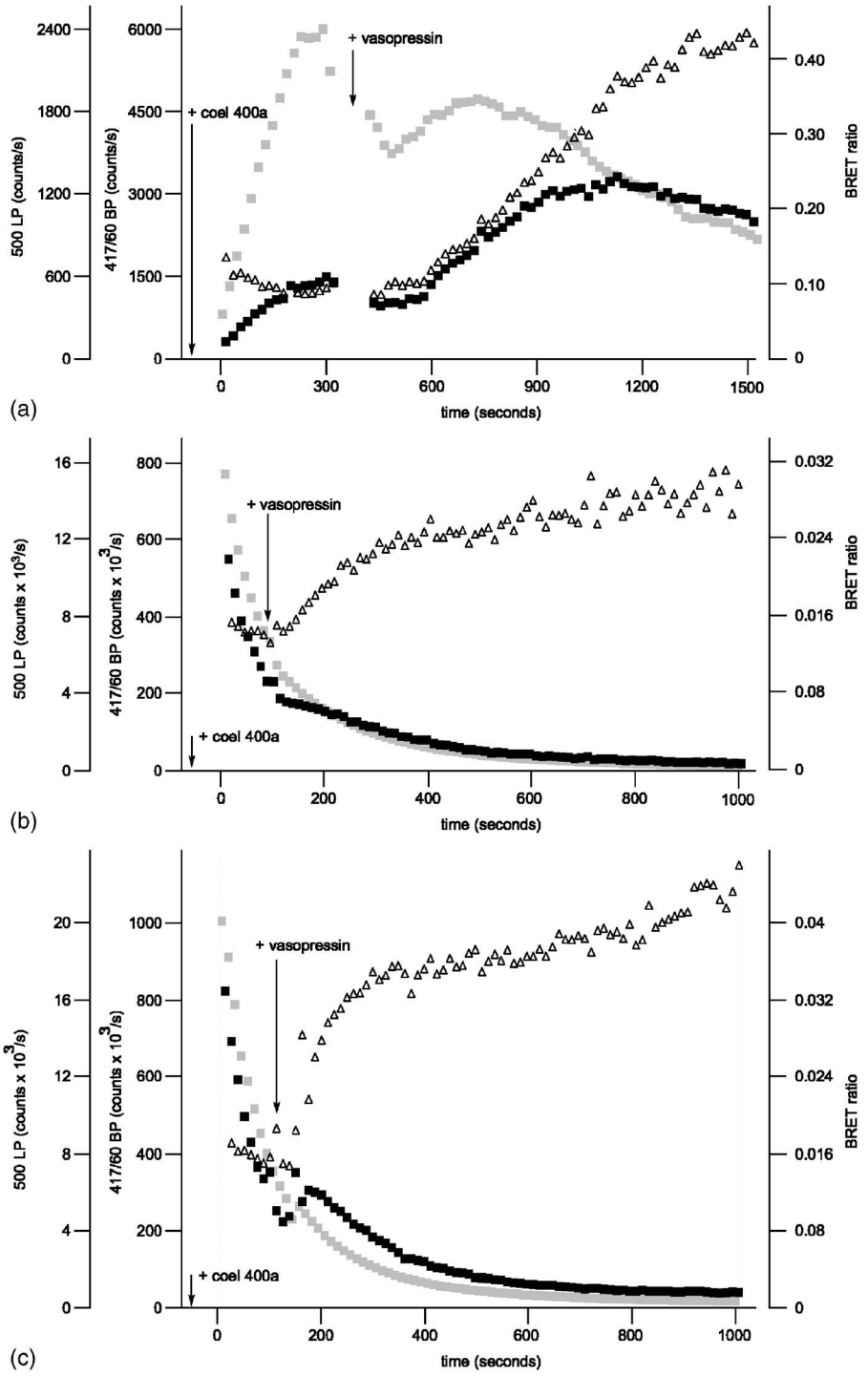

Fig. 5 Observations on BRET in biosensor cell transplants and in cell suspensions *in vitro*. (a) Vasopressin-sensitive biosensor cells were prepared as described in Materials and Methods in Sec. 2 and were transplanted beneath the kidney capsule of immunodeficient mice. Cells express two fusion proteins—GFP^2-β-arrestin2 and V2-vasopressin-receptor-RLuc8. Following an intraperitoneal injection of 2-mg/kg coelenterazine 400a, light emission was measured using 417/60 BP and 500 LP filters (gray and black symbols, respectively). The triangles show the ratio of light detected using the two filters [BRET ratio=(500 LP)/(417/60 BP)]. After ~350 s, light emission acquisition was stopped temporarily and vasopressin (0.5 mg/kg) was injected intraperitoneally into the animal. Light emission using the two filters was then measured for another ~1100 s. (b) Vasopressin-sensitive biosensor cells were studied *in vitro*. A suspension of 100,000 cells was used in an incubation with coelenterazine 400a at 25 °C (see Materials and Methods in Sec. 2). Light emission using 417/60 BP and 500 LP filters and the BRET ratio are plotted. After 100 s, vasopressin was added to the cells without interrupting light acquisition. (c) The experiment was performed as described in (b), except that the cell suspension was maintained at 37 °C throughout the experiment.

nal portion of the window, thus providing a way to temporarily attach the animal to the light detection apparatus. Here we implanted cells in the kidney, which is an ideal organ for the placement of biosensor cells because of its very high blood flow. Biosensor cells are exposed to any circulating compounds to which the cells are engineered to detect. The technology could be adapted to other internal organs as required.

Windows of various types have been used in mice for visualization of transplanted cells and tissues and for observation of internal organs.[17–25] In one recent report, CCD camera imaging of bioluminescence in the olfactory bulb was used in conjunction with a glass window replacing a small section of the skull in a mouse.[42] Windows have also been used more frequently with fluorescence imaging.[24] Novel features of the window used in the present experiments are that it is formed of lightweight plastic, is easy to construct, is disposable, and has an attached woven polyester collar, allowing it to be tightly integrated into tissues of the mouse between the skin and body wall. Light transmission was $>80\%$ over the range 350 to 1200 nm. The attached polyester collar is the key to successful use of this form of window in animals. It solves the main problem encountered with earlier forms of windows used in this laboratory, which was detachment of the window from the surrounding skin.[25] In the present experiments, we noticed no loss of functionality of windows over at least 40 days.

The window removes the barrier of tissues between the bioluminescent cells and the light detection apparatus. This should also remove sources of changes in wavelength of light emitted by the cells, i.e., tissue autofluorescence and selective absorption of light at some wavelengths. That the window did indeed fulfill these requirements was shown by the fact that the ratio (light detected with correct filter)/(light detected with incorrect filter) differed only slightly between measurements made *in vitro* and measurements made in the animal. In the case of RLuc/native coelenterazine, in the animal $\sim 3\%$ of emitted light was detected with the 580 LP filter versus the 475/50 BP filter. It was $\sim 2\%$ *in vitro*. In the case of FLuc/luciferin, $\sim 1\%$ of emitted light was detected with the 475/50 BP filter versus the 580 LP filter, both *in vitro* and in the animal. Thus it was simple to discriminate light produced by RLuc versus light produced by FLuc following coinjection of coelenterazine and luciferin.

A fiber optic light guide was used here in conjunction with the plastic window as a convenient method to link the animal to a photon counter, and thereby provide a means of continuous light measurement. Fiber optics have been more extensively used in fluorescence measurements in animals[43] and have been relatively little used with bioluminescence. However, in one experiment, bioluminescence was measured in the mouse brain via an indwelling optical fiber;[44] in this case, therefore, the animal was permanently attached to the light-measuring apparatus. Fiber optics were used in the present experiments, because the aim was accurate measurement of light at different wavelengths, not spatial imaging of the bioluminescent cells. Fiber optics are ideal in situations where measurements need to be made at multiple wavelengths and where light emission may change rapidly. Temporal, as opposed to spatial, information about light emission is readily obtained with this apparatus.

We demonstrated the utility of the skin-body wall window/fiber optics combination in simultaneous measurements of RLuc and FLuc bioluminescence and in detection of BRET. In previous studies using CCD camera imaging, animals were either sequentially injected with coelenterazine and luciferin (for measurement of bioluminescence from cells expressing both RLuc and Fluc) or were coinjected with coelenterazine and luciferin (for cells expressing only one of the two luciferases, but implanted in different anatomical locations).[34] In the present experiments, we showed that the skin-body wall window/fiber optics combination in conjunction with the appropriate optical filters allowed the simultaneous measurement of RLuc and FLuc within the same cells. This ability should be very useful for studies in which one of the two luciferases is used to sense an event within the animal, and the other is used to normalize the measurements.[45]

We also showed the potential for the skin/body wall window plus fiber optics for the detection of BRET resulting from the interaction of two fusion proteins, V2-vasopressin-receptor-RLuc and GFP^2-β-arrestin2. These BRET measurements require administration of coelenterazine 400a. If coelenterazine 400a was repeatedly administered to animals, there would be toxicity from methanol/ethanol, in which the compound is normally dissolved. One solution to this problem is the use of coelenterazine/cyclodextrin complexes, which enable coelenterazines to be administered in an aqueous solution.[31] Cyclodextrin complexes worked well in these experiments for the administration of native coelenterazine, and should work well for repeated administration of coelenterazine 400a. Rapid utilization of coelenterazine substrate necessitates imaging immediately following administration, which places constraints on imaging protocols.[34,46] Cyclodextrin/coelenterazine complexes produced light emission that was lower and more stable over time than that produced by the injection of solutions in methanol. Cyclodextrin complexes should be useful in biosensor applications in living animals.

We conclude that the combination of a skin/body wall window and fiber optic light detection is well suited for rapid simultaneous measurements of different bioluminescent proteins in internal organs in the mouse, and that it should be readily adaptable for a variety of cell-based biosensor applications in intact animals.

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