Construction and Validation of Improved Triple Fusion Reporter Gene Vectors for Molecular Imaging of Living Subjects

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Abstract

Multimodality imaging using several reporter genes and imaging technologies has become an increasingly important tool in determining the location(s), magnitude, and time variation of reporter gene expression in small animals. We have reported construction and validation of several triple fusion genes composed of a bioluminescent, a fluorescent, and a positron emission tomography (PET) reporter gene in cell culture and in living subjects. However, the bioluminescent and fluorescent components of fusion reporter proteins encoded by these vectors possess lesser activities when compared with the bioluminescent and fluorescent components of the nonfusions. In this study, we first created a mutant (mtfl) of a thermostable firefly luciferase (fl) bearing the peroxisome localization signal to have greater cytoplasmic localization and improved access for its substrate, D-luciferin. Comparison between the three luciferases [mtfl, fl, and firefly luciferase (fl)] both in cell culture and in living mice revealed that mtfl possessed 6- to 10-fold (in vitro) and 2-fold (in vivo) higher activity than fl. The improved version of the triple fusion vector carrying mtfl as the bioluminescent reporter component showed significantly (P < 0.05) higher bioluminescence than the previous triple fusion vectors. Of the three different red fluorescent reporter genes (jred, hcred, and mrfp1), isolated from jellyfish chromophore, coral Heteractis crispa, and coral Discosoma, respectively, evaluated, mrfp1 was able to preserve highest expression as a component of the triple fusion reporter gene for in vivo fluorescence imaging. A truncated version of wild-type herpes simplex virus 1 (HSV1) thymidine kinase gene (wttk) retained a higher expression level than the truncated mutant HSV1-sr39 TK (ttk) as the third reporter component of this improved triple fusion vector. Multimodality imaging of tumor-bearing mice using bioluminescence and microPET showed higher luciferase activity [(2.7 ± 0.1 versus 1.9 ± 0.1) × (106 p/s/cm²/sr)] but similar level of fluorine-18–labeled 2-fluoro-2-deoxyxarabinofuranosyl-5-ethyluracil (18F-FEAU) uptake (1.37 ± 0.15 versus 1.37 ± 0.2) percentage injected dose per gram] by mtfl-mrfp1-wttk–expressing tumors compared with the fl-mrfp1-wttk–expressing tumors. Both tumors showed 4- to 5-fold higher accumulation (P < 0.05) of 18F-FEAU than fluorine-18–labeled 9-(4-fluoro-3-hydroxymethylbutyl)guanine. This improved triple fusion reporter vector will enable high sensitivity detection of lower numbers of cells from living animals using the combined bioluminescence, fluorescence, and microPET imaging techniques. [Cancer Res 2007;67(7):3085–93]

Introduction

Convergence of conventional molecular biology and noninvasive imaging technologies is leading to significant advancements in the field of molecular imaging. Molecular imaging is intrinsically multimodality in nature and involves several radionuclide [positron emission tomography (PET) and single-photon emission computed tomography] and nonradionuclide technologies [magnetic resonance imaging, ultrasound-guided imaging, bioluminescence, and fluorescence optical imaging]. Biomedical science extensively uses these multimodality imaging strategies to analyze the molecular pathways of cancer initiation and progression and drug therapy in living subjects (1). Fusion reporter genes encoding for fusion reporter proteins have emerged as an important tool for molecular imaging specially working with small animals. The most common fusion reporter proteins for molecular imaging involve fusions between a fluorescent and a PET reporter protein that can translate information from a single cell to living animals with high resolution and in a tomographic manner (2–5). However, these fusions lack the sensitivity (i.e., detection of lower number of cells from a living animal) for in vivo imaging and require highly sophisticated and a costly cyclotron for tracer production. Bioluminescent reporter genes [e.g., firefly luciferase (fl) and Renilla luciferase (rl)], on the other hand, have emerged as a very sensitive detection tool (~500 cells) for small animals from superficial depths (1–3 mm; ref. 6). Fusion proteins encoded by fusion genes composed of a bioluminescent, a fluorescent, and a PET reporter gene are thus ideal for bridging imaging gene expression from a single cell to living animal with sensitivity and from greater depths. We have reported previously the construction and validation of several multimodality bifusion and triple fusion reporter genes in living subjects using fluorescence microscopy, a cooled charge-coupled device (CCD) camera, and microPET (7, 8). Others have also reported on fusion reporters (3–5, 9). These triple fusion reporters can preserve relatively high thymidine kinase (TK) activity but moderate luciferase and fluorescence activities and are therefore less sensitive for in vivo studies specially where detection of signal from the lowest possible cells are required.

fl (note that fl refers to the gene and FL to the enzyme; i.e., protein) is one of the most widely used reporter genes in modern molecular biology and in molecular imaging. This well-characterized 62-kDa luciferase enzyme [isolated from North American firefly (Photinus pyralis)] emits light in the yellow-green region
through oxidation of β-luciferin (10, 11). The wild-type (WT) fl is thermolabile with an in vitro half-life of 2 to 3 min at 37°C and in vivo half-life of 1 to 4 h (12). Although thermolability is advantageous for time-sensitive studies, such as induction or repression of gene expression, drug response etc., a more thermostable enzyme results in accumulation of more enzyme over time, thus producing much higher light output. Tisi et al. (13) thus constructed a thermostable fl (fl bearing the mutations E354K, I232A, T214A, and F295L), which has an in vitro half-life of 15 min. However, the COOH-terminal end of the luciferase directs the enzyme to the peroxisome (14). Localization of the luciferase to the peroxisomes could interfere with luciferase expression and normal cellular physiology in various ways. First, availability of ATP, O₂, and luciferin and the stability of FL within peroxisomes have not been investigated in detail, which could affect the in vivo imaging measurements. Second, large amounts of a foreign protein in the peroxisomes could impair the normal function and interfere with the import of other peroxisomal targeting proteins.

In the current study, we first constructed a mutant (mutfl) of flf by replacing the SKL sequence with the isoleucine-valine-lysine (IAV) sequence to achieve better cytoplasmic distribution and better access to its substrate β-luciferin (15). Comparison between flf, tff, and mutfl gene expression level in cell culture and living animal showed that mutfl possesses the highest expression among all the three luciferases. At the purified protein level, mTFL could preserve a 10- to 15-fold higher activity than TFL at 37°C. To improve the sensitivity of our existing triple fusion vector (fl-mtfl-1-ttk), we replaced the flf with mutfl and validated the vector both in cell culture and in living mice. We also compared the activity of three red fluorescent reporter proteins [JRED (16), HcRED (17), and mRFP1 (18)] and two PET reporter proteins [truncated herpes simplex virus 1 (HSV1)-sr39 mutant TK (1 TK) and truncated HSV1-WT TK (wtTK)] as other components of the triple fusion reporter vector by in vitro assays and in vivo imaging techniques (note: names of all the genes are italicized and corresponding proteins are capitalized). Among all these second-generation triple fusion reporter proteins, the mTFL-mRFP1-wtTK was able to preserve the highest activity of all the three reporter proteins both in cell culture and in living animals.

Materials and Methods

Chemicals. [8-3H]Enciclovir and 3H-labeled 2'-fluoro-2'-deoxyarabinono-furanosyl-5-ethyluracil (FEAU) were obtained from Moravek Biochemicals (Brea, CA). Fluorine-18-labeled FEAU (18F-FAEU) and fluorine-18-labeled 9-(4-fluoro-3-hydroxymethylbutyl)guanine (18F-FHBG) were synthesized at Stanford University (19, 20). β-luciferin was purchased from BioSynth International (Naperville, IL). The Jred and Hered (t-Hered) enzyme activity was done as described previously (8).

Cloning of flf and mutfl in eukaryotic and prokaryotic expression vectors purification of luciferase enzymes. PCR amplification and standard cloning techniques were used to insert the gff gene from plasmid pCDNA3.1-CMV-β-gal into the prokaryotic expression vector pBAD-Myc-HisA. The SKL (tcacaatg) peptide sequence present at the end of the flf gene was modified to IAV (atgcgcgtg) by PCR (using a 3′ primer-GGGAGCTT-CAGGGCGATTCGCGC), and the PCR product was cloned in the pBAD-Myc-HisA and in the pCDNA3.1 (mammalian expression vector; modified bases are in bold). For protein purification, Escherichia coli strain Top10 cells were transformed with the pBAD-Myc-HisA-flf/mutfl vectors and the proteins were purified using a Ni-NTA agarose (Qiagen, Valencia, CA) column following the protocol of the manufacturer. Fractions with high activity (measured in a luminometer by adding 100 μL LAR reagent to 5 μL protein of different fractions) were pooled together, desalted (using PD10 columns), and concentrated by spinning through a concentrator (Millipore, Billerica, MA). Protein estimation was done following the Bradford assay protocol. To measure the enzyme activities of the purified TFL and mTFL at different temperatures, 1 μg of each protein was incubated at 23°C, 37°C, and 40°C and luciferase activities were measured at specific time point. The proteins were stored on ice at 0 min time point.

Construction of mutfl-mrflj-wtfl and other fusion genes. PCR amplification and standard cloning techniques were used to generate the CMV-flf/tfl-mrflj/pred/hered-wtfl/tkt fusion plasmids. Briefly, tfl and mutfl bioluminescence reporter genes, mrflj, pred, and hered fluorescence reporter genes and tkt and wtfl PET reporter genes were amplified from the respective plasmids and cloned in frame in the existing triple fusion reporter vector CMV-flf-mrflj-egfp1-tkt (8) to construct the second-generation triple fusion vectors.

Cell lines, transient transfection, and stable cell generation. Chinese hamster ovary (CHO) cancer cells, 293T human embryonic kidney cells, MDA-MB human breast cancer cells, and C6 rat glioma cells were used (American Type Culture Collection, Manassas, VA). The C6, CHO, and MDA-MB cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The 293T cells were cultured as described earlier (8). All transient transfections were carried out using the Superfect transfection reagent (Qiagen). For cell culture generation, the C6 cells were transfected with either β-fl-mrflj-wtfl or mTFL-mrflj-wtfl and then single clones were selected using G418 containing DMEM. For both plasmids, clones exhibiting highest activity for all the three reporters were selected and used further in vivo studies.

Bioluminescence imaging of mutant FL expression in living mice. For in vivo bioluminescence imaging, mice were implanted with 293T cells transfected with different single, and fusion reporter genes were anesthetized and injected with 100 μL (15 mg/mL in PBS) of β-luciferin i.p. Mice were then scanned for 1 min using the Xenogen IVIS-200 optical imaging system (Xenogen Corp., Alameda, CA). Regions of interest (ROI) were drawn over the implanted cell area(s) and quantified by using the Living Image software version 2.5. Bioluminescence signal was recorded as maximum (photons/s/cm²/sr).

Fluorescence imaging of fusion vectors containing pred and mrflj reporter gene expression in living mice. All images were acquired using a Maestro in vivo imaging system (Cambridge Research and Instrumentation, Woburn, MA). The mice were anesthetized, injected with various numbers of cells transiently expressing the mTFL-mrflj-wtfl and mTFL-mrflj-wtfl fusion genes, and then placed inside the imaging system. Multispectral imaging (MSI) data sets (cubes) were acquired with imaged spaced every 10 mm spectral interval in the 550 to 700 nm spectral range (excitation filter, 503–550 nm; emission filter, 600–700 nm). All the images were corrected for background autofluorescence.

Bioluminescence and microPET imaging of tumor xenograft-bearing mice. Animal care and euthanasia were done with the approval of the Administrative Panels on Laboratory Animal Care of Stanford University. Four- to 6-week-old nude mice (nu/nu; n = 6) were implanted s.c. with C6 cells stably expressing mTFL-mrflj-wtfl and mTFL-mrflj-wtfl fusion reporters on the two shoulders. When the tumors were of 6 to 8 mm size, mice were first imaged for bioluminescence and signals were measured as described above. The mice were then scanned using a microPET (Vista, GE, London, Canada) for 18F-FAEU uptake and 24 h after for 18F-FHBG uptake. Briefly, each mouse was injected with ~200 μCi of each tracer i.v. and scanned for 10 min after 1 h. The microPET images were reconstructed with the ordered-subsets expectation maximization algorithm (21) and analyzed using a Medical Imaging Data Examiner (22). Volumetric ROIs were drawn over the tumors and the mean activities were recorded from the entire ROI. The percentage injected dose per gram (% ID/g) was calculated by dividing the ROI counts by the injected dose (decay corrected).
Results

A mutant thermostable luciferase shows increased activity over thermostable and WT FL in both cell culture and in living mice. Tisi et al. (13) reported the construction and characterization of a thermostable firefly (fl) bearing a COOH-terminal peroxisome targeting sequence (SKL) that obstructs the cytoplasmic distribution of the enzyme and access to its substrate, d-luciferin. We mutated the SKL sequence of fl to IAV to achieve better cytoplasmic localization, which should lead to higher light output from cells due to better access to its substrate. To compare the levels of enzyme activity of both the mtFL, TFL with the FL, three different cell lines (293T, CHO, and MDA-MB) were transiently transfected with the CMV-mtfl, CMV-tfl, and CMV-fl plasmids along with negative controls (mock-transfected cells). Each cell line was also cotransfected with the CMV-β-gal reporter gene to normalize for transfection efficiency. After 24 h, the luciferase activities were assayed from all the cells lysated and normalized to luciferase activities were assayed from all the cells lysated and normalized to luciferase activity. Both mtFL (6-fold in 293T and 15-fold in CHO) and TFL (4-fold in 293T and 10-fold in CHO) proteins showed significantly higher bioluminescence compared with FL (Fig. 1A).

To evaluate the stability of the tfl and mtfl proteins, luciferase activities of the purified proteins were measured after incubating at different temperatures (23°C, 37°C, and 40°C). The mtFL shows 12 to 18 time higher activity at 23°C and 37°C, and about 8- to 10-fold higher activity at 40°C than the TFL protein (Fig. 1B). For both proteins, the activity remains stable over 30 min when incubated at 23°C but drops significantly over time. At 37°C, a slightly decline in activity is observed at 30 min for both protein fractions, indicating that IAV mutation does not affect the thermostable nature of these luciferases (Fig. 1B).

To compare the increased expression of mtf and tfl over the fl in living mice, we implanted five million 293T cells transiently transfected with 5 μg DNA of CMV-mtfl, CMV-tfl, and CMV-fl plasmids on the backs of three (each group) nude mice. The mice were then scanned for 1 min at 24 and 48 h using a cooled CCD camera after i.p. injection of d-luciferin. Bioluminescence imaging of the cells expressing mtf plasmid show 1.4-fold higher expression [4.4 × 10⁷ maximum (p/s/cm²/sr)] than cells expressing fl plasmid [3.06 × 10⁷ maximum (p/s/cm²/sr); P = nonsignificant (NS)] and 2-fold higher than cells expressing tfl plasmid [2.13 × 10⁷ maximum (p/s/cm²/sr); P = NS] at 24 h from all three mice of each group (Fig. 2A). The trend of increased bioluminescence activity among the three luciferases (mtFL>TFL>FL) is observed but statistical significance is not reached. At 48 h, bioluminescence signals decrease for each group of mice; however, the average fold difference of signals remains approximately constant (mtfl/tfl/fl, 2.6:1:3:1; P = NS; Fig. 2B).

Figure 1. In vitro comparison of the luciferase activities between WT and mutant luciferases in cell culture and purified protein extracts. A, comparison of luciferase activity of mutated thermostable (mtfl), thermostable (tfl), and WT FLs (fl) in three different cell lines: 293T, CHO, and MDA-MB cells were transiently transfected with equal amounts of CMV-mtfl, CMV-tfl, and CMV-fl plasmids and harvested after 24 h and luciferase assays were done using 1 μg protein from each cell lysate. Each cell line was also cotransfected with CMV-β-gal and values for FL activity were normalized with β-Gal activity. The FL activity is expressed as relative light unit (RLU) per microgram protein. All the experiments were done in triplicate. *, P > 0.05. Note that Y-axis is presented in logarithmic scale. B, time course FL activity of the purified fractions of mtFL and TFL at different temperature: equal amounts (1 μg) of purified fractions of mtFL and TFL proteins were incubated at different temperatures (23°C, 37°C, and 40°C), and luciferase activity was measured at various time points (2, 4, 6, 8, 10, 12, 14, and 30 min). Luciferase activity of mtFL protein fraction is significantly higher than the TFL fraction at all temperatures but shows a rapid decline in activity at 40°C than at 23°C and 37°C.
The monomeric red fluorescent protein (mrfp1) exhibits highest fluorescence among three different red fluorescent proteins in cell culture. To achieve improvement for in vivo fluorescence imaging, we compared the expression in different cell lines of three different red fluorescent protein genes (mrfp1, jred, and hcred) with different excitation, emission spectra, and biochemical properties. mRFP1 exhibits the highest fluorescence intensity among all the three fluorescent proteins (data not shown) when transiently expressed in 293T, CHO, and MDA-MB cells by fluorescence microscopy and by a fluorometer and was therefore further pursued as a component of the new-generation vectors. However, we did construct several triple fusion vectors using hcred and jred reporter genes to evaluate the individual activity of the bioluminescence and PET reporter components (Table 1) in a fusion background.

New generation of triple fusion reporter gene vectors shows higher bioluminescence compared with the earlier generation of triple fusion vectors in cell culture. To improve the sensitivity of the previously described triple fusion vectors (8), new fusion vectors were constructed by replacing the fl with the mtfl, mrfp1 with the jred, and the ttk with the wttk reporter genes. The order of the reporter genes, spacer sequence, and the vector backbone (pCDNA3.1) were kept constant as described previously (8). Two more triple fusion vectors carrying the mtfl, mrfp1, or egfp and wttk (CMV-mtfl-mrfp1/egfp-wttk) were also constructed. Transient transfection of the new triple fusion reporter vectors (CMV-mtfl-mrfp1-wttk and CMV-mtfl-jred-wttk) along with the control vectors (CMV-mtfl, CMV-wttk, CMV-jred, CMV-hcred, and CMV-β-gal) and the existing triple fusion vector (CMV-fl-rfp-wttk) reveals that all new fusion vectors possess much higher luciferase activity (4- to 5-fold higher compared with CMV-fl-rfp-wttk; P < 0.05; Fig. 3). The fusion vectors have 4- to 5-fold times lower expression (varying with specific cell line) than CMV-mtfl but have similar level of expression as CMV-fl (Fig. 3). Whereas the level of luciferase expression is significantly higher when using the new fusion vectors, fusions bearing the truncated sr39 tk show decreased level

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Figure 2. Comparison of FL activity obtained from CMV-mtfl, CMV-tfl, and CMV-fl vectors from 293T cells implanted in living mice. A, bioluminescence imaging of 293T cells transiently expressing the three different forms of FL in living mice: 1 × 10⁶ of 293T cells were transiently transfected with CMV-mtfl (a), CMV-tfl (b), and CMV-fl (c) plasmids and were implanted s.c. in three groups of living mice (n = 3) and were Image was adjusted to same scale, and signal is calculated as max (pixel/s/cm²/steradian). B, comparison of mean light output from 293T cells expressing CMV-mtfl, CMV-tfl, and CMV-fl vectors implanted in living mice: total light output from the 293T cells expressing the three different luciferase vectors were calculated from the ROIs drawn over the sites of cell implantation from three groups (n = 3) of living mice at 24 and 48 h. At both 24 and 48 h, mtfl showed 2-fold higher signal than fl and 1.5-fold higher signal than tfl luciferase. Representative of triplicate experiments. Columns, (p/sec/cm²/steradian) maximum × 10⁶; bars, SE. n.s., statistically nonsignificant.
of tk expression compared with the previous vectors (CMV-fl-mrfp1-ttk and CMV-mtfl-egfp-ttk). In contrast, the new triple fusion vectors (CMV-mtfl-mrfp1-wttk or CMV-mtfl-jred-wttk) carrying the wttk gene are able to preserve equal expression level when compared with CMV-fl-mrfp1-wttk and CMV-wttk alone (Fig. 3). Surprisingly, the CMV-mtfl-hcred-wttk vector exhibits lower wttk expression compared with CMV-mtfl-mrfp1-wttk or CMV-mtfl-jred-wttk vectors. Detailed comparisons of the activities of all three components of the newly constructed fusions with appropriate controls are shown in Table 1.

Fluorescence microscopy of 293T cells transiently transfected with the new and existing triple fusion vectors reveal that both mrfp1 and hcred retain higher fluorescence signal than jred reporter as fusion partners of the triple reporter genes.

The mtfl-mrfp1-wttk triple fusion vector exhibits the highest level of bioluminescence and fluorescence in living mice compared with all vectors tested. Among the five new fusion vectors constructed in this study, we chose to use the CMV-mtfl-mrfp1-wttk and CMV-mtfl-jred-wttk vectors for further evaluation in living subjects as they preserve the highest activity of both bioluminescence (offers highest sensitivity among the three modalities) and PET (capable of tomographic and depth-independent information) reporter components. Two and four million 293T cells transiently expressing either CMV-mtfl-mrfp1-wttk or CMV-mtfl-jred-wttk fusion vectors (along with CMV-β-gal) were implanted s.c. on the dorsal (left and right) side of four nude mice. The mice were first imaged for fluorescence and then for bioluminescence using a cooled CCD camera. Faint fluorescence signals are visible from the cells expressing CMV-mtfl-mrfp1-wttk vector but not from the CMV-mtfl-jred-wttk. However, the bioluminescence imaging of the same mice exhibit equal and proportionate signals by both two million [mtfl-mrfp1-wttk versus mtfl-jred-wttk (2.47 ± 0.6 versus 2.37 ± 0.8) × 10^7 maximum (p/s/cm^2/sr)] and four million [mtfl-mrfp1-wttk versus mtfl-jred-wttk (3.53 ± 0.1 versus 3.11 ± 0.1) × 10^7 maximum (p/s/cm^2/sr); P = NS] cells expressing the two different vectors (Fig. 4B).

Using a spectral imaging system, we also tried to image the expression level of both CMV-mtfl-mrfp1-wttk and CMV-mtfl-jred-wttk fusion vectors in living mice. Before bioluminescence imaging, the same group of mice was imaged for 2,000 to 2,500 ms for fluorescence using 550 to 570 excitation and 600 to 700 nm emission filters. The raw image was then normalized for back ground autofluorescence using software. Both the two and four million cells expressing CMV-mtfl-mrfp1-wttk fusion reporter are well visualized in all four mice (Fig. 4I). However, only very faint signal is visible from the four million cells expressing CMV-mtfl-jred-wttk fusion vector indicating a lower sensitivity of the jred fluorescence reporter gene for in vivo fluorescence imaging.

To determine the absolute level of the fusion reporter proteins, equal number of cells from the above mentioned two groups were lysed and assayed for luciferase, TK, and β-Gal activity. Both the cells expressing CMV-mtfl-mrfp1-wttk and CMV-mtfl-jred-wttk plasmids exhibit similar levels of mTFL and TK activity after normalization with β-Gal activity (Fig. 4C).

**Tumors expressing the mtfl-mrfp1-wttk fusion reporter show equal level of TK but higher FL activity than tumors expressing the mtfl-mrfp1-wttk fusion reporter in living mice.** To compare the strength of these second-generation and previous fusion vectors for preclinical studies, we used a tumor xenograft model. We also used different 18F-labeled tracers to optimize reporter gene: reporter probe combination for molecular imaging. Five million C6 cells stably expressing either mtfl-mrfp1-wttk or mtfl-mrfp1-wttk fusion reporters were implanted on two shoulders of
six nude mice. When the tumors reached 6 to 8 mm in diameter, mice were imaged for bioluminescence 10 min after i.p. injection of D-luciferin (Fig. 5A). Tumors expressing mtfl-mrfp1-wtk vector show 1.4-fold higher bioluminescence signal (P = NS) than the tumors expressing fl-mrfp1-wtk vector (2.75 ± 0.1 versus 1.96 ± 0.15 × 10^6 maximum (p/sec/cm^2/sr); Fig. 5C). MicroPET imaging of the same tumor-bearing mice with 18F-FEAU (f200 Ci) shows moderate and similar levels of uptake (1.37 ± 0.15 and 1.37 ± 0.22% ID/g) between the mtfl-mrfp1-wtk-expressing tumors and fl-mrfp1-wtk-expressing tumors (P = NS). Because WT TK has been shown to have preferential accumulation of the pyrimidine nucleoside analogues than the acycloguanosine analogues, we chose to compare the uptake level of 18F-FEAU and 18F-FHBG using the same group of mice. Twenty-four hours after following the scan with 18F-FEAU, mice were injected with 18F-FHBG (f200 µCi/mouse) and scanned for 10 min using the microPET (Fig. 5B). Again, both the mtfl-mrfp1-wtk and the fl-mrfp1-wtk tumors show near to equal level of 18F-FHBG uptake (0.26 ± 0.02 and 0.3 ± 0.06% ID/g, P = NS), which is 4- to 5-fold lower than the 18F-FEAU uptake (Fig. 5D). The luciferase activity and 18F-FEAU uptake of the tumors of these mice are well correlated (R^2 = 0.69 for mtfl-mrfp1-wtk tumors; R^2 = 0.59 for fl-mrfp1-wtk tumors). Due to very low accumulation of 18F-FHBG in the tumors, we did not obtain a good correlation between bioluminescence and microPET signal for both tumors. In agreement with our earlier study (19), 18F-FEAU showed very low activity by the gastrointestinal tract compared with 18F-FHBG (Fig. 5B).

**Discussion**

In this study, we report the construction and validation of an improved bioluminescence reporter gene (mtfl) and an improved version of a triple fusion reporter (CMV-mtfl-mrfp1-wtk) for use with three different imaging modalities. Several triple fusion reporter genes have been constructed by us and other investigators and are being extensively used for multimodality imaging in living subjects (8, 23–25). Our previous triple fusion vector CMV-hrl-mrfp1-wtk retains very high TK but moderate levels of RL and mRFP1 activity and thus is less sensitive for optical imaging.

![Figure 4. Comparison of the expression of CMV-mtfl-mrfp1-wtk and CMV-mtfl-jred-wtk vectors by fluorescence and bioluminescence imaging in living mice. A and B, fluorescence and bioluminescence imaging of mice: two and four million 293T cells were transiently transfected with CMV-mtfl-jred-wtk (left) and CMV-mtfl-mrfp1-wtk (right) vectors, respectively, and implanted on the dorsal side of a nude mouse (sites A1 and A2 have two million cells and sites A3 and A4 have four million cells) and imaged for bioluminescence (A) using Maestro system. The same mouse was then injected with D-luciferin and imaged for bioluminescence (B) in a Xenogen optical system. Fluorescence signal from cells expressing CMV-mtfl-mrfp1-wtk was clearly visible (A2 and A4). However, cells expressing CMV-mtfl-jred-wtk (A1 and A3) plasmid show faint fluorescence signal. Corresponding bioluminescence signal showed equal signal intensity by both groups of cells (B2 and B4 versus B1 and B3). C, FL and TK activity of the implanted cells expressing the CMV-mtfl-mrfp1-wtk and CMV-mtfl-jred-wtk vectors: 293T cells transfected with CMV-mtfl-mrfp1-wtk and CMV-mtfl-jred-wtk plasmids and used for imaging were separately assayed for FL and TK activity to ensure comparable level of reporter gene expression for both groups. The FL and TK activities of both groups were not significantly different. Note: both TK and FL activities were normalized with β-Gal activity cotransfected in the same cells.](https://cancerres.aacrjournals.org/content/67/7/3090.full#suppl-data)
Moreover, the emission spectrum of hrl (470 nm) is less favorable than fl (570 nm) for in vivo imaging due to attenuation of light while traveling through tissues. Use of the mtfl and the corresponding triple fusion reporters will thus increase the sensitivity of bioluminescence imaging of these multimodality fusion vectors.

fl and rl are the most popular bioluminescent reporter genes for small animal imaging. Although both enzymes are capable of generating light after reacting with specific substrates, their sequence, substrate specificity reaction conditions, and rate of kinetics are very different. Higher emission wavelength and more stable light production always favor FL over RL for in vivo imaging. Nevertheless, the smaller monomeric nature of RL can better tolerate added molecular engineering (fusion or splitting) than FL, aimed for specific research. Active research to generate stable and red-shifted mutants of RL is going on our laboratory (26). The hrl-mrfp1-ttk constructed earlier had the capability of retaining highest activity for all the three reporter proteins among all the eight fusion vectors (8). However, this vector did poorly in certain studies (for e.g., cardiac imaging) where greater depth leads to less transmitted light. To improve the sensitivity of in vivo bioluminescence imaging, we emphasized the use of mtfl as the bioluminescent component of the next-generation triple fusion reporters. FL has turnover rate of 3 min at 37°C in vitro and 49.3 min in vivo in the presence of D-luciferin. Researchers have been actively mutating critical amino acids of FL to produce a more thermostable enzyme (12, 27). Tisi et al. (13) reported a thermostable mutant of fl (tfl bearing the mutations E354K, I232A, T214A, and F295L).

Figure 5. Multimodality imaging of the tumor xenografts expressing mtfl-mrfp1-wttk and fl-mrfp1-wttk fusion reporter genes by bioluminescence and microPET imaging in living mice. A, bioluminescence imaging of a living mouse implanted with C6 tumors stably expressing the mtfl-mrfp1-wttk and fl-mrfp1-wttk triple fusion reporter genes: 5 x 10^6 C6 cells stably expressing either CMV-mtfl-mrfp1-wttk fusion (A) or CMV-fl-mrfp1-wttk fusion (B) were implanted on two shoulders of a nude mouse and the tumors were allowed to reach a diameter of 6 to 8 mm. The mouse was injected with α-luciferin (100 μg/mouse) and imaged for bioluminescence. The mtfl-mrfp1-wttk tumor showed higher luciferase signal (P = NS) than the fl-mrfp1-wttk fusion tumors. B, microPET imaging of a living mouse implanted with tumor-expressing C6 cells stably expressing the mtfl-mrfp1-wttk and fl-mrfp1-wttk triple fusion reporter genes for 18F-FEAU and 18F-FHBG uptake: the same mouse described in (A) was first injected with ~200 μCi 18F-FEAU, and microPET imaging was done after 1 h for 10 min. Specific uptake of 18F-FEAU was seen at both (A and B) tumors (P = NS) with very low activity in the gastrointestinal tract (GI). After 24 h, the mouse was again injected with ~200 μCi 18F-FHBG observed in both tumors (A and B) with high nonspecific accumulation in the gastrointestinal tract. C, graphical representation of the bioluminescence signal exhibited by the mtfl-mrfp1-wttk (A) and fl-mrfp1-wttk (B) tumors of six nude mice. Bioluminescence signals were calculated for the respective ROIs drawn over the sites of tumors (A and B). Representative of six experiments. Columns, (p/sec/cm^2/sr) maximum x 10^3; bars, SE. D, comparison between the uptake of 18F-FEAU and 18F-FHBG by tumors expressing mtfl-mrfp1-wttk and fl-mrfp1-wttk genes in six nude mice. % ID/g of 18F-FEAU and 18F-FHBG uptakes were calculated for the respective ROIs drawn over the tumors. (A and B) showed similar uptake for both tracers (P = NS). However, 18F-FEAU accumulation was significantly higher than 18F-FHBG (P > 0.05) accumulation.
and evaluated its thermostability and kinetics of light emission at the protein activity level. We used tfl to replace fl domain of our existing triple fusion genes (fl-mrfp1-ttk and fl-egfp-ttk) and achieve higher sensitivity for bioluminescence imaging. Although these new fusions (tfl-mrfp1/egfp-wttk/ttk) show 3- to 4-fold higher FL activity than the triple fusions (data not shown), the presence of the peroxisome targeting sequence (SKL) in tfl raised concerns about accumulation of the fusion protein in peroxisomes. For efficient expression and better access to the substrates, fusion proteins should be devoid of any organelle-specific localization sequence. We therefore mutated the SKL sequence of tfl to IAV to retain the fusion protein into cytoplasm. This mTFL shows a gain of 3- to 4-fold higher expression level in cell culture and 2-fold higher expression from living animals. Following the same trend found in cell culture, the purified mTFL shows 4- to 5-fold higher activity than purified TFl at 37°C. The crystal structure of Fl shows that the SKL sequence in the luciferase structure remains disordered and exposed for allowing interaction with its receptor (10). We assume that a change in the peroxisome sequence might have altered the tertiary structure of FL ensuing enhanced interaction with the substrate. However, this change does not affect the thermostability of the enzyme. Further investigation is going on to uncover the specific reason of this enhanced activity. As expected, the triple fusions carrying the mTFL reporter show much higher bioluminescence activity than the previous fusion vectors both in vitro and in vivo.

We also tried to improve the other two components of the triple fusion construct, the PET and the fluorescent domains. Fluorescence imaging in vivo has generally been limited by the autofluorescence of tissues, which reduces the sensitivity of detection and accuracy of quantification of the labeled target (28). Using a multispectral instrumentation, we attempted to determine the sensitivity of these second-generation fusions for fluorescence imaging by implanting fewer numbers of cells expressing the fusion reporter. We were able to detect fluorescence signal from as low as one million cells s.c. implanted and expressing CMV-mtfl-mrfp1/wttk vector; this is an important improvement over our earlier study (requiring a minimum of five million cells). Replacement of mrfp1 with jred or hered does not result in overall improvement of the fusion vectors for fluorescent imaging. Several newer mutants of mrfp1 with increased photosensitivity and brightness (mCherry and tdTomato) or far-red emission spectra (mPlum and mRaspberry at 649 and 625 nm) have been reported (29). Based on recent tests in xenograft models of metastatic breast cancer (30), tdTomato would be the most likely to provide a further improvement over mrfp1.

Our second-generation triple fusion vectors bearing mTFL, mrfp1/jred, and ttk show decrease in TK activity in cell culture compared with the first-generation constructs. However, the second-generation triple fusions with wtTK can preserve high activity as confirmed by in vitro assays and microPET imaging. HSV1-TK phosphorylates a range of substrates, including acycloguanosines (e.g., acyclovir, ganciclovir, and penciclovir) and uracil derivatives (e.g., FEAU). Several amino acids in HSV1-TK (Gln125, Arg176, Tyr172, and Met179) are critical for binding to either purine-like or pyrimidine-based nucleosides (30). The nucleobase moiety (either a thymine or a guanine ring) is stabilized by direct hydrogen bonds with the highly conserved Gln125 and Arg176 and the pyrimidine ring is further fixed by interaction with Tyr172 and Met128 in a sandwich-like orientation (31). Any change in the final tertiary structure caused by fusion of two or more polypeptide might result in change in activity. It could be possible that fusion of mTFL and mrfp1/jred to the mutant sr39 ttk alters the folding properties of the triple fusions leading to a decrease in TK activity either due to reduced binding to penciclovir or FHBG or overall reduction in the enzymatic property of TK. However, fusion of mTFL and mrfp1/jred with the wttk probably does not adversely modify the tertiary structure of the triple reporter proteins so that TK activity is maintained. An active area of research is to optimize the reporter gene/reporter probe combination for monitoring the HSV1-tk gene expression (32). Thus, we also compared the uptake level of 18F-FEAU and 18F-FHBG by these second-generation fusions carrying wttk. As shown earlier (19), 18F-FEAU exhibited 4- to 5-fold higher accumulation than 18F-FHBG in the tumors expressing mTFL/mrfp1-wttk fusions. Because 18F-FEAU has a much lower activity in the gastrointestinal tract compared with 18F-FHBG (19), these second-generation fusions will be useful for studies involving gene expression near gastrointestinal tract. These current fusions with wttk reporter gene will also serve as important tools for assessing the specificity and pharmacokinetics of different radiotracers in living subjects.

The role of molecular imaging is continuously evolving. Instead of only being a conventional tool for translating cell-based approaches to living subjects, it is playing a proactive role in all stages of oncology diagnosis and therapeutic treatment and it is detecting intricate mechanisms of cell-cell interactions in physiologic milieu. These improved triple fusion vectors will allow us (a) monitor tumor progression as a very early stage, (b) detect micrometastases, and (c) image trafficking of fewer number of cells with greater sensitivity and higher spatial resolution, using bioluminescence and microPET imaging. Although the fluorescent component of these fusions needs improvement to gain higher quantum yield at far red-shifted wavelength for better in vivo fluorescence imaging, it will be useful for cellular imaging and fluorescence-activated cell sorting. These improved triple fusions will thus likely be a preferred choice for future imaging of early progression of tumors, cell-based therapies, and cell trafficking with greater sensitivity and specificity.

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References


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