Chapter 2

Luciferase Protein Complementation Assays for Bioluminescence Imaging of Cells and Mice

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Abstract

Protein fragment complementation assays (PCAs) with luciferase reporters currently are the preferred method for detecting and quantifying protein–protein interactions in living animals. At the most basic level, PCAs involve fusion of two proteins of interest to enzymatically inactive fragments of luciferase. Upon association of the proteins of interest, the luciferase fragments are capable of reconstituting enzymatic activity to generate luminescence in vivo. In addition to bi-molecular luciferase PCAs, unimolecular biosensors for hormones, kinases, and proteases also have been developed using target peptides inserted between inactive luciferase fragments. Luciferase PCAs offer unprecedented opportunities to quantify dynamics of protein–protein interactions in intact cells and living animals, but successful use of luciferase PCAs in cells and mice involves careful consideration of many technical factors. This chapter discusses the design of luciferase PCAs appropriate for animal imaging, including construction of reporters, incorporation of reporters into cells and mice, imaging techniques, and data analysis.

Key words: Molecular imaging, optical imaging, split luciferase, bioluminescence, protein complementation assay, PCA.

1. Introduction

Bioluminescence imaging of intact animals is a powerful technology for detecting and quantifying the spatial and temporal occurrence of cellular and molecular events using luminescent enzyme reporters. Traditionally, luciferase enzymes have been used as reporters of promoter activity and, in the case of firefly luciferase, as an assay for ATP. In contrast to these methods, luciferase protein complementation assays (PCAs) were developed to measure post-translational events such as protein interactions,

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phosphorylation, and enzymatic cleavage of substrates. Luciferase PCAs have been developed for three major luciferases from firefly (1, 2), sea pansy (*Renilla reniformis*) (3, 4), and the copepod *Gaussia* (*Gaussia princeps*) (5). Several firefly and *Renilla* luciferase PCAs have been published for use in animals. Although in vivo *Gaussia* luciferase PCAs have been published only for live cells, this technology should be easily translatable to animal work. This chapter details the steps in developing an in vivo assay based on luciferase protein complementation, including reporter vector design, introduction of reporters into cells and animals, and bioluminescence imaging of the luciferase PCA in live cells and mice, with an emphasis on firefly luciferase.

2. Materials

2.1. Molecular Biology Reagents

1. Plasmids with open reading frames coding for proteins of interest and luciferases
2. Expression vectors suitable for PCA expression
3. Molecular biology reagents and equipment for PCR, restriction digests, and ligations.

2.2. Cells Culture Reagents

1. HEK-293 or other cell line with high transfection efficiency
2. Tumor cell line or other biologically relevant cell lines of interest
3. General cell culture reagents and plasticware.

2.3. Reagents for Cell Imaging

1. 96-well plates, black plate, clear bottom with lid, tissue culture treated (Costar #3603)
2. Multichannel pipettes suitable for delivering 1–10 and 20–200 μl
3. Low adherence sterile pipette tips (Maxymum Recovery from Axygen, or similar)
4. Sterile commercial 1× phosphate buffered saline (PBS) solution
5. Luciferin solution 15 mg/ml in PBS (sterile filtered, store at –20°C) (firefly luciferase substrate)
6. Coelenterazine 1 mg/ml stock in acidified MeOH (0.5% HCL (v/v)), store at –20°C (substrate for *Renilla* and *Gaussia* luciferases)
7. IVIS-200 or IVIS-100 (Caliper) or similar bioluminescence imaging system with software for region of interest analysis.

2.4. Animal Imaging Materials

In addition to the items listed above in **Section 2.3**, the following items are required or suggested for animal imaging.

1. Mice for construction of animal models (nude or SCID for xenografts)
2. Coelenterazine, 10 mg/ml stock in acidified MeOH (0.5% HCL (v/v)), store at –20°C, dilute to desired concentration immediately before imaging in 40% DMSO/PBS for animal imaging (needed only for *Renilla* and *Gaussia* luciferases)
3. Small animal shaver (Wahl compact cordless trimmer recommended) (optional)
4. Depiliatory lotion such as Nair or Neet (optional).

3. Methods

3.1. Constructing a Luciferase PCA

1. Select a suitable target for the luciferase PCA. *See* **Note 1** for suggestions regarding PCA target selection.
2. Plan relevant orientation of fusion constructs. It is best to test all reasonable orientations of the fusions, keeping in mind the cellular location of the fusion. *See* **Note 2** for examples of bimolecular protein interaction assays and *see* **Note 3** for unimolecular luciferase PCAs.
3. Select an appropriate luciferase for your assay. Several properties of firefly *Renilla*, and *Gaussia* luciferases should be considered in choosing a luciferase enzyme for the PCA (*see* **Table 2.1** and **Note 4**).
4. Design a linker to insert between protein folding domains. Linkers may be used to control the spacing and freedom of motion for the enzyme fragments relative to the proteins of interests. *See* **Note 5** on linker design.
5. Design control constructs. Control constructs that are identical to experimental constructs with the exception of the control feature, such as mutation of a phosphorylation or cleavage site, or substitution of a non-interacting protein should be prepared.
6. Choose a vector to express the constructs. Fusion constructs should be inserted in a mammalian expression vector consistent with the selection method for producing stable cell lines. For pairs of vectors, orthogonal selection methods will be required. *See* **Note 7** for additional information related to generating stable cell lines.
7. Verify constructs. Engineered open reading frames should be fully sequenced. To efficiently choose clones to sequence,

Table 2.1
Properties of intact luciferases

Luciferase	Amino acids	Cofactors	λ max (nm) (in solution)	λ range (nm)	Relative intensity (intracellular)[a]	Relative intensity (mouse tissue)[a]
Firefly	550	ATP, Mg^+, O_2	578 at 37°C[b]	520–740 at 37°C[b]	1	1
Renilla	311	O_2	475[a]	420–580[a]	1	ND[c]
Gaussia	185 (secreted form)	O_2	480[a]	440–590[a]	Approx 100	2

Sources: [a]Tannous et al. (15).
[b]Zhao et al. (23).
[c]Reference (1) includes data for secreted *Gaussia* luciferase in mice compared with *Renilla* luciferase, but this data is not applicable to intracellular *Gaussia* complementation assays.

it is sometimes helpful to test plasmid minipreps in transient transfections (*see* **Section 2.1**) to identify clones that produce luminescence signals.

3.2. Introduction of PCA Constructs into Cells

1. Test reporters in transient transfections. Seed HEK-293 cells in 6-well plates at 150,000 per well. Transfect cells the next day with 1 μg plasmid DNA for each PCA or control construct (0.5 μg, each for pairs of constructs). On the day after transfection, split cells into black 96-well plates (1×10^5 cells/well) to be used the next day for imaging. *See* **Section 3.3** for cell imaging. Prepare parallel sets of transfected cells for western blotting or other relevant biochemical assays. These assays are important assure that the bioluminescence output reflects relevant biochemical events. *See* **Note 6** for suggested controls for cell assays.
2. Produce stable reporter cell lines. In a relevant tumor cell line, prepare a batch of stable transfectants using standard methods and isolate clonal sublines expressing the PCA reporters. *See* **Note 7** for suggested strategies.
3. Test clonal lines. Test tumor cell PCA sublines in culture to identify a small number of lines that exhibit a good signal-to-background ratio and produce relatively bright bioluminescence in the PCA. *See* **Section 3.3** for cell imaging.

3.3. Bioluminescence Imaging of Cells

1. Seed cells in black-walled, clear bottom 96-well tissue culture plates at a density of 10,000–20,000 cells per well. Permit cells to adhere overnight.

2. Remove media next day and treat as needed in a minimum volume of fresh media (e.g., 50 μl). To construct a time course of drug treatments, it may be helpful to replace the media then add treatments at 10× to the cells on a single plate in reverse time order. The plate may then be imaged at the completion of the course.
3. For imaging firefly luciferase, add luciferin (for firefly luciferase) to the medium in 1/10 volume at a final concentration of 0.15 mg/ml using a multichannel pipette and low adherence tips. A low volume multichannel reservoir is helpful for reducing luciferin waste. Tip the plate and insert the pipette tip under the fluid level before expelling the luciferin to assure full delivery of the substrate. Rock the plate in a figure-8 motion.
4. For *Gaussia* or *Renilla* luciferase imaging in cells, albumin in media will cause considerable background luminescence with coelenterazine. To eliminate albumin background with native coelenterazine, short-term treatments may be performed in media lacking albumin, or media may be removed and cells washed with PBS prior to imaging. Add coelenterazine (1:1,000 of 1 mg/ml MeOH stock) in 50 μl PBS per well.
5. After addition of luciferase substrate, take a brief test image of 1–10 s on the IVIS. Use this image to estimate the exposure and binning needed for subsequent images. Typical settings for luciferase complementation imaging would be 0.5- to 1-min exposure at maximum binning. Take subsequent images with the appropriate exposure and binning in serial mode to assure capture of the peak luminescence signal. For firefly luciferase, peak light production from intact cells occurs approximately 5–10 min after substrate addition. Peak bioluminescence from *Gaussia* or *Renilla* luciferases occurs rapidly within the first minute after adding coelenterazine. *See* **Section 3.6** for data analysis.

3.4. Construction of Mouse Tumor Model

Tumor cells ($\approx 1 \times 10^6$) stably expressing the PCA reporter or control reporter are injected in contralateral flanks of the mouse. Tumor cells may be injected subcutaneously or orthotopically, such as in the mammary fat pad. After palpable tumors form, mice should be imaged as below. For alternative approaches to construction of mouse models, *see* **Note 8**.

3.5. Mouse Imaging

For firefly luciferase imaging, mice should be injected intraperitoneally with luciferin (15 mg/ml stock in PBS, 150 μg/g mouse) 10 min before imaging is to begin. Mice are placed in the isoflurane induction chamber 5 min before imaging, then transferred to the IVIS imaging chamber after they are fully

anesthetized. Imaging is performed essentially as for cells (**Section 3.3**). For *Renilla* or *Gaussia* luciferase PCAs, we recommend injection of coelenterazine (2.5 mg/kg dissolved in 40% DMSO in PBS, 50 μl) by tail vein, followed by immediate imaging for 1–5 min (*see* **Note 9**).

3.6. Region of Interest Analysis

Quantify luminescence (photons/s) in a region of interest (ROI) which encompasses the area of luminescence, keeping a standard ROI for all the mice in the experiment (*see* **Note 10**).

4. Notes

1. Luciferase PCAs could be designed for almost any biochemical event that involves a change in the conformation or association of proteins. Luciferase PCAs can be designed to measure either association or dissociation of the luciferase fragments. (In contrast, only association can be measured with PCAs based on fluorescent proteins such as GFP (6), since this method traps the fragments irreversibly in the bound state.) One important consideration is the availability of agents targeting the pathway of interest, or other strategies (such as mutant forms of the proteins of interest) that will result in an "on" and an "off" state for the PCA. For investigation of new biochemical targets, it is also helpful to prepare a control PCA with related, well-studied proteins that can be used to help validate the PCA for the new target. PCAs may be more easily designed for proteins that have been demonstrated to permit fusions, such as GFP fusions, without significant perturbation of function.

   One very useful application of the luciferase PCA is to adapt a FRET or BRET assay for bioluminescence imaging in mice. The user should note that luciferase PCAs differ from FRET and BRET in that PCAs depend on direct contact of the luciferase fragments to reconstitute enzymatic activity. FRET and BRET, in contrast, generate signals based on proximity of fusion proteins. Consequently, some adjustment of the design of the fusion constructs, such as length of linkers, may be required to convert a FRET or BRET assay to a luciferase PCA.

   Our experience with luciferase PCAs indicates that they perform optimally at moderate expression levels, such as those typically achieved in stable cell lines. PCAs necessarily require expression of non-native proteins and should be designed to minimize any impact of the reporter on cell

function. Gross over-expression of PCA constructs could, in principle, induce artificially high protein–protein association or enzyme-substrate interactions, for example. As with any fusion protein or transgene, the user should validate by methods other than bioluminescence that the luciferase PCA constructs perform as intended.

2. Two separate open reading frames are used to express the two fusion proteins in a bimolecular PCA. These may be incorporated into two vectors (which facilitates a mix-and-match strategy for co-transfections with other PCA reporters) or in a single plasmid construct (to more closely link expression of the two reporters). **Figure 2.1** illustrates features of bimolecular luciferase reporters. Both rational design and empiric experience govern the construction of luciferase PCA constructs. In many cases, a review of the literature will reveal previous fusions, such as those to fluorescent proteins, which provide valuable insight into the impact of fusions on protein localization and function. Sites

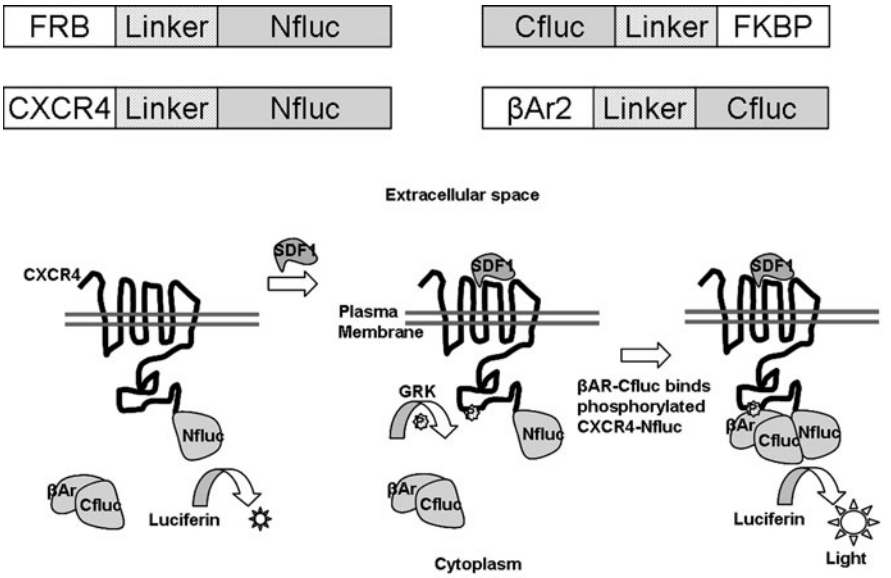

Fig. 2.1. Bimolecular constructs for luciferase PCAs. The *upper pair* of constructs shows two interacting cytoplasmic proteins (FRB domain from the mammalian target of rapaymycin (mTOR) and FK506 binding protein (FKBP)), which associate upon binding of the compound rapamycin. This association brings Nfluc and Cfluc into close proximity, reconstituting firefly luciferase activity. In this case, the linkers are 16 amino acids long and contain the motif GGGSSSGGG with restriction sites (2). The *lower pair* of constructs shows the 7-transmembrane receptor CXCR4 fused as above to Nfluc, while its cytoplasmic binding partner, β-arrestin 2, is fused to Cfluc with a similar linker consisting of 14 amino acids (20). Note that the optimal construct orientation for this PCA was with the interacting protein at the N-terminus of Cfluc (20). The schematic diagram illustrates the concept of bimolecular luciferase complementation for CXCR4-Nfluc and β-arrestin 2-Cfluc. CXCR4-Nfluc binds the chemokine SDF-1, resulting in phosphorylation of the intracellular C-terminal domain of CXCR4 by a G-protein receptor kinase (GRK) and subsequent recruitment of β-arrestin2-Cfluc to reconstitute luciferase activity.

of post-translational modification and important interactions with other binding partners should also be considered. Finally, it is advisable to attempt more than one orientation for fusions identify an optimal design.

3. Luciferase PCAs constructed as a single open reading frame have been used to image of several kinds of biochemical events, including ligand binding, phosphorylation, and proteolytic cleavage events (**Fig. 2.2**). *See* **Note 2** for additional advice regarding general construction of PCAs.
4. We generally prefer firefly luciferase PCA for intracellular protein interactions and biosensors because for reasons related to mouse imaging in particular. The more red-shifted light of firefly luciferase (**Table 2.1**) penetrates mouse tissues better, improving signal detection, and

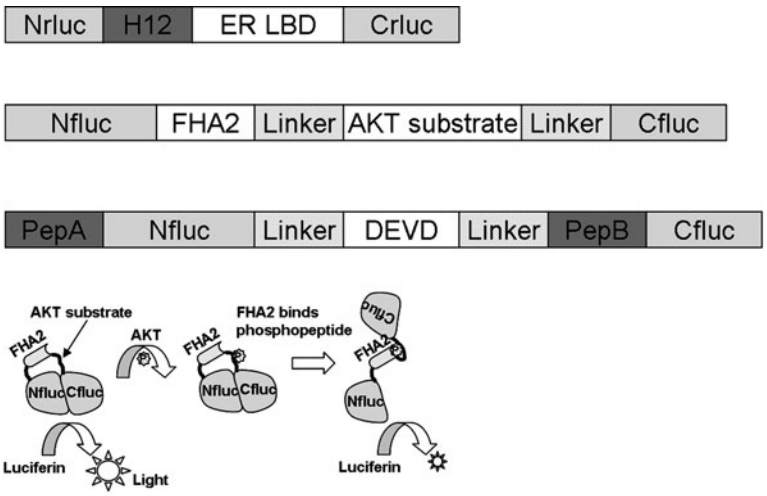

Fig. 2.2. Unimolecular constructs for luciferase PCAs. The *upper* construct contains a single protein fragment for the human estrogen receptor with amino acids 281–549 encompassing helix 12 (H12) and the ligand binding domain (LBD) of the receptor, flanked by inactive fragments of *Renilla* luciferase (Nrluc and Crluc) (3). This sensor for estrogen agonists and antagonists produces strong luminescence activity upon ligand-induced interaction of H12 with the LBD. The *middle* construct contains two interacting protein/peptide regions, an FHA2 phosphopeptide binding domain, and an AKT substrate peptide, separated by short linkers and flanked by inactive fragments of firefly luciferase. This sensor for active (21) produces luminescence upon AKT phosphorylation of the substrate peptide, which binds to the FHA2 region of the protein and disrupts complementation of the luciferase fragments. (*See* diagram.) The *third* construct codes for a single protein which is cleaved to form a bimolecular product. The construct includes three functional regions: PepA and PepB are proteins with a strong constitutive interaction which is disrupted in the intact fusion protein, and DEVD is a substrate for Caspase-3, an enzyme which is activated early in apoptosis. This apoptosis sensor produces increased luminescence upon cleavage of the DEVD peptide by Caspase-3 and subsequent complementation of the luciferase fragments driven by association of PepA and PepB (22).

bioluminescence from this luciferase is more stable over the course of imaging. Luciferin, the substrate for firefly luciferase, has more favorable biodistribution than coelenterazine. In addition, i.p. injection of luciferin is much more reproducible for many investigators than tail vein or intracardiac injection required for coelenterazine.

Nevertheless, there are several applications that might benefit from other bioluminescent PCAs. For example, in situations where the firefly luciferase cofactors Mg^+ and ATP may be limiting, *Renilla* or *Gaussia* luciferase may be used successfully, although as yet no specific examples of this exist in the literature. Steric bulk can also be reduced by employing *Renilla* and especially *Gaussia* PCAs due to their smaller sizes. Finally, it should be noted that luciferase PCAs are not the only bioluminescent imaging approach to PCAs. β-galactosidase PCAs have been adapted for bioluminescence imaging by using intact firefly luciferase as a secondary reporter for β-galactosidase activity (7, 8).

Several different strategies have emerged for bisecting various luciferase enzymes. For firefly luciferase, we used a library screening approach to identify fragments of NLuc 2–416 and CLuc 398–550 as the best overall combination of low background and high signal, and we continue to find that this pair is optimal for our assays (2). Recently, Paulmurugan et al. reported alternative firefly luciferase PCA fragments NLuc 2–398 and CLuc 394–550 that may provide higher signal-to-background for some protein interactions (9). Two pairs of *Renilla* luciferase enzyme fragments have been recommended for their performance in PCAs: 1–229 and 230–311 (10), and 1–110 and 111–311 (4). A single recommended *Gaussia* PCA pair has been reported (5), consisting of NGluc 1–93, CGluc 94–169. (Because native *Gaussia* luciferase contains a 16 amino acid secretion signal that was removed for the study, the first three amino acids of the Ngluc are MKP, the final three are GIG.) Performance of PCAs is heavily impacted by many factors, so the user should consider testing different pairs of luciferase fragments to optimize signal-to-background for a particular application.

5. Linkers serve to provide points for restriction site in the DNA construct and to control distance and flexibility between protein domains in the reporter. Flexible linkers (such as GGGSSGGG flanked by restriction sites necessary for cloning) may be helpful in reducing the interference between separate folding domains. For more constraint, a short tri-glycine linker, or no linker at all, may produce good results. The user should consider hydrophilicity of

amino acids, steric bulk, and tendency for secondary structure formation in selecting a linker sequence.

6. To distinguish a true PCA signal from effects that impact luciferase enzyme function, the PCA conditions may be tested on full length luciferase in parallel with the PCA. PCA reporter function then can be normalized to function of the intact enzyme to eliminate general effects on enzyme function. In 96-well plate assays, it may also be necessary to include a transfection control, such as β-galactosidase, or to normalize to total protein in a well by an assay such as sulforhodamine B (11), crystal violet, or BCA (Pierce).
7. Our own experience is that lentiviral constructs tagged with fluorescent proteins can be used to rapidly and efficiently obtain batch transductants, from which clonal sublines can be generated if needed for optimal performance. Transductions can be performed with two viral vectors simultaneously to generate stable reporter cell lines more rapidly. Lentiviruses significantly reduce the time required for obtaining stable lines compared with traditional plasmid strategies.

   Use of a selectable marker, linked to the reporter through an IRES, can help assure retention of the reporter in cell culture, as well as provide a means to select for cells expressing the reporter at higher levels. Bimolecular reporters may be introduced with tri-cistronic expression vectors (the third position being occupied by a selection marker), although this strategy commonly results in attenuated expression of mRNA molecules further removed from the promoter. Available drug selection markers include neomycin, hygromycin, blasticidin, and zeocin, among others. Of course, use of selectable markers is not possible in mice, so poorly tolerated constructs may not be retained when these constructs are incorporated into solid tumor models.

   To rapidly isolate clonal reporter lines, a batch of cells stably expressing the PCA reporter is seeded in 15-mm dishes at 100–300 cells/dish. After colonies of 50 or more cells form, a grid is drawn on the bottom of the dish with heavy black marker, and the dish is imaged in the IVIS under basal conditions or conditions that activate the PCA to identify luminescent colonies. Placing a layer of aluminum foil, nitrocellulose, or other non-bioluminescent material under the dish in the IVIS enables the grid on the dish to be seen, so that colonies are easily located. Using the grid system, locate the luminescent colony and mark it on the underside of the dish with a colored marker. Clones can be harvested from the dish using cloning rings and trypsin, or by direct “picking” of colonies. A second round

of colony selection is helpful to insure stability of the clonal line.

8. For short-term testing of a luciferase PCA, the simplest mouse experiment is to conduct treatment tests within minutes or hours after implanting PCA-expressing cells. This approach depends only on the short-term survival of cells in the animal, so that non-tumorigenic cell lines and transient transfectants can be employed. For this approach we implant (i.p. or s.q.) 5–10 $\times$ 10^6 cells in PBS. To produce a more confined locus of cell deposition, cells can be injected in a 1:1 mixture of PBS (or DMEM) and matrigel. Cells bearing a relevant control PCA (non-interacting protein, uncleavable peptide, etc.) should be implanted in a parallel set of mice (for i.p.) or in the contralateral flanks of the mice (for s.q.).

   Stable cell lines expressing PCAs can be easily used to construct mouse models by implanting luciferase PCA cell lines to form tumors. Cells may be injected subcutaneously to form flank tumors, or in relevant physiologic sites, such as the mammary fat pad for breast tumor lines. In principle, PCA constructs could also be introduced to mice directly, by construction of transgenic animals or infection with viral vectors, for example, although such assays are not reported in the literature as yet.

   In calculating numbers of cells needed to obtain a detectable signal, the user should consider the depth and optical properties of the tissue through which the light will pass. As a rule of thumb, firefly luciferase light will be attenuated approximately tenfold for each centimeter of tissue, but optically dense tissues such as liver will attenuate light much more than skin, bone, or lung. Best results generally will be obtained by maximizing the number of cells and the perfusion of those cells in the mouse.

9. One of the strengths of luciferase imaging in mice is that a mouse can be imaged repetitively, such as before and after a defined pharmacologic intervention. This strategy allows each mouse to serve as its own control, which reduces experimental variations. To perform repetitive imaging of mice, the user should take into account that luciferase levels in mice peak approximately 10 min after i.p. injection, then decline slowly to background levels by $\approx$ 6 h post injection (12). Luciferin biodistribution for imaging firefly luciferase PCA can be stabilized for hours or days by using an osmotic pump (Alzet) (13). This method has the advantage of producing a relatively constant bioluminescence signal in mice, though i.p. injection of luciferin results in higher tissue concentrations of luciferin and increased signal.

Coelenterazine has a more rapid kinetic course in mice. Therefore, maximum imaging signal for *Renilla* or *Gaussia* luciferases is obtained immediately after injecting coelenterazine through intravenous or intracardiac routes (14–16). In our hands, coelenterazine is not soluble in PBS unless a co-solvent such as DMSO or ethanol is used, though several publications recommend only PBS as a solvent. Injection of a coelenterazine suspension in PBS is not recommended. Biodistribution of coelenterazine in some tissues, such as the intact brain, is limited by drug transport proteins, which can hinder in vivo imaging of *Renilla* or *Gaussia* PCAs in some tissues (17).

Mouse fur attenuates and scatters light, and this effect is most pronounced in black mice. This problem may be overcome by using nude mice or shaving animals over the region(s) of interest for imaging. For black mice, further hair removal with a depiliatory lotion (Nair, Neet) after shaving is helpful. Care should be taken with depiliatories to limit exposure time and abrasion as mouse skin is quite delicate.

10. Software accompanying the imaging equipment is used to perform the region of interest (ROI) analysis. **Figure 2.3** shows an example image and quantified luminescence obtained with a firefly PCA in cell culture. The photons detected by the machine are summed over the

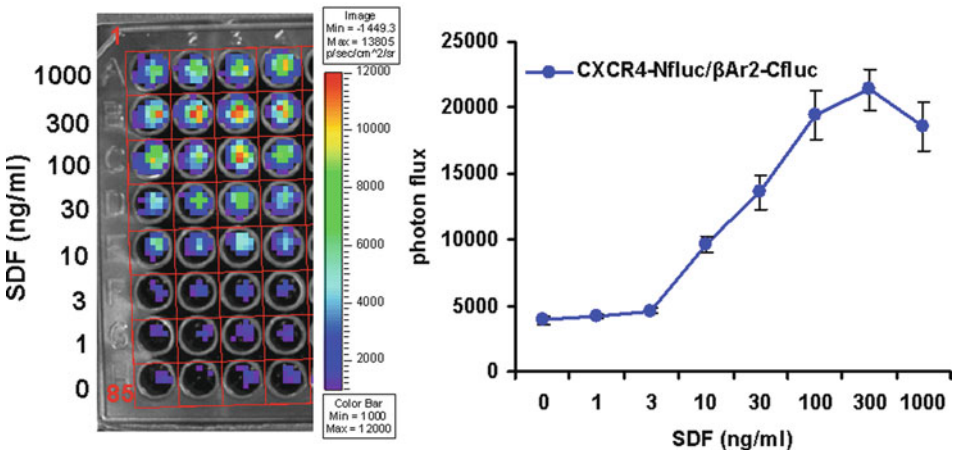

Fig. 2.3. Sample firefly luciferase PCA in cell culture. The assay was prepared as described in **Section 3.3**, using the CXCR4-Nfluc/βAr2-Cfluc pair diagramed in **Fig. 2.1**. In this image, the luminescence data appear as a pseudocolor overlay on the plate photo and is adjusted to illustrate the range of luminescence in the plate. The *red grid* marks superimposed on the image denote the regions of interest, one for each well. Total photons/s are summed over each region and averaged over the quadruplicate samples, to obtain the graphical data on the *right* of the figure. In this case, the data illustrate the association of the PCA pair upon incubation for 10 min with the chemokine SDF.

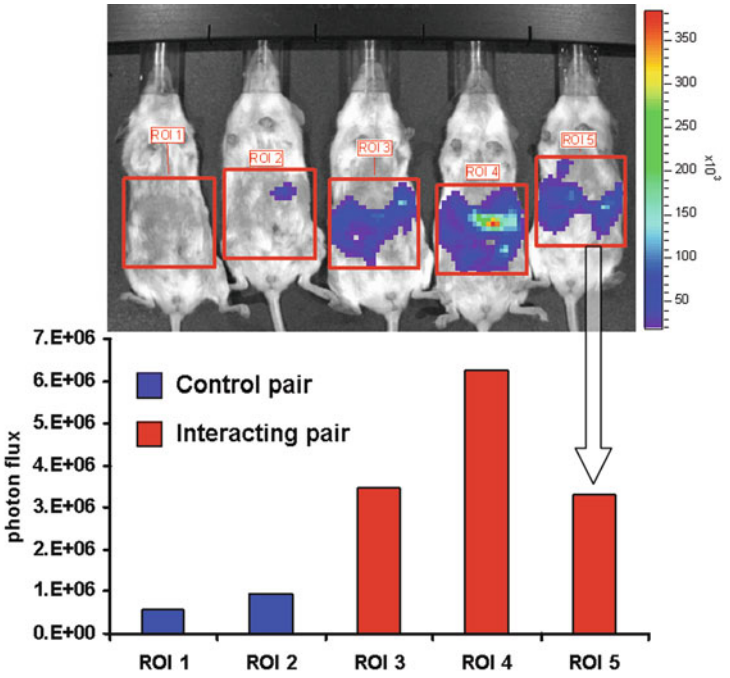

Fig. 2.4. Sample mouse imaging data. These sample data illustrate the region of interest analysis, denoted by the *identical red squares* on each mouse. Photon flux in each area is quantified in the graph below the image. These data illustrate results for a control firefly luciferase PCA, and a constitutively interacting PCA pair in transiently transfected cells (5×10^6) implanted into the peritoneal cavity of the mouse.

area of the ROI to obtain data with units of photons/time (s), or photon flux. Maintaining a standard region of interest within an experiment (or series of experiments) is important to facilitate comparison of mouse imaging data. A simple circular, oval, or square ROI is normally sufficient to be used for all the mice within a set (*see* **Fig. 2.4**). It is helpful to image a "blank" mouse injected with luciferin to obtain a background reading if background subtraction is desired. It is important to note that manipulation of the pseudocolor image display does not alter the quantitative data set for emitted photons. The user should manipulate the display range of pseudocolor image to best highlight the luminescence qualities of their sample.

To control for mouse-to-mouse variations, bioluminescence data may be normalized to one of several markers. For firefly luciferase PCAs, data may be normalized to intact *Renilla* or *Gaussia* luciferase incorporated into PCA cells. Other useful markers could include fluorescent proteins, such as mPlum or tdTomato (18–20), which can be detected using the fluorescence imaging capabilities

of the IVIS and appropriate filters. (Care should be taken in fluorescence imaging to account for the red shift imposed by mouse tissue when selecting excitation and emission filters.) Fluorescence imaging is best performed prior to injection of substrates for bioluminescence imaging. Finally, for solid tumors, simple tumor volumes can suffice for normalizing luminescence signals.

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