

RESEARCH ARTICLE

A Method to Rapidly and Accurately Compare the Relative Efficacies of Non-invasive Imaging Reporter Genes in a Mouse Model and its Application to Luciferase Reporters

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Abstract

Purpose: Our goal is to develop a simple, quantitative, robust method to compare the efficacy of imaging reporter genes in culture and *in vivo*. We describe an adenoviral vector–liver transduction procedure and compare the luciferase reporter efficacies.

Procedures: Alternative reporter genes are expressed in a common adenoviral vector. Vector amounts used *in vivo* are based on cell culture titrations, ensuring that the same transduction efficacy is used for each vector. After imaging, *in vivo* and *in vitro* values are normalized to hepatic vector transduction using quantitative real-time PCR.

Results: We assayed standard firefly luciferase (FLuc), enhanced firefly luciferase (EFLuc), luciferase 2 (Luc2), humanized *Renilla* luciferase (hRLuc), *Renilla* luciferase 8.6-535 (RLuc8.6), and a membrane-bound *Gaussia* luciferase variant (extGLuc) in cell culture and *in vivo*. We observed greater than 100-fold increase in bioluminescent signal for both EFLuc and Luc2 when compared to FLuc and greater than 10⁶-fold increase for RLuc8.6 when compared to hRLuc. ExtGLuc was not detectable in liver.

Conclusions: Our findings contrast, in some cases, with conclusions drawn in prior comparisons of these reporter genes and demonstrate the need for a standardized method to evaluate alternative reporter genes *in vivo*. Our procedure can be adapted for reporter genes that utilize alternative imaging modalities (fluorescence, bioluminescence, MRI, SPECT, PET).

Key Words: Adenovirus, *In vivo* imaging, Non-invasive imaging, Molecular imaging, Bioluminescence imaging, Firefly luciferase, *Renilla* luciferase, *Gaussia* luciferase, Reporter gene efficacy, Reporter gene comparison

Introduction

Bioluminescent luciferase enzymes have been used as reporter genes in a wide variety of biological assays that

include (but are not limited to) monitoring gene expression in transgenic and knock-in animals [1–5], tumor growth [6–9], tumor metastasis [6], progress of bacterial [9, 10], viral [11], and parasitic [12] infections, distribution of transplanted cells both for therapy [13, 14] and for regenerative medicine [15], distribution of potentially therapeutic naked DNA, viruses, bacteria, nanoparticles and other delivery vehicles, and protein–protein interactions (using “split

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luciferase” complementation assays and other “molecular sensors”) [16]. Early studies employed firefly (*Photinus pyralis*) luciferase (FLuc). As an alternative, the luciferase derived from the sea pansy *Renilla reniformis* (RLuc) later gained significant popularity.

Luciferases oxidize a substrate to emit light; D-luciferin is oxidized to oxyluciferin by FLuc; coelenterazine is oxidized to coelenteramide by RLuc [17, 18]. Luciferase reporters gained popularity with the development of bioluminescence imaging (BLI) as an important tool in basic and pre-clinical research. Due to the semi-transparent nature of animal tissue, bioluminescent reporters can be used in live animal studies, without the need to first euthanize the animal and section tissues [3, 4, 19]. BLI presents some advantages over other non-invasive whole animal imaging modalities due to its low cost, ease of imaging, high sensitivity, lack of requirement for radioisotopes, and readily available, stable substrates.

As the advantages of luciferase-based BLI imaging in living animals became recognized and incorporated into basic and pre-clinical research, a variety of genetically modified firefly [20, 21] and *Renilla* luciferase [22] molecules, as well as luciferases from other biological sources (e.g., *Gaussia* luciferase [23]), have been developed. Although many luciferase reporter variants have been developed, each one claiming advantages over their predecessors for non-invasive imaging in living animals, it is difficult for researchers to compare one luciferase reporter to another by consulting the literature due to the variety of vectors, cellular targets, and manner in which the luciferases have been expressed in experimental models. One common approach to evaluating the BLI efficacy of various luciferase reporters *in vivo* has been to transfect or retrovirally transduce these genes into tumor cell lines, prepare murine xenografts and image the resulting tumors after they reach palpable dimensions, but before presumed tumor necrosis has begun [9, 21–27]. However, these comparisons are compromised by a variety of potential complications, for example: (1) differing chromosomal integration sites may affect the expression of the reporter proteins, (2) numbers of reporter genomes stably integrated into the cells may differ, (3) tumors may grow at different rates in alternative hosts, and (4) vascularization may vary greatly among tumors, resulting in differences in substrate availability. The resulting variability makes it difficult to determine accurately the comparative efficacy for alternative reporter genes.

In this report we describe a simple, robust method to compare different imaging reporter genes and demonstrate its utility with six different luciferase BLI vectors. We use a common non-integrating adenoviral vector, transcriptional promoter, 3' untranslated region, and polyadenylation signal to express the six reporter genes in liver, following intravenous injection. Episomal adenoviral vectors are not influenced by the local control elements or chromatin topography that may be encountered when using an

integrating vector. The viral delivery vectors are titrated in cell culture prior to evaluating *in vivo* efficacy for reporter gene expression. Following BLI analysis, the mice are euthanized and the numbers of viral genomes per liver cell and the levels of reporter gene activity are measured in liver extracts. Reporter gene activity measured *in vivo* is normalized by viral genome analysis to correct for any mouse-to-mouse differences in hepatic transduction. This assay methodology eliminates variability due to differences in delivery vector, transduction target size, copy number for reporter genomes, integration site silencing/modulation of reporter gene expression, and target vascularity. The only variables in the analysis are the reporter gene and the reporter probe; all other variables are minimized. Quantifying viral genomes permits post-imaging normalization.

We present this report with two aims: The first aim is a direct comparison of the six luciferase reporter genes. The second aim is to describe a simple, reliable, reproducible, quantifiable means to compare other reporter gene imaging systems, including both additional BLI reporter genes as well as fluorescent, SPECT, PET, and MRI reporter-based genes as they are developed.

Materials and Methods

Adenovirus Vector Construction

The plasmids and adenoviral vectors used in this study are listed in Table 1. All primers are listed in Table 2. All adenovirus vectors were constructed in a pENTR™ vector backbone to allow LR recombination into the pAd/CMV/V5-DEST vector, using the Gateway System (Invitrogen, Carlsbad, CA, USA). A schematic representation of the construction of these vectors is shown in Fig. 1. Initially, we created the primary vector, pENTRHM, by removing the chloramphenicol and *ccdB* genes from the pENTR4D vector (Invitrogen) with *Sall* and *EcoRI* restriction and inserting a small DNA fragment containing multiple cloning sites (*Sall*–*HindIII*–*KpnI*–*SmaI*–*SacI*–*BamHI*–*EcoRI*). All luciferase genes were amplified using high-fidelity DNA polymerase (Phusion polymerase, Finnzymes/New England Biolabs) according to the manufacturer's instructions. The firefly luciferase (FLuc) gene was amplified from pBI-GL (Clontech, Mountain View, CA, USA). The luciferase 2 (*Luc2*) and the humanized *Renilla* luciferase (hRLuc) genes were amplified from pGL4.13 and pRL-TK, respectively (Promega, Madison, WI, USA). The enhanced firefly luciferase (EFLuc) [21], the red-shifted *Renilla* luciferase RLuc8.6-535 (RLuc8.6 hereafter) [22], and the membrane-anchored *Gaussia* luciferase (extGLuc) [26] were amplified from plasmids pRV100G, pRV2011, and pSL1180, respectively. These three plasmids were gifts from Dr. Caius Radu (UCLA). The oligonucleotides used for PCR for each luciferase gene included the *HindIII* and *XhoI* restriction sites, inserted immediately upstream of the Kozak sequence and downstream of the termination codon, respectively. The amplified DNA fragments were treated with *HindIII/XhoI* and inserted into pENTRHM cleaved with the same enzymes to generate

Table 1. Plasmids and adenoviral vectors used in this study

Plasmid or virus	Description or relevant characteristics	Source
Plasmids		
pENTR4D	Gateway™ shuttle vector	Invitrogen
pAd/CMV/V5-DEST	Gateway™ destination adenoviral vector	Invitrogen
pBI-GL	Firefly luciferase FLuc gene donor vector	Clontech
pGL4.13	Firefly Luc2 gene donor vector	Promega
phRL-Tk	<i>Renilla</i> hRLuc gene donor vector	Promega
pRV100G	EFLuc gene donor vector	C. Radu [21]
pV2011	RLuc8.6 gene donor vector	C. Radu [22]
pSL1180	extGLuc gene donor vector	C. Radu [26]
pEGFP-N2	EGFP gene donor vector	Clontech
pENTRHM	pENTR4D with <i>Cm^R</i> and <i>ccdB</i> genes replaced by a synthetic multiple cloning site	This study
pENTRHM-FLUC	pENTRHM containing FLuc gene	This study
pENTRHM-EFLUC	pENTRHM containing EFLuc gene	This study
pENTRHM-LUC2	pENTRHM containing Luc2 gene	This study
pENTRHM-hRLUC	pENTRHM containing hRLuc gene	This study
pENTRHM-RLUC8.6	pENTRHM containing RLuc8.6 gene	This study
pENTRHM-extGLUC	pENTRHM containing extGLuc gene	This study
pENTRHM-EGFP	pENTRHM containing EGFP gene	This study
pAdHM-FLUC	pAd/CMV/V5/DEST vector after recombination with pENTRHM-FLUC	This study
pAdHM-EFLUC	pAd/CMV/V5/DEST vector after recombination with pENTRHM-EFLUC	This study
pAdHM-LUC2	pAd/CMV/V5/DEST vector after recombination with pENTRHM-LUC2	This study
pAdHM-hRLUC	pAd/CMV/V5/DEST vector after recombination with pENTRHM-hRLUC	This study
pAdHM-RLUC8.6	pAd/CMV/V5/DEST vector after recombination with pENTRHM-RLUC8.6	This study
pAdHM-extGLUC	pAd/CMV/V5/DEST vector after recombination with pENTRHM-extGLUC	This study
pAdHM-EGFP	pAd/CMV/V5/DEST vector after recombination with pENTRHM-EGFP	This study
Adenovirus		
AdHM.FLUC	Human adenovirus type 5 (Ad5) from Gateway System™ expressing FLuc from a CMV promoter.	This study
AdHM.EFLUC	Ad5 expressing EFLuc from a CMV promoter	This study
AdHM.LUC2	Ad5 expressing Luc2 from a CMV promoter	This study
AdHM.hRLUC	Ad5 expressing hRLuc from a CMV promoter	This study
AdHM.RLUC8.6	Ad5 expressing RLuc8.6 from a CMV promoter	This study
AdHM.extGLUC	Ad5 expressing extGLuc8.6 from a CMV promoter	This study
AdHM.EGFP	Ad5 expressing EGFP from a CMV promoter	This study

pENTRHM-LUC plasmids. These constructs were then recombined with pAd/CMV/V5-DEST, using LR Clonase II (Invitrogen), according to the manufacturer's protocol to generate the pAdHM-LUC's plasmids. The enhanced green-fluorescent protein gene (EGFP) was used as negative control for the bioluminescence experiments; the EGFP coding region was cloned as a *KpnI/XbaI* fragment from pEGFP-N2 (Clontech)

and inserted into pENTRHM linearized with the same enzymes.

To generate adenoviral vector particles, plasmid DNA from each construct was linearized with *PacI* and 1 µg was used to transfect HEK293A cells seeded on six-well cell culture plates, using Lipofectamine 2000 (Invitrogen). After 10–14 days, cells from wells showing cytopathic effects were collected by centrifugation, suspended in 1 ml

Table 2. Primers used in this study

Primer sequence	Description	Source
<i>Fwd:</i> TCGACCAAGCTTTCGGTACCAGCCCGGGAGAGCTCAAGGATCCAAG; <i>Rev:</i> AATCTTGATCCTTGAGCTCTCCCGGGCTGGTACCGAAAGCTTGG	Multiple cloning site for pENTR4D: <i>SaI</i> – <i>HindIII</i> – <i>KpnI</i> – <i>SmaI</i> – <i>SacI</i> – <i>BamHI</i> – <i>EcoRI</i>	This study
<i>Fwd:</i> ACTTAAGCTTGCCACCATGGAAGACGCCAAAAACATA; <i>Rev:</i> AACTCG AGAATTATTACATTTTACAATTTGGACT	FLuc from pBI-GL. <i>HindIII/XhoI</i> directional cloning	This study
<i>Fwd:</i> TTAAGCTTGCCACCATGGAAGATGCCAAGAACA; <i>Rev:</i> TTCTCGAGCTA CTTGCCGCCCTTCTT	EFLuc from pRV100G, removing the eGFP fused protein at the 5' end. <i>HindIII/XhoI</i> directional cloning	This study
<i>Fwd:</i> TTAAGCTTGCGACGATGGCCATGGCTCTCCAG; <i>Rev:</i> TTCTCGAGCT ATTATTGAATCCGCTGTG	ExtGLuc from pRSL1180. <i>HindIII/XhoI</i> directional cloning	This study
<i>Fwd:</i> TAAAGCTTAGCGACGATGGCCCTCCAAG; <i>Rev:</i> TTCTCGAGCTACTGTT GTTCTTCAGC	RLuc8.6-535 from pRV2011. <i>HindIII/XhoI</i> directional cloning	This study
<i>Fwd:</i> ACTTAAGCTTGCCACCATGGAAGATGCCAAAAACATT; <i>Rev:</i> AACTCGA GAATTATTACAGGCGATCTTGC	Luc2 from pGL4.13. <i>HindIII/XhoI</i> directional cloning	This study
<i>Fwd:</i> ACTTAAGCTTGCCACCATGGCTTCCAAGGTGACGAC; <i>Rev:</i> AACTCGA GAATTATTACTGCTCGTTCTTCAGCAC	hRLuc from phRL-TK. <i>HindIII/XhoI</i> directional cloning	This study
<i>Fwd:</i> CAAGGGTTGAGTACTTGTGTTAGGGTTA; <i>Rev:</i> GGTGGGTAGAGAGAAG AAATATCTGACT; <i>Probe:</i> TAMRA-AGGACAATGGCCCTTGGCTGGACAA-BHQ2A	TaqMan qPCR primer against mouse Oct4 promoter region	Gallaher et al. [31]
<i>Fwd:</i> TTGTGGTTCTTGACATATGGC; <i>Rev:</i> TCGGAATCCCGGCACC; <i>Probe:</i> FAM-CTCACCTGCCGCTCCGTTTC-TAMRA	Taqman qPCR primer against adenovirus type 5 pX gene	This study

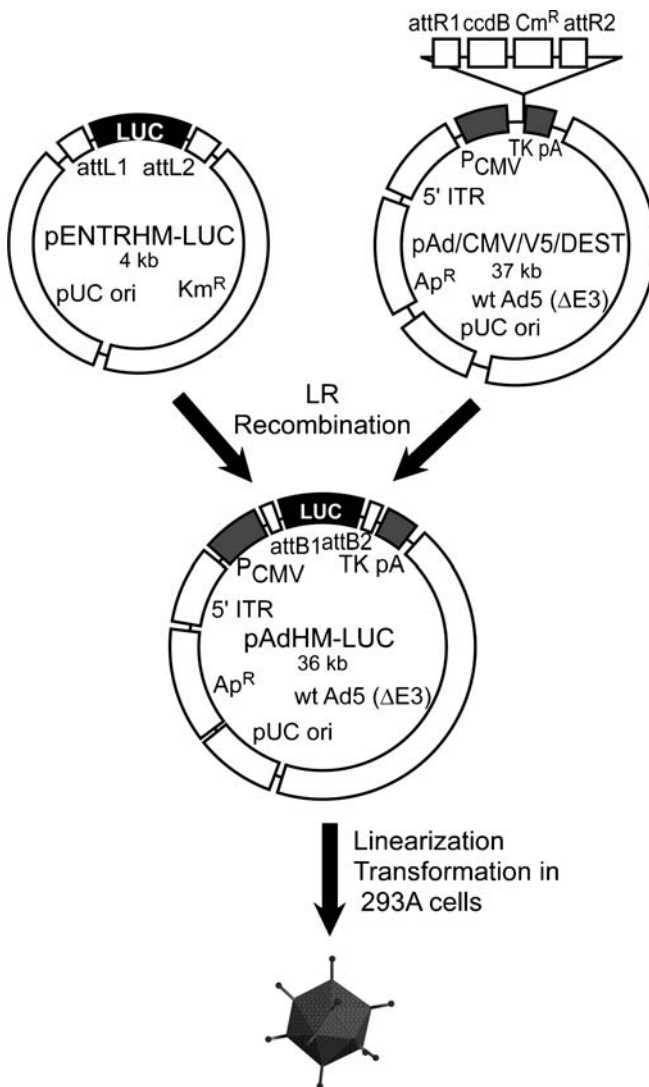


Fig. 1. Construction and propagation of adenoviral vectors. Invitrogen's Gateway® Cloning System was used to construct adenoviral expression vectors for the FLuc, Luc2, EFLuc, hRLuc, RLuc8.6, and extGLuc luciferase reporter genes and EGFP as a negative control. The coding regions of the seven proteins were first cloned into an "entry" vector to create the pENTR-LUC plasmids then transferred to the pAd/CMV/V5/DEST vector using the LR recombination reaction to create the seven pAdHM vectors. After linearizing, the Ad vectors were transformed into 293A cells and vector preparations were prepared. *attR1/attR2/attL1/attL2* are the initial recombination regions; *attB1/attB2* are the recombination regions after the LR recombination reaction. *Cm^R* chloramphenicol resistance gene; *Km^R* kanamycin resistance gene; *Ap^R* ampicillin resistance gene; *ccdB* the coding region for the cytotoxic protein CcdB, used as positive-selection marker in recombined clones; *P_{CMV}* the CMV promoter; *TKpA* the thymidine kinase polyadenylation signal; *5' ITR* the viral 5' inverted terminal repeats, *wt Ad5 (ΔE3)* Ad5 sequences that include a 3' ITR and packaging signal.

of conditioned medium, and subjected to three cycles of freeze/thawing. The lysed suspension was centrifuged, and the supernatant was used to propagate the adenovector in HEK293A cells.

Adenovector Propagation and Titration

Infected HEK293A cell pellets were lysed using deoxycholate [28]. Cell pellets were resuspended in 200 mM Tris·HCl, pH 8.0, and 0.5% deoxycholate and incubated at room temperature for 30 min, followed by a 1-h DNase treatment at 37°C, then subjected to a clarification centrifugation. The lysates were loaded onto a 50-mM Tris·HCl, pH 8.0, cesium chloride buoyant density step gradients with 1.35 and 1.25 g/ml densities. Gradients were ultracentrifuged for 90 min at 180,000 rcf and the vector bands at the interface were collected. The vector suspension was further purified on a linear cesium chloride gradient with a hinge density of 1.33 g/ml then centrifuged for 24 h at 180,000 rcf. Purified adenoviral vectors were dialyzed against a 3% sucrose storage buffer (10 mM Tris·HCl, pH 7.4; 150 mM NaCl; 2 mM MgCl₂) and snap-frozen in liquid nitrogen for storage.

The adenoviral particle concentration for each stock preparation was determined as previously described [29]. Aliquots of the adenovector suspension were lysed in adenovirus lysis solution (0.1% SDS; 10 mM Tris, pH 7.4; 1 mM EDTA) for 30 min at room temperature and the absorbance at 260 nm was determined on a μ Quant plate reader spectrophotometer (BioTek Instruments, Winooski, VT, USA) ($\text{particles/ml} = \text{OD}_{260} / 9.09 \times 10^{-13}$).

Biological titration was performed using the infectious genome protocol [30, 31], using TaqMan to quantify vector genomes reaching HeLa cell nuclei. To determine the number of infectious genome units (IGUs), the cells were washed with PBS 3 h after infection and nuclei were purified using NP-40 lysis buffer (0.65% NP-40; 150 mM NaCl; 10 mM Tris·HCl, pH 8.0; 1.5 mM MgCl₂) to ensure that only infectious vector genomes are counted. DNA was purified using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA) and subjected to TaqMan quantitative real-time PCR (Applied Biosystems, Foster City, CA, USA); primers and probe specific for the adenoviral pX protein gene were used for absolute quantization using a plasmid standard. The number of IGUs was determined by comparing the amount of PCR product from the nuclear extracts, normalized to one copy of the amplification region in the plasmid standard. In addition, Tissue Culture Infectious Dose 50 (TCID₅₀) assays were performed [32] as an added measure to evaluate vector integrity and efficacy. TCID₅₀ and IGU assays resulted in similar titers, but with greater variability in the TCID₅₀ values. We have used the IGU titers for normalization.

Tissue Culture Infections to Titer Vector

Adherent HeLa cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μ g/ml). HeLa cells were seeded onto either 6- or 12-well cell culture plates 1 day prior to adenovector infection. Wells were infected with the adenoviral vectors by directly diluting vector stocks into the cell culture medium (1 ml medium for 12-well plates, 2 ml medium for six-well plates). At 24 h post-infection, bioluminescent activity was determined by imaging the intact cells in the presence of appropriate substrate (500 μ g luciferin or 3.3 μ g coelenterazine) in an IVIS® Lumina imaging system (Caliper Life Science, Hopkinton, MA, USA). After imaging, HeLa cells were removed

with trypsin and split into samples used for TaqMan-based adenovector transduction assays and standard luminometer-based luciferase enzyme assays (Promega, Madison, WI, USA).

HeLa cells were first infected with AdHM.FLUC at multiplicities of infection (MOI) of 0, 4, 16, 64, 256, and 1024 to determine the optimal MOI (data not shown). For AdHM comparisons, HeLa cells were then infected at a MOI of 256 for all six vectors (AdHM.FLUC, AdHM.LUC2, AdHM.EFLUC, AdHM.hRLUC, AdHM.RLUC8.6, AdHM.extGLUC) and the AdHM.EGFP control vector.

In Vivo Studies

Female hairless SKH1 mice (Charles River, San Diego, CA, USA) were housed in accordance with the UCLA Division of Laboratory Animal Medicine institutional guidelines. Experiments were performed in mice at 16 weeks of age to determine the optimal MOI and at 12 weeks of age for the experiments comparing the alternative BLI reporter genes.

Initial titration studies were performed by injecting 1×10^9 , 3×10^9 , and 1×10^{10} infectious genome units of AdHM.FLUC into mice to determine the optimal vector dose. At 72 h after injection, the mice were anesthetized (2.5 mg ketamine+0.25 mg xylazine in 100 μ L saline, i.p., per animal). The mice were then imaged using 3 mg of luciferin in 100 μ L administered intraperitoneally in the IVIS® Lumina imaging system. Specific activity (bioluminescence/vector genome) was equal for the two highest adenovirus doses. The lowest adenovirus dose demonstrated the non-linear dose response typical of adenoviral vector transduction when virus levels drop below the Kupffer cell barrier, where a large proportion of vector is taken up by these liver macrophages. We observed the same specific activity at the two highest doses and chose the higher dose for the greatest sensitivity in the experiments to compare the efficacy of the various luciferase reporter genes.

To compare the efficacy of the six luciferase reporter genes, groups of three animals were then injected via the tail vein with 10^{10} IGU of the seven AdHM vectors. At 72 h after injection, the mice were anesthetized with ketamine/xylazine. Mice receiving AdHM.FLUC, AdHM.LUC2 and AdHM.EFLUC were imaged as described above. Mice receiving AdHM.hRLUC, AdHM.RLUC8.6, and AdHM.extGLUC were injected intravenously with 20 μ g of coelenterazine in 100 μ L and then imaged in the IVIS® Lumina imaging system.

These substrate concentrations have previously been shown in our laboratory to provide optimal imaging characteristics for firefly and Renilla luciferases (unpublished data). The intensity for firefly luciferase-based reporters typically takes 5 to 10 min to peak and demonstrates broad, steady activity, while Renilla luciferase bioluminescence peaks and begins to drop within 2 min. For this set of experiments, we took multiple 10-s measurements post-injection to ensure that we sampled during the peak of bioluminescence activity.

Mice injected with AdHM.EGFP were imaged first with coelenterazine. One hour later, these mice were injected with luciferin and re-imaged. The values for coelenterazine-imaged AdHM.EGFP mice, which do not express an enzyme that metabolizes coelenterazine, were subtracted from the values obtained for mice receiving AdHM.hRLUC, AdHM.RLUC8.6, and AdHM.extGLUC vectors. The values for luciferin-imaged AdHM.EGFP mice, which do not express an enzyme that

metabolizes luciferin, were subtracted from the values obtained for mice receiving AdHM.FLUC and AdHM.LUC2 and AdHM.EFLUC.

Total flux for the mice receiving the luciferase vectors was determined by defining a 2.5-cm region of interest over the abdominal area corresponding to the liver. Bioluminescence was corrected by subtracting the appropriate value for the mice that received AdHM.EGFP. Mice were then euthanized, the livers were removed and weighed, and samples were taken for vector and genomic DNA analysis, *in vitro* luciferase IVIS® bioluminescence assay, and conventional luciferase enzyme assay.

Both genomic and adenoviral vector DNAs were isolated from the liver samples using the DNeasy protocol (Quiagen, Thousand Oaks, CA, USA). Absolute quantization for vector genomes and liver cell genomes was performed with TaqMan PCR using the same adenovector primers and TaqMan probe used in the cell culture experiments and primers and probe specific for the mouse *Oct4* gene. Standard curves to measure the number of liver genomes were prepared from pre-weighed liver samples based on an estimation of 1.35×10^8 hepatocytes per gram [33].

For *in vitro* luciferase assay, liver samples were homogenized in Passive Lysis Buffer (Promega). One portion of the supernatants was used in Promega's standard firefly luciferase or *Renilla* luciferase assays with a Lumat LB9501 luminometer (Berthold, Oak Ridge, TN, USA). For these assays, 100 μ L of Promega Firefly Luciferase Assay Reagent or 100 μ L of Promega Renilla Luciferase Assay Reagent was used for 10 μ L of appropriately diluted liver homogenate. Duplicate samples from a second portion of the liver supernatants were placed in wells of multiwell plates and analyzed for bioluminescence in the IVIS® Lumina imaging system using assay conditions identical to those used for the luminometer analyses. Data are expressed as photons per second. Under these assay conditions, substrates (luciferin and coelenterazine) are saturating and enzyme is limiting for product production. Additional control experiments demonstrated that residual substrate in the liver extract does not contribute significantly to the bioluminescence observed in the liver homogenate assays.

Results

Adenovirus Vector Construction and Preparation

One of the confounding issues in comparing different reporter genes is the use, by different researchers, of alternative expression vectors, promoters, and 5'- and 3'- untranslated mRNA regions. To maintain similar vector structures for each luciferase reporter in this study, we opted for the versatility of the commercially available Gateway System (Invitrogen). All luciferase genes were cloned as *HindIII/XhoI* inserts into an "entry" vector and then recombined into pAd/CMV/V5-DEST. The use of this commercially available, easily manipulable cloning system will permit other researchers to easily create identical vectors with which to evaluate new reporter genes as they are developed and utilize our vectors as controls for comparison.

The adenoviral vector stocks were titrated for infectious genome units in culture as described in "Materials and Methods". The vector concentrations were AdHM.FLUC,

2.43×10^{11} IGU/ml; AdHM.LUC2; 1.43×10^{11} IGU/ml; AdHM.EFLUC, 7.61×10^{10} IGU/ml; AdHM.hRLUC; 8.24×10^{10} IGU/ml; AdHM.RLUC8.6, 2.05×10^{11} IGU/ml; AdHM.extGLUC, 8.07×10^{10} IGU/ml; and AdHM.EGFP; 9.19×10^{10} IGU/ml (Fig. 2a). The vector particle concentrations, determined by lysis and optical density measurement, were AdHM.FLUC, 9.37×10^{11} particles/ml; AdHM.LUC2; 3.78×10^{11} particles/ml; AdHM.EFLUC; 2.39×10^{11} particles/ml; AdHM.hRLUC, 2.67×10^{11} particles/ml; AdHM.RLUC8.6; 5.87×10^{11} particles/ml; AdHM.extGLUC; 2.29×10^{11} particles/ml; and AdHM.EGFP, 2.33×10^{11} particles/ml (Fig. 2b). The ratio of vector particle concentration to infectious genome units may be used as a marker for vector quality; a very high ratio implies many defective particles. The average ratio for all seven vectors was 3.02 ± 0.17 particles/IGU (mean \pm SEM) (Fig. 2c). The low particle/IGU ratio indicates high quality for these adenoviral vectors; the small SEM demonstrates the reproducibility of the viral preparation method with a variety of inserted reporter gene-coding sequences.

Bioluminescence Luciferase Reporter Gene Activities in Cultured Cells

Four wells of HeLa cells were infected (MOI, 256 IGU/cell) with each of the seven adenoviral vectors. After 24 h, the substrates (luciferase or coelenterazine) were added to the wells, and the plates were imaged in the IVIS® imaging system. The quadruplicate samples for each Ad vector are shown in Fig. 3a; the quantification of the bioluminescence data from these adenoviral vector-transduced HeLa cells is shown in Fig. 3b. The normalized (per vector genome) activities of both Luc2 (1.8×10^5 photons/second/vector genome) and EFLuc (1.3×10^5 photons/second/vector genome), both derived from FLuc, were more than two orders of magnitude greater than that of the original

FLuc reporter (9.6×10^2 photons/second/vector genome) using these standardized conditions (Fig. 3b). For the luciferase reporters that utilize coelenterazine as substrate, RLuc8.6 (1.4×10^5 photons/second/vector genome) and extGLuc (5.4×10^4 photons/second/vector genome) were also greater than two orders of magnitude more active than the reference hRLuc reporter (3.3×10^2 photons/second/vector genome).

Hepatic Transduction Efficacy of the Adenoviral Vectors

Groups of three mice were injected intravenously via the tail vein with 10^{10} IGU for each of the six AdHM.LUC vectors and the control AdHM.EGFP vector. At 3 days after adenovector administration, the mice were injected with the appropriate substrate and imaged with the IVIS imaging system (see the following section). After imaging, the mice were euthanized and liver homogenates were prepared. The number of vector genomes per cell was determined (Fig. 4) by TaqMan quantitative PCR analyses of adenovector DNA as described previously [31] and quantitative PCR for the murine *Oct4* gene using a standard curve prepared from a known number of liver cells. The mean transduction frequencies for the seven transduction vectors were AdHM.FLUC, 37 Ad genomes/cell; AdHM.LUC2, 35 Ad genomes/cell; AdHM.EFLUC; 91 Ad genomes/cell; AdHM.hRLUC, 64 Ad genomes/cell; AdHM.RLUC8.6, 74 Ad genomes/cell; AdHM.extGLUC, 69 Ad genomes/cell; and AdHM.EGFP, 54 Ad genomes/cell. The seven viral vector preparations differed in hepatic transduction efficacy by only threefold in their means, demonstrating the consistency of the vector preparation procedure, the vector IGU titrations in culture, and the hepatic delivery following intravenous adenovirus vector injections. One-way ANOVA test showed no significant differences between the means of these groups (P value=0.176).

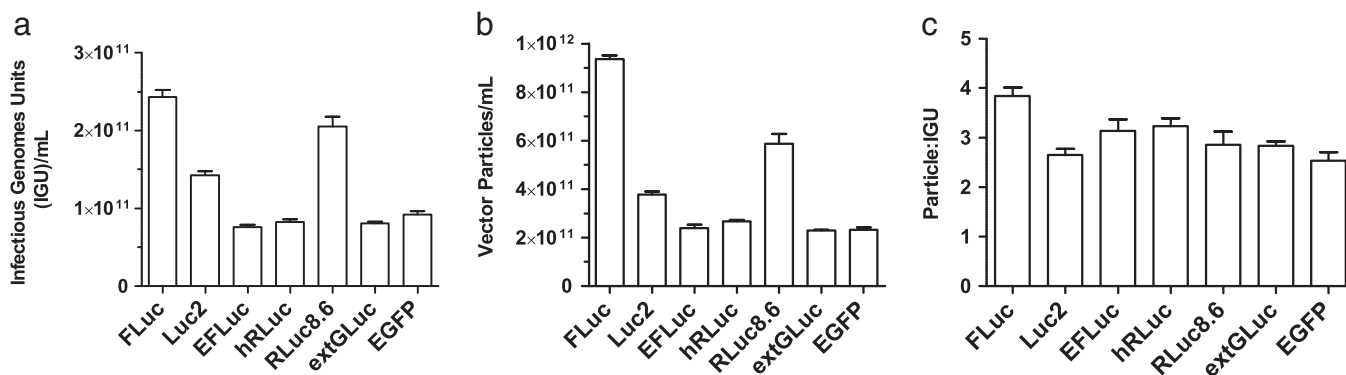


Fig. 2. Comparison of infectious genome units (IGUs) and vector particle counts for the AdHM.LUC transduction vectors. **a** IGU values for the seven Ad vector preparations were determined following HeLa cell transduction. Nuclei were harvested 3 h after vector addition. Vector genomes were measured by quantitative PCR as described in “Materials and Methods”. Data are means \pm SEM of triplicate assays for duplicate transductions. (Panel B) Particle concentrations were determined by OD₂₆₀ readings of SDS disrupted vector stocks. (Panel c) Ratios of vector particles:IGUs for the seven adenovector preparations. Data are means \pm SEM.

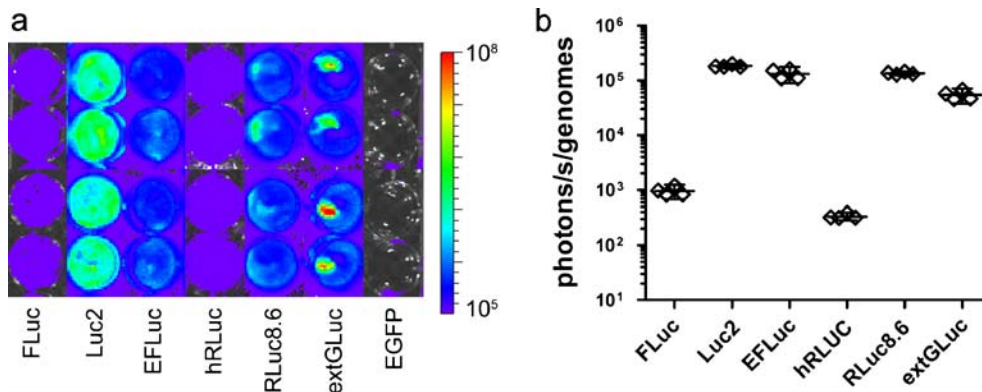


Fig. 3. Comparison of luciferase reporter gene/reporter probe activities in HeLa cells. **a** Four wells of HeLa cells were infected with 256 IGU/cell for each vector. After 24 h, D-luciferin was added to the wells transduced with the FLuc, Luc2, EFLuc, and EGFP vectors, and coelenterazine was added to the wells transduced with hRLuc, RLuc8.6 and extGLuc vectors. Bioluminescence images were then obtained for the cultured cells with the IVIS imaging instrument. **b** Quantification of the bioluminescence images. After imaging, the cells were lysed and the number of transduced viral genomes in each well was determined by quantitative PCR. Total flux (photons per second) was normalized to transduced vector genomes for each reporter gene. Data are means \pm SEM from four wells for each vector.

Bioluminescence Luciferase Reporter Gene Activities in Livers Following Adenoviral Vector Administration

Each mouse in the groups described above was imaged *in vivo* with the IVIS® imaging system, following an injection of appropriate substrate, to detect luciferase reporter gene activity over the abdominal area corresponding to the liver. Figure 5a shows examples of bioluminescence for the Luc2/luciferin and RLuc8.6/coelenterazine reporter gene/reporter probe systems, with bioluminescence values shown on the same scale. FLuc, hRLuc, and extGLuc bioluminescence are not detectable on this scale. Quantitative region of interest bioluminescence measurements from the living animals (Fig. 5b) demonstrated robust activity for Luc2 (3.4×10^7

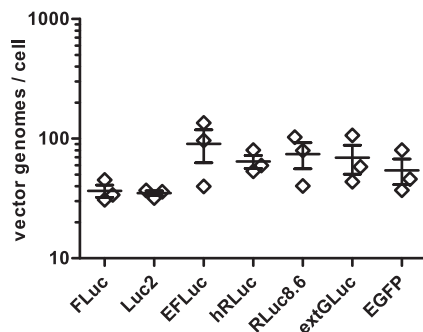


Fig. 4. Comparison of hepatic transduction efficacy of the AdHM.LUC vectors. Three mice per group were injected intravenously with 10^{10} IGU of each AdHM.LUC vector. After 3 days, the luciferase reporter activity of each mouse was measured by BLI (see legend to Fig. 5). After imaging, the mice were euthanized and vector genomes present in DNA samples from liver homogenates were measured and normalized to cellular genomes (measured by TaqMan PCR for the Oct4 gene). Each point is the mean of duplicate samples from a liver homogenate. Error bars indicate means \pm SEM.

photons/s per 10^6 vector genomes), EFLuc (9.0×10^6 photons/s per 10^6 vector genomes), and RLuc8.6 (3.6×10^6 photons/s per 10^6 vector genomes). A somewhat low activity was observed for the FLuc reporter gene (3.3×10^4 photons/s per 10^6 vector genomes) and a substantially lower activity for the hRLuc reporter gene (57 photons per second per 10^6 vector genomes). No luciferase activity could be detected for the extGLuc reporter gene above that observed for the mice that received the EGFP control gene + coelenterazine in the *in vivo* bioluminescence assay despite the presence of comparable numbers of vector genomes in the liver (Fig. 4).

To compare the luciferase activities of the liver lysates with the bioluminescence values observed with the living mice, duplicate samples of each liver extract were placed in cell culture wells, appropriate substrate (luciferase or coelenterazine) was added, and the plates were analyzed in the IVIS® imaging system (Fig. 5c). The luciferase activity profiles of the liver lysates, following normalization for vector genomes (Fig. 5c), had similar activity profiles to those seen in the cell culture studies of Fig. 3; hepatic extracts of mice receiving AdHM.LUC2 (7.6×10^6 photons/second/vector genome) and AdHM.EFLUC (2.0×10^6 photons/second/vector genome) showed substantially more reporter gene activity than hepatic extracts from mice receiving AdHM.FLUC (2.0×10^2 photons/second/vector genome). Similarly, hepatic extracts of mice receiving AdHM.RLuc8.6 (2.6×10^7 photons/second/vector genome) and AdHM.extGLUC (1.7×10^3 photons/second/vector genome) showed substantially greater activity than did hepatic extracts of mice receiving AdHM.hRLUC (5.1 photons/second/vector genome). In the *in vivo* hepatic assay, the Luc2/luciferin reporter gene/reporter probe assay has the greatest sensitivity by a factor of ~ 3.8 (compared to EFLuc, the next most sensitive reporter gene); in the *in vitro* assay of liver extracts, the RLuc8.6/coelenterazine reporter gene/reporter probe assay is the most sensitive by a factor of

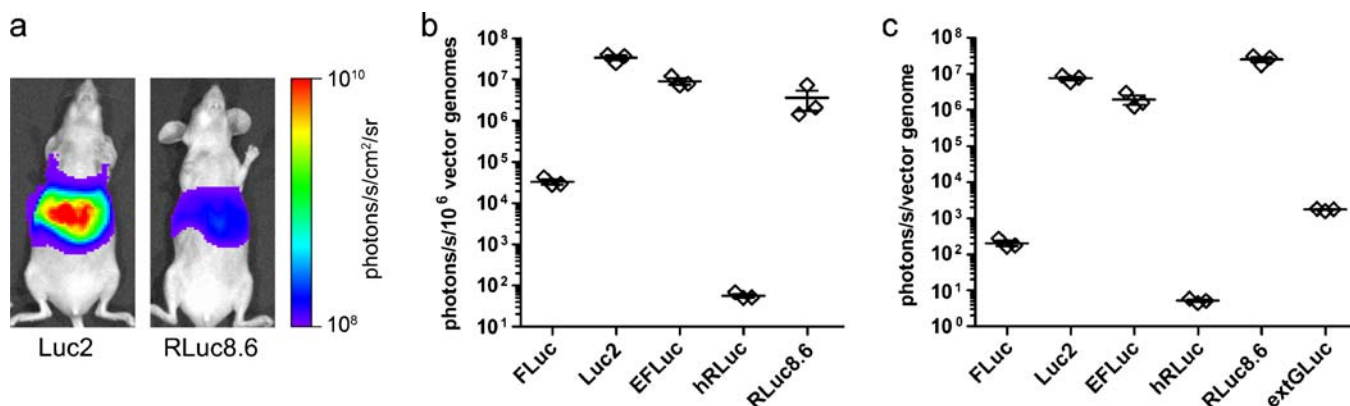


Fig. 5. Comparison of the luciferase reporter gene/reporter probe activities in liver. **a** Representative bioluminescence images, measured with the IVIS[®] instrument, for a mouse injected with 10^{10} IGU of AdHM.LUC2 then imaged 3 days later with luciferin and a mouse injected with 10^{10} IGU of AdHM.RLUC8.6 then imaged 3 days later with coelenterazine. **b** Bioluminescent efficacy of the luciferase reporter genes *in vivo*. Three mice were injected with 10^{10} IGU of each adenovector. Three days after vector administration, the mice received appropriate substrate (D-luciferin for the AdHM.FLUC, AdHM.LUC2, and AdHM.EFLUC vectors; coelenterazine for the AdHM.hRLUC, AdHM.RLUC8.6, and AdHM.extGLuc vectors), and bioluminescence was measured in the living mice. Mice injected with AdHM.EGFP were imaged successively with coelenterazine then with luciferin, as described in “Materials and Methods”. After measuring *in vivo* bioluminescence, the mice were euthanized, liver extracts were prepared, and vector genomes present in the liver extracts were measured (Fig. 4). The total flux (photons/s) values measured by bioluminescence imaging of the living mice were normalized to vector genomes present in the liver. Data are means \pm SEM. **c** Bioluminescent efficacy of the luciferase reporter genes in liver extracts. Duplicate samples from liver extracts of each of the three mice for each adenovector were placed in cell culture wells, appropriate substrates were added, and total flux (photons/s) values were measured by bioluminescence for the liver homogenates. The means of each duplicate sample, normalized for viral genomes, are presented as single data points; the range bars are SEMs for these means.

~ 3.4 (compared to Luc2, the next most sensitive reporter gene). Because of the great difference in the sensitivity of the *in vivo* assay (Fig. 5b) versus the liver lysate assay (Fig. 5c), bioluminescence is expressed as “per 10^6 vector genomes” in Fig. 5b and “per vector genome” in Fig. 5c.

We also assayed the liver extracts for luciferase activity using conventional luminometer luciferase assays. The bioluminescence assays using the multi-well IVIS procedure and the conventional luminometer assays correlated completely (data not shown).

Discussion

Because of the utility of BLI, a number of mutated luciferase reporters designed to overcome differences in energy/wavelength of the emitted light, codon usage, mRNA stability, protein stability, and other limitations have been created. Common approaches have included bioprospecting, codon optimization, mutagenesis to red-shift the emitted light, and mutagenesis to modify stability at mammalian physiological temperatures.

To facilitate comparisons of the existing BLI reporter genes, as well as both additional BLI reporter genes to be developed in the future and reporter genes that use other imaging modalities to monitor gene expression, we developed a standardized protocol in which we minimize variability from such sources as vector construction, transfection level, target variability, vascularization, etc. To ensure comparability, we inserted each reporter gene into

an identical adenoviral vector. We used a commercially available vector to facilitate the ability of other investigators to create new vectors for additional reporter genes and obtain results that can be compared with our data. Adenoviral vectors have the advantage of high transduction efficiency. Their tropism for liver cells provides a target tissue/organ that is well defined and well vascularized and exhibits substantial availability for substrate uptake. Post-imaging normalization, by measuring reporter gene transduction levels in liver, allows correction both for animal-to-animal differences and variability in vector delivery.

Wild-type firefly luciferase contains a peroxisome targeting sequence at its C-terminus. Sherf and Wood [20] produced “luc+,” a cytoplasmic version of FLuc which was also codon-optimized. This luciferase was further modified by removing consensus sequences for transcription factor binding sites to generate a newer version, Luc2 [33]. Rabinovich *et al.* [21] described another enhanced FLuc gene—EFLuc—that also employed codon optimization along with removal of cryptic splice sites. They report greater than 100-fold increase in sensitivity, compared to FLuc, in transfected T cells in culture and 200–400 greater sensitivity with EFLuc following a subcutaneous injection of these cells. Because of the differences in cells, vectors, and assays, one cannot draw conclusions about the relative efficacies/sensitivities of FLuc, Luc2, and EFLuc from these studies. Using our standardized cell culture assays, we found a similar 100–200-fold increase for both EFLuc and Luc2 relative to FLuc (Fig. 3). When tested *in vivo* with our

standardized adenoviral vector–liver assay, we observe nearly three logs greater sensitivity for EFLuc and Luc2 relative to FLuc, with Luc2 having about a fivefold greater sensitivity than EFLuc (Fig. 5).

Because *Renilla* and *Gaussia* luciferases use coelenterazine, a substrate distinct from that used by the firefly luciferases, these reporter enzymes can be used with FLuc to multiplex in non-invasive reporter gene expression studies [34]. As with FLuc, the humanized *Renilla* luciferase (hRLuc) has been codon-optimized to remove cryptic sites and improve expression over the native gene [35]. We used this optimized version, hRLuc, in our studies to compare the coelenterazine luciferases. For BLI, the amount of attenuation of signal through tissue is dependent on the wavelength of the emitted light; wavelengths under 600 nm are absorbed much more effectively than light further in the red spectrum, mainly due to absorption by hemoglobin [25, 36]. Firefly luciferase has a peak emission wavelength of 562 nm, and 17% of its emitted spectrum is greater than 600 nm. However, photon emission for RLuc peaks at 481 nm [37], a wavelength substantially lower than that of FLuc, and only 3% of its emitted spectrum is greater than 600 nm. Thus, FLuc is predicted to have a significant advantage for *in vivo* imaging, where substantial light absorption will occur, assuming equal substrate availability for both luciferases. RLuc8.6 was developed by Loening *et al.* [22] to address this limitation by mutationally red-shifting the peak emission of RLuc. Loening *et al.* [22] did not directly compare RLuc and RLuc8.6 because RLuc8.6 was created by further mutation of an intermediate improvement in RLuc for BLI use [38]. In our standardized HeLa cell culture assay, the activity of RLuc8.6 was ~500 greater than that of hRLuc in HeLa cells (Fig. 3). Using the IVIS® bioluminescence measurements, the RLuc8.6 reporter gene was six and five orders of magnitude greater than hRLuc in the adenovector–liver lysate assay and *in vivo* imaging, respectively (Fig. 5).

Gaussia luciferase (GLuc) is a secreted enzyme [23]. Santos *et al.* [26] developed a membrane-bound version, extGLuc, by fusing the CD8 transmembrane domain to the GLuc C-terminal domain. They report a ninefold increase in signal, compared to secreted GLuc, in cell culture and a 15-fold increase *in vivo* using injected T cells. In our culture experiments, extGLuc activity was ~200-fold greater than hRLuc (similar to the results observed by Santos *et al.* [26] for retrovirally infected fibroblasts), but not as active as RLuc8.6 (Fig. 3). In head-to-head comparisons for *in vivo* efficacy of the three luciferases that use coelenterazine (hRLuc, hRLuc8.6, and extGLuc) in our adenovector–liver *in vivo* imaging protocol, we were unable to detect extGLuc activity in liver by *in vivo* imaging (Fig. 5). These data with six luciferase BLI reporter genes illustrate the utility, in comparing the efficacy of alternative imaging reporter genes, of an assay that uses a common vector, is amenable to both cell culture and *in vivo* analysis with well-defined target tissues, and in which the levels of the reporter gene can be

measured after imaging to normalize the imaging values for transduction efficiency.

In summary, we compared six luciferase reporter genes by BLI, both in cell culture and *in vivo*. For firefly luciferase, both Luc2 and EFLuc show a marked increase in detectability over standard FLuc. For the coelenterazine-based luciferases, RLuc8.6 is far more sensitive than hRLuc. Although extGLuc works well as a reporter gene in cell culture, it was undetectable in liver by *in vivo* imaging. For cell culture assays, where tissue absorption is not a significant factor, Luc2, EFLuc, RLuc8.6, and extGLuc all appear to have about the same efficacy for BLI experiments (Fig. 3b). Although the appropriate experiments have not been done, we would speculate that, for subcutaneous tumor models with small tumors, there will not be substantial differences in imaging efficacy due to the choice of the Luc2, EFLuc, or RLuc8.6 BLI reporter genes. Because of a lack of knowledge about the extGLuc reporter, we cannot speculate about its utility for subcutaneous xenograft experiments. For imaging of tissues within the mouse, our data suggest that, for equivalent numbers of imaging gene genomes, the order of sensitivity is Luc2>EFLuc>RLuc8.6>>FLuc>>hRLuc>>extGLuc. These results should better inform researchers when choosing luciferase reporter genes in future studies. More importantly, in our view, we provide a standardized protocol that can be reliably applied to compare the efficacy of non-invasive reporter genes for any imaging modality, minimizing the contributions of all variables other than the reporter gene and the reporter probe.

Acknowledgements. We thank Arthur Catapang for technical help, David Stout and Waldemar Ladno for advice and assistance with the optical imaging experiments, and Arion Chantzioannou and the members of the Herschman lab for helpful discussions. This study was funded by the National Cancer Institute *In Vivo* Cellular and Molecular Imaging Center (ICMIC) award P50 CA086306 (HRH). JG is supported by a Scholars in Oncologic Medical Imaging (SOMI) fellowship from the National Cancer Institute (Award R25T CA098010).

Conflicts of Interest. The authors declare that they have no conflicts of interest.

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