

Visualizing fewer than 10 mouse T cells with an enhanced firefly luciferase in immunocompetent mouse models of cancer

Brian A. Rabinovich^{*†}, Yang Ye^{*}, Tamara Etto^{*}, Jie Qing Chen^{*}, Hyam I. Levitsky[†], Willem W. Overwijk^{*}, Laurence J. N. Cooper^{*}, Juri Gelovani^{*}, and Patrick Hwu^{*}

^{*}M. D. Anderson Cancer Center, 7455 Fannin Street, Houston, TX 77054; and [†]Johns Hopkins University School of Medicine, Sidney Kimmel Comprehensive Cancer Center, 1650 Orleans Street, Baltimore, MD 21231

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Antigen specific T cell migration to sites of infection or cancer is critical for an effective immune response. In mouse models of cancer, the number of lymphocytes reaching the tumor is typically only a few hundred, yet technology capable of imaging these cells using bioluminescence has yet to be achieved. A combination of codon optimization, removal of cryptic splice sites and retroviral modification was used to engineer an enhanced firefly luciferase (ffLuc) vector. Compared with ffLuc, T cells expressing our construct generated >100 times more light, permitting detection of as few as three cells implanted s.c. while maintaining long term coexpression of a reporter gene (Thy1.1). Expression of enhanced ffLuc in mouse T cells permitted the tracking of $<3 \times 10^4$ adoptively transferred T cells infiltrating sites of vaccination and preestablished tumors. Penetration of light through deep tissues, including the liver and spleen, was also observed. Finally, we were able to enumerate infiltrating mouse lymphocytes constituting <0.3% of total tumor cellularity, representing a significant improvement over standard methods of quantitation including flow cytometry.

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Complete resolution of infection or cancer often depends on an effective T cell mediated immune response. In many cases, in particular that of a tumor setting, only hundreds of tumor-specific T cells traffic to the tumor. To date, the signal intensity generated by bioluminescent (BLI) reporter genes has been insufficient to track fewer than tens of thousands of T cells in living animals. This has made it very difficult to study strategies for improving tumor homing.

In addition to BLI, positron emission tomography (PET) (1) and intravital microscopy (IVM) (2, 3) offer dynamic imaging but are less applicable to routine preclinical studies because of their expense and laborious nature. PET also requires the use of radioactive substrates and IVM is highly invasive and generally requires that studied animals be euthanized. Recently, IVM was used to image tumor specific T cells at tumor sites indicating that tumor cell killing by an individual T cell occurs slowly (> 6 h) (4).

Pioneered by Contag *et al.* and using bioluminescent bacteria (5), BLI is among the most commonly used modalities for noninvasive imaging of small animals. To date, the luciferase genes from *Photinus pyralis* (American firefly; ffLuc), *Pyrophorus plagiophthalmus* (CB-Luc), *Renilla reniformis* (rLuc), and *Gaussia princeps* (gLuc) are the most commonly used light emitting reporters. Of these, only ffLuc and CB-Luc catabolize a substrate (D-Luciferin) that is relatively stable *in vivo*. gLuc and rLuc catabolize coelenterazine but their use is limited by background luminescence (6), rapid clearance of their substrate, and flash kinetics and blue/green emission, respectively (7, 8).

Mouse T cells expressing ffLuc have been obtained from transgenic (tg) mice (9–11), bone marrow (BM) chimeric mice (obtained after lentiviral transduction of donor BM) (12), and direct retroviral transduction of T cells *in vitro* (13, 14). Unfortunately, ffLuc is expressed poorly in mouse T cells resulting in

poor sensitivity of detection (at the level of thousands of cells), requiring investigators to acquire images with high binning densities [8–16 pixels (px) grouped together] to capture weak signals in general areas (9, 13–15). In digital imaging, a px is the smallest piece of data in an image. For review, see ref. 16.

Experience from human clinical trials employing the adoptive transfer of expanded autologous tumor-infiltrating T cells into cancer patients indicates that very small numbers of lymphocytes ($<0.005\%$ per gram of tumor) reach the tumor (17). To date, most reports studying T cell trafficking to tumors have been performed in xenograft models because expression of ffLuc in human T cells provides sufficient sensitivity for BLI (18–23). These models, however, do not consider the complex interplay of T cells with an autologous immune system. Several investigators have thus attempted to use BLI for T cell trafficking in immunocompetent models of cancer, but because of the weak signal intensity generated by ffLuc-expressing T cells, images had to be acquired using high binning density (ρ) ($\leq 256 \times 256$ px) (15, 24). Comparison of xenogeneic and syngeneic systems has been recently performed in a model of mammary carcinoma in which BLI was clearly superior in the xenogeneic system (24). Other models investigating the trafficking of ffLuc-expressing mouse T cells have also necessitated high px ρ , thus limiting spatial analysis. These include collagen-induced arthritis (CIA) (14), experimental autoimmune encephalitis (EAE) (13) and graft versus host disease (GVHD) (10, 12, 25).

We engineered an enhanced version of ffLuc (effLuc) encoded within a retroviral vector to facilitate detection of <10 , 000 T cells at a given site in living animals. Retroviral transduction was chosen because it is a rapid procedure (3 days) for achieving stable expression of a given gene, which facilitates subsequent experimentation within days to weeks. Compared with mouse T cells expressing ffLuc, those expressing effLuc generated >100 times as much detectable light. Further, the augmented signal intensity provided the means to image as few as three effLuc-expressing T cells and to examine the limits of detection of cells expressing effLuc in models of vaccination and adoptive immunotherapy of established tumors.

Results

Construction of an Enhanced Firefly Luciferase and Expression in Mouse T Cells. We made modifications to an ffLuc encoding retroviral vector (pMSCV-ffLuc-pIRES2-Thy1.1 [v-ffLuc]) to

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[†]To whom correspondence should be addressed. E-mail: brabinov@mdanderson.org.

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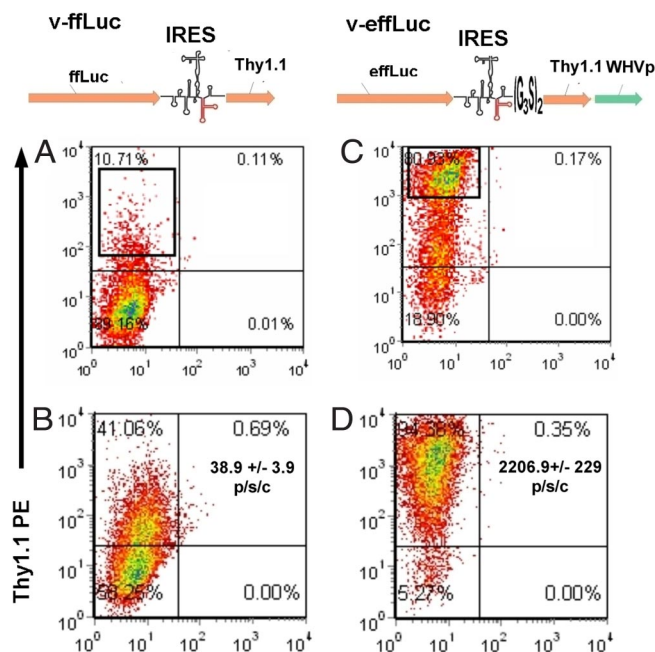


Fig. 1. Comparison of coreporter expression and *in vitro* luminescent activity of T cells expressing standard versus enhanced retroviral firefly luciferase constructs. OT-1 T cells were transduced with the retroviruses v-ffLuc (A and B) or v-effLuc (C and D) (illustrations of vector coding regions are shown above each column) and assessed for Thy1.1 expression 3 days later via flow cytometry (A and C). The boxed areas represent the regions chosen for sorting. 7 days after sorting, transduced T cells were reassessed for longevity of Thy1.1 expression. (B and D) OT-1 T cells (1×10^5) transduced with the indicated retroviruses were assessed for bioluminescent activity before sorting and after addition of D-Luciferin. Signal intensity (photons per second per cell) is in the top right quadrant of B and D.

improve expression of the reporter transgene, in particular for mouse T cells. Thy1.1 was chosen as the coreporter because it differs from its allelic variant Thy1.2 sufficiently to be detected by a specific monoclonal antibody but is not immunogenic in Thy1.2⁺ strains. Mouse CD8 OT-1 TCR tg T cells specific for Ovalbumin peptide 257–264 in the context of H2-kb (26) transduced with v-ffLuc demonstrated weak Thy1.1 (flow cytometry) and luciferase activity (*in vitro*). (Fig. 1A and B). To generate an improved construct, effLuc, we added a (G₃S)₂ linker to the 3' end of the pIRES2 [supporting information (SI) Fig. S1], codons were optimized to the highest frequency of *Mus musculus* and a Woodchuck Hepatitis Virus (WHV) pre element was added downstream of Thy1.1 to augment export of the viral mRNA into the cytosol (27). Construct illustrations are shown in Fig. 1. Codon optimization lead to the modification of three consensus acceptor splice sites (positions 131, 894, and 1004), 9 cryptic acceptor sites (positions 194, 305, 551, 639, 669, 801, 1011, 1112, and 1403) and five cryptic donor sites (positions 334, 349, 1083, 1169, and 1173). One consensus (position 1433) and two cryptic donor splice sites (positions 334 and 1173) from the ffLuc ORF were eliminated by the GeneOptimizer software. Removal of cryptic splice sites was considered important for mouse T cells because excessive aberrant splicing has been reported as a protective tactic used by mouse T cells against retroviral infection (28).

These modifications resulted in >80% of effLuc-transduced T cells expressing Thy1.1 (a 6-fold increase) and a >100-fold increase in Thy1.1 mean fluorescent intensity in the unsorted population. Less than 25% of effLuc OT-1 lost Thy1.1 expression over 7 days of culture compared with >85% for ffLuc. During this time, relative to ffLuc-expressing T cells, luciferase activity

from effLuc-expressing OT-1 T cells increased from 60% to >250% (Fig. S2B). After sorting, we found that effLuc-transduced (compared with ffLuc-transduced) T cells demonstrated a >55-fold increase in intensity [photon flux (the radiance (photons per second) in each px integrated over the ROI area (cm²) $\times 4\pi$ at a given cell number)]. The signal intensity of OT-1 effLuc versus OT-1 ffLuc was $2,206.9 \pm 229$ photons per second per cell and 38.9 ± 3.9 photons per second per cell, respectively (Fig. 1B and D). We also observed a 100- to 110-fold increase in sensitivity (number of cells detected at the same photon flux) (Table S1). Viral integration, measured on days 3 and 5 after transduction, was 4- to 6-fold better for v-effLuc versus v-ffLuc (Fig. S3), thus the increase in luciferase activity is a function of both integration efficiency and expression efficiency.

Because the levels of firefly luciferase expression reported here are superior to previously observed, we compared several parameters of *in vitro* immunological function between sorted v-ffLuc, v-effLuc, and untransduced OT-1 cells. We found no differences in proliferation, cytotoxicity, or IFN- γ production (Fig. S4). Interestingly, in the unsorted population, we found that, at 24 h after transduction, the number of cells in the v-ffLuc population was approximately half that in the v-effLuc. The proliferation curves normalized after 24 h (Fig. S5). Because there was no difference in cell viability, we speculate that the initial difference was due to diminished proliferation of ffLuc OT-1 cells that were forced to handle the expression of ffLuc, which utilizes many rare codons.

effLuc was designed for mouse T cells. Nevertheless, we hypothesized that effLuc should provide enhanced sensitivity when transduced into a variety of tissues. We transduced a panel of human and mouse cell lines to investigate the universal applicability of this technology. We found that enhanced intensity and sensitivity of effLuc versus ffLuc ranged from 10- to 250-fold and 10- to >400-fold, respectively (Table S1).

Imaging effLuc-Expressing T Cells *in Vivo*. C57BL/6 T cells were transduced with each of the two constructs, and T cell numbers ranging from 3 to 30,000 were injected s.c. into C57BL/6 (B6) Albino mice. The limit of detection for T cells transduced with ffLuc was between 10,000 and 30,000 cells. We found that <300 T cells expressing effLuc were required to match the photon flux of 30,000 T cells expressing ffLuc ($2.7 \times 10^5 \pm 1.3 \times 10^4$ versus $8 \times 10^4 \pm 8.3 \times 10^3$ photons/sec/ROI). Thus, effLuc resulted in an increase in sensitivity of 200–400 times (Fig. 2A). Strikingly, we found that as few as three T cells expressing effLuc could be imaged even when acquisition was performed using a binning setting of 4 (512×512 px) (Fig. 2B). These results suggest that effLuc should permit the study of detailed trafficking patterns of even small populations of lymphocytes. Moreover, although there was 40% loss in signal, ≤ 300 effLuc-expressing T cells could still be detected through the pigmented skin of wild type C57BL/6 mice. This will allow for imaging studies in a wide variety of tg and knockout animals not available on albino strains (Fig. S6).

Our *in vivo* images of effLuc T cells were acquired using a small px ρ (binning of 4), allowing us to pinpoint T cell location with better confidence than has been historically possible with ffLuc T cells, which requires larger px ρ settings (binning of 8–16 corresponding to 256×256 and 128×128 px, respectively) to raise the signal above the noise (9, 13, 14).

We examined the difference in sensitivity between T cells transduced with v-ffLuc versus v-effLuc in two different immunocompetent animal models. The first was a model of vaccination whereby T cells migrate into the s.c. tissue of the vaccine site. Ova peptide-pulsed or DCs (10^5 cells) were injected s.c. into the left or right inguinal region, respectively. On the same day, ffLuc- or effLuc-expressing OT-1 T cells ranging from 3×10^4 to 10^6 in

Packaging of Retrovirus. Retroviral vectors were packaged into ecotropic virus as described in ref. 37 with the noted addition of pCLEco (38) (Addgene) to the transfection mix. Supernatants were concentrated between 25 $\times$ and 100 $\times$, using Centricon Plus-20 Centrifugal Filter Units (Millipore), and then titered on NIH 3T3 cells.

Transduction of Mouse T Cells. Splenocytes from C57BL/6 wild type mice or OT-I TCR tg mice were cultured in X-Vivo-15 (Lonza), Normocin (Invivogen), 200 IU Proleukin (Chiron), and 0.1 μ g/ml anti-mouse CD3 (clone 2C11; BD Biosciences). After 22–24 h, retrovirus was added at an MOI of 5 in the presence of polybrene (Sigma) and Lipofectamine 2000 (Invitrogen) at 1.6 μ g/ml and 2 μ g/ml, respectively, and spinfected at 850 $\times$ g for 2 h. The following day, cells were washed and expanded in Alpha-MEM, 10% FBS, Normocin, and 200 IU/ml Proleukin. Cells were stained three days after transduction with anti-mouse Thy1.1 PE (BD-PharMingen) and sorted using a FACSVantage (BD Biosciences).

In Vitro Bioluminescence Assay. Transduced T cells were washed in PBS and 1 $\times$ 10⁵ cells tested for light emission in a 96-well format in triplicate in 200 μ l OPTI-MEM/150 μ g/ml D-Luciferin (Xenogen), using a Topcount apparatus (Perkin-Elmer).

Ova Vaccination Model. To generate CD11b⁺ DCs, Bone marrow was harvested from C57BL/6 wild type mice and seeded in 6-well plates at 1 $\times$ 10⁶/ml (5 ml) in Alpha-MEM, 10% FBS, Normocin, and 200 μ g/ml Flt3-L and cultured at 37°C in 7.5% CO₂. On day 8, GM-CSF was added to 20 μ g/ml. On day 10, LPS was

added at to 100 ng/ml. On day 11, DCs were pulsed with 1 μ g/ml Ova peptide 257–264 (Ovap) and washed, and 1 $\times$ 10⁵ cells were injected into the left inguinal region of C57BL/6 Albino mice. The same number of nonpulsed DCs was injected on the right side. Immediately after DCs injection, 3 $\times$ 10⁴ – 1 $\times$ 10⁶ OT-I TCR tg T cells expressing ffluc or effLuc were injected i.v. via retro-orbital injection. Imaging was performed on days 1, 2, 4, and 5.

Model of Adoptive Immunotherapy for Cancer. EL4 or EG.7 tumor cells (2 $\times$ 10⁶) were injected into the left and right flank of C57BL/6 Albino mice. Seven days later, 3 $\times$ 10⁴ – 10⁶ OT-I TCR tg T cells expressing effLuc were injected IV via retro-orbital injection. Imaging was performed on days 4 and 5. In some cases, tumors were resected and examined for Thy1.1 expression either via standard IHC of formalin fixed paraffin embedded sections or via flow cytometry.

In Vivo Bioluminescence Imaging. Isoflurane-anesthetized animals were imaged using an IVIS 200 system (Xenogen) 8 min after i.p. injection of 2 mg of D-luciferin according to the manufacturer's specifications. Living Image software was used to analyze the data.

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