Longitudinal, Noninvasive Imaging of T-Cell Effector Function and Proliferation in Living Subjects

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Abstract
Adoptive immunotherapy is evolving to assume an increasing role in treating cancer. Most imaging studies in adoptive immunotherapy to date have focused primarily on locating tumor-specific T cells rather than understanding their effector functions. In this study, we report the development of a noninvasive imaging strategy to monitor T-cell activation in living subjects by linking a reporter gene to the Granzyme B promoter (pGB), whose transcriptional activity is known to increase during T-cell activation. Because pGB is relatively weak and does not lead to sufficient reporter gene expression for noninvasive imaging, we specifically employed 2 signal amplification strategies, namely the Two Step Transcription Amplification (TSTA) strategy and the cytomegalovirus enhancer (CMVe) strategy, to maximize firefly luciferase reporter gene expression. Although both amplification strategies were capable of increasing pGB activity in activated primary murine splenocytes, only the level of bioluminescence activity achieved with the CMVe strategy was adequate for noninvasive imaging in mice. Using T cells transduced with a reporter vector containing the hybrid pGB–CMVe promoter, we were able to optically image T-cell effector function longitudinally in response to tumor antigens in living mice. This methodology has the potential to accelerate the study of adoptive immunotherapy in preclinical cancer models. Cancer Res; 70(24); 10141–9. ©2010 AACR.

Introduction
Cellular immunity plays a key role in immunosurveillance of early cancers, prevention of relapse from minimal residual disease, and these roles are being exploited in the design of cancer vaccines (1) and adoptive immune cell therapies (2–4). To achieve full therapeutic potential for adoptively transferred T cells, large numbers need to be injected into patients (5, 6). Current culture techniques needed to reach these numbers can cause negative effects on the functional characteristics of the modified T cells, making them less effective in patients (5). Therefore, it is important to monitor the function of cytotoxic and/or helper T cells prior to and after adoptive transfer.

Currently, cytotoxic T-cell function is measured through cell killing assays on target cells, whereas helper T cells are analyzed for their cytokine production during exposure to target antigens. In living subjects, however, T-cell function is measured by therapeutic outcome (e.g., reduction in tumor volume). Although tumor-specific T cells may have great efficacy in CTL assays, they are often ineffective against target tumor cells when injected in living subjects (reviewed in ref. 7). Research into the cell culture conditions and retargeting of T cells using chimeric T-cell receptors has improved the efficacy of tumor-specific T cells in living subjects. Recently, Morgan et al. (8) found partial success in treating melanoma patients using retargeted T cells partly due to culturing retargeted T cells for less than a week. Various strategies for improving the efficacy of immunoadoptive therapy, such as modifications of cell culture conditions, alterations in T-cell repertoire, and identifying the cell population(s) responsible for tumor eradication, would greatly benefit from an imaging tool to noninvasively visualize the function of transferred T cells in living subjects.

In this study, we pursued this goal by linking the promoters of T-cell activation markers to reporter genes commonly employed for molecular imaging. Markers of T-cell activation, such as interleukin-2 (IL-2) and Granzyme B, are routinely used to gauge the activation status of T cells in cell culture and ex vivo (9). The cytokine IL-2 is produced mainly by CD4+ T cells in the early stages of activation. In contrast, the expression of Granzyme B in CD8+ T cells signifies their full differentiation and acquisition of killing potential (10). The full-length Granzyme B promoter (pGB; ~9 kb) has been well characterized and contains in its distal end binding sites for
the AP-1, CBF, and CRE transcription factors (11, 12) which are induced during T-cell activation (13) and can drive reporter gene expression during T-cell activation at a level sufficient for in vitro detection (12). By coupling this promoter to a bioluminescence reporter gene, we inferred that CTL function could be visualized in live, small animals. However, our initial experiments showed the bioluminescence signal generated by this promoter to be too weak for detection in living animals.

Detection of reporter gene expression from tissue-specific promoters can indeed be difficult in living animals such as mice and rats (14, 15). Several methods have been reported to increase promoter activity to enhance the production of reporter or therapeutic proteins—these include multimerizing promoters, using full-length promoters, creating hybrid promoters, and using 2-step systems based on the yeast 2-hybrid system (14, 16). Hybrid promoters that use the cytomegalovirus enhancer (CMVe), a strong transcriptional enhancer, in conjunction with the tissue-specific promoter (17, 18) and the Two Step Transcription Amplification method (TSTA; see Supplementary Fig. S1 online; ref. 14) have been used to increase reporter gene expression and enable visualization in small animals. These two methods are applied here to the pGB and we compare these two approaches for the ability to visualize T-cell effector function in small animals using bioluminescence imaging (BLI).

Materials and Methods

Animals

Mice were purchased from Jackson Laboratories and housed under pathogen-free conditions at the Stanford Research Animal Facility. All animal procedures were approved by the Institutional Administrative Panel on Laboratory Animal Care at Stanford University (APLAC 9759).

Cells

The tumor cell line EL4 (C57BL/6, H2b, thymoma) and its derivative E.G7 (EL4 cells stably expressing chicken OVA cDNA; ref. 19) were purchased from ATCC.

Lentivirus production and transduction

High-titer lentiviral vectors were produced using a modified version of the protocol presented in Zhang et al. (20). For details, see Supplemental Methods.

Primary murine splenocytes from C57BL/6 and OT1 mice were first depleted of B cells (only for cell culture studies) using Easy Sep Reagent (Stem Cell Tech) then prestimulated for 36 hours with 2.5 µg/mL of concanavalin A (Calbiochem) in cRPMI, 50 µmol/L of βME, 10 ng/mL of IL-7 (R & D Systems). A total of 5 × 10⁶ cells were then transduced for 3 to 4 hours in 500 µL OptiMEM + 8µg/mL polybrene (Sigma) in 12-well tissue culture plates. Cells were then cultured in a total of 2.5 mL per well cRPMI, 50 µmol/L of βME, 10 µg/mL of IL-15 (R & D Systems) 10 U/mL of IL-2 (Chiron), and 50 mmol/L of α-methyl mannoside (Calbiochem) for 48 hours prior to further study. For in vitro studies, murine T cells were activated with anti-CD3 and anti-CD28 antibodies (1 µg/mL; BD Biosciences).

Adoptive transfer

Forty-eight hours posttransduction, cells were labeled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) as previously described (21). A total of 10 × 10⁶ labeled T cells in 200 µL of PBS were injected via the tail vein into congenic albino C57BL/6 mice bearing EL4 (nontarget) and E.G7 (target) tumor cells (1 × 10⁶ cells in the left and right shoulder, respectively) inoculated subcutaneously 6 days prior to adoptive T-cell transfer.

Cell isolation and flow cytometry

E.G7 and EL4 tumors, spleens, and lymph nodes were excised from mice. For E.G7 and EL4 tumors, single-cell suspensions were created by dissociation of tumors with Liberase Blendzymes (Roche) as described (22) and the dissociated tumor cells were stained with various antibodies. Antibodies against CD4, CD8, CD69, Vs2, and Vβ5.1 were purchased from Biolegend. Samples were acquired on a FACScalibur (BD Biosciences). In Figure 2 E and F, mean fluorescence intensity (MFI) for green fluorescent protein

![Figure 1. CMVε-coupled promoter significantly amplifies reporter gene expression compared with TSTA and full-length CMV promoter in primary murine splenocytes. A, map of pGBTSTA and pGBeFG lentiviral vectors. B, splenocytes were transduced as described in Materials and Methods with lentiviral vectors carrying the 828 bp pGB driving expression of fluc–egfp fusion, pGB in the TSTA system (pGBTSTA) driving fluc expression, pGB with CMVe (pGBe) driving expression of fluc–egfp (pGBeFG) or fluc2-tomato (pGBeLT), or pCMV driving expression of fluc–egfp (pCMVFG). Forty-eight hours after transduction, cells were stimulated with anti-CD3 and anti-CD28 antibodies or left unstimulated. Twenty-four hours later, cells were harvested and analyzed for luciferase activity using an in vitro luciferase assay. Data are normalized to total protein per sample. n = 3, Student’s t test. Asterisks above bars denote statistical significance; *, P < 0.05.](image-url)
(GFP) was quantified by gating on either CD4^+ or CD8^+ cells. In Figures 3 and 4, cells were gated on CFSE and CD8 double positive cells. Data were analyzed using FlowJo Software (TreeStar).

**Statistical analysis**

Two-tailed Student’s t tests were used to evaluate the significant difference or P values between samples in Figures 1 and 2. Standard deviations of mean values are depicted as error bars in Figures 1 and 2. ANOVA and the Bonferroni multiple comparison test (α = 0.05) were used to evaluate significant difference in Figures 3 and 4. Standard error of mean (SEM) is depicted as error bars in Figures 3 and 4.

**Results**

**Two transcription enhancer strategies increase the activity of the ~828 bp pGB**

In vitro, the ~828 bp pGB drives low firefly luciferase (fluc) reporter gene expression when transiently transcted into primary murine splenocytes and activated with anti-CD3 and anti-CD28 antibodies (1 μg/mL) as compared with the CMV promoter [pCMV; 250 relative light units (RLU)/μg of protein for pGB compared with 9,930 RLU/μg for pCMV; see Supplementary Fig. S2A]. A similar comparison was observed in transiently transfected Jurkat cells after normalization to control vector (see Supplementary Fig. S2B). A minimum of 10,000 RLU/μg of protein is needed in cell culture to detect fluc activity in living animals (data not shown). To increase reporter gene expression driven by pGB, we created two lentiviral vectors that carried either pGB in the TSTA system to drive fluc expression (pGBTSTAF) or a hybrid promoter, in which the CMVe was cloned upstream of pGB (pGBe) to drive the expression of a fluc–EGFP (FG) fusion protein (pGBeFG; Fig. 1A). As controls, we also created vectors in which FG expression was under control of pGB only (pGBFG) or pCMV (pCMVFG). Splenocytes were transduced with lentiviral vectors carrying pGBFG, pGBTSTAF, pGBeFG, or pCMVFG. Upon activation of splenocytes using anti-CD3 and anti-CD28 antibodies, fluc activity from cells transduced with pGBeFG was 10-fold higher than cells transduced with pGBFG (P < 0.05) and 5-fold higher than pGBTSTAF. As all the lentiviral vectors we generated were from the same backbone and they transduced primary splenocytes with equal efficiency (35%; Sup-
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LT activity, we replaced the FG reporter gene with a fusion gene, change in transduction efficiency. To further elevate reporter expression was due to differences in the promoters and not a complementary Fig. 3), we concluded the difference in luciferase expression was due to differences in the promoters and not a change in transduction efficiency. To further elevate reporter activity, we replaced the FG reporter gene with a fusion gene, LT, composed of a synthetic firefly luciferase (luc2, see Materials and Methods) and the tandem tomato red fluorescent protein to create pGBeLT (Fig. 1B). In subsequent experiments, we transduced splenocytes with the pGBeLT vector.

Hybrid promoter maintains characteristics of the endogenous pGb for driving Granzyme B expression

In primary murine splenocytes transduced with the pGBeG, the MFI peaked at 72 hours of sustained activation with anti-CD3 and anti-CD28 antibodies (Fig. 2A). Granzyme B protein expression in the same splenocytes transduced with pGBeG (Fig. 2B and C) directly correlated with fluorescence expressed from reporter gene ($R^2 = 0.92$; Fig. 2D). To further characterize the hybrid promoter by determining its expression in T-cell subsets, we transduced B cell–depleted murine splenocytes with pGBeG and analyzed cells by flow cytometry. eGFP expression was detected in both activated CD4$^+$ and CD8$^+$ T cells (Fig. 2E); however, eGFP expression was significantly higher in CD8$^+$ T cells than in CD4$^+$ T cells ($P = 0.008$). Also, splenocytes transduced with pGBeG had a 7-fold higher eGFP expression when compared with splenocytes transduced with the pGBTSTA-eGFP lentiviral vector ($P = 0.0004$; Fig. 2E and F).

Activated T cells are detected in target tumor using bioluminescence imaging

As proof of principle for the detection of T-cell activation in living animals, we used OT1 transgenic mice whose CD8$^+$ T cells express a T-cell receptor specific for a chicken ovalbumin peptide (residues 257–264) in the context of H2-K$^b$ (23). Purified CD8$^+$ T cells from OT1 transgenic mice were transduced with the pGBeLT lentiviral vector. Transduced T cells were then injected via the tail vein into congenic albino C57BL/6 mice bearing 6-day-old target (E.G7) and nontargeted (EL4) tumors respectively ($n = 2$).
respectively. These mice are congeneric to wild-type C57BL/6 mice. Twenty-four hours after adoptive transfer, bioluminescence signal was detected in the area of the E.G7 tumor but not where EL4 cells had been injected (Fig. 3A). Similar to the cell culture kinetics, this signal peaked 72 hours postadoptive transfer (Fig. 3B) and was significantly larger than the signal from the EL4 tumor (P = 0.009). In tumor-bearing animals in which syngeneic polyclonal, nontargeted T cells labeled with pGBeLT were adoptively transferred, no signal could be detected from the sites of EL4 or E.G7 tumor grafts at any time during the study period (Fig. 3B), even though T cells could be detected in these tumors when they were transduced with a lentiviral vector carrying a constitutively active promoter (see Supplementary Fig. S4 online). The peak signal intensity from the E.G7 tumor coincided with tumor regression (Fig. 3C). CD8⁺ T cells isolated from E.G7 tumors had a higher percentage of cells expressing CD69 than CD8⁺ T cells isolated from EL4 tumors as early as 24 hours postadoptive transfer and remained higher at 72 and 120 hours postadoptive therapy (Fig. 3D). In the draining lymph nodes, we were not able to detect any fLuc expression until day 3 postadoptive therapy (Supplementary Fig. S5A online) even though we were able to detect OT1 T cells 24 hours postadoptive therapy in draining lymph nodes from both tumors by flow cytometry (Supplementary Fig. S5B-D online).

**Bioluminescent signal from target tumor is due to CTL effector function and not T-cell proliferation**

To determine whether the signal in the E.G7 tumor was a result of CTL effector function or stemmed from a mere increase in the number of intratumoral T cells, we compared...
the bioluminescence signal from pGBeLT transduced T cells to the signal from T-cells transduced with a lentiviral vector carrying a fusion gene, LG, composed of luc2 and egfp driven by the constitutive human Ubiquitin C promoter (pUbiLG). The human Ubiquitin C promoter (pUbi) is not influenced by T-cell activation through the T-cell receptor (data not shown) and has been used to study T-cell proliferation in a tumor model (24, 25). Because the spleen serves as a site of T-cell filtration and is not a site of CTL effector function, we did not expect to detect bioluminescence from pGBeLT transduced OT1 T cells in the spleens of tumor-bearing mice. We observed an increase in the signal from the spleen in tumor-bearing mice injected with pUbiLG transduced OT1 T cells (Supplementary Fig. S6 online). Because both pGB and pUbi have different promoter strength, we represented the bioluminescent signal from both promoters as fold increase from day 0 to compare reporter gene expression from both promoters on the same graph (Fig. 4A). There was a significant difference between the bioluminescent signal from pGB and pUbi transduced OT1 T cells in the spleen. The fold increase in bioluminescence from pUbiLG transduced T cells in the spleen increased over the course of the experiment. In contrast, signal from pGBeLT transduced T cells in the spleen did not change significantly (P > 0.05) through the course of the experiment (Fig. 4B). This was confirmed with flow cytometric analysis on spleens from mice that received adoptively transferred OT1 T cells that were transduced with the lentiviral vector and labeled with CFSE (Fig. 4B). CFSE-labeled splenocytes of non–tumor-burdened mice showed only 1 round of cell division and no CD69 expression. CFSE-labeled cells isolated from splenocytes of tumor-carrying mice had undergone at least 4 rounds of cell division, as indicated by the decrease of CFSE label. However, they did not show any expression of CD69. In the tumor, we would expect increased pUbiLG signal before the peak of pGBeLT signal because of the dynamics T-cell response, in which proliferation precedes differentiation into effector cells (reviewed in ref. 26). There was a statistically significant increase in the fold induction between day 3 and day 2 in T cells transduced with pUbiLG in the target tumor (P = 0.003). However, this occurred at a similar time as the peak fold induction in pGBeLT transduced T cells in the target tumor (Fig. 4C). In the target tumor, by day 5, most of the T cells had complete loss of CFSE (Fig. 4D), indicating that they had undergone several rounds of division. In addition, the signal from pGBeLT transduced T cells displays similar kinetics as shown in Figure 4, after normalizing the signal by the number of OT1 T cells isolated from the spleen or tumor (Supplementary Fig. S7 online).

**Discussion**

In this study of T-cell receptor–dependent T-cell activation *in vivo*, we used the minimal pGB for expressing reporter genes for *in vivo* imaging because it is well characterized (11, 12), and there has also been success using the promoter’s human homologue to detect activated T cells in transgenic mice via flow cytometry (27). Furthermore, it can be detected by flow cytometry by staining for CD107a expression, which has been used to detect antigen-specific CD8+ T cells (28) and NK (natural killer) cell activity (29). However, when we transiently transfected primary T cells with pGB-Fluc, the amount of reporter gene expression was weak compared with that of pCMV-Fluc and insufficient for long-term *in vivo* imaging (data not shown). We therefore chose two enhancer strategies, CMVe and TSTA, to increase pGB activity.

Both CMVe and TSTA elements enhanced pGB activity, with CMVe being superior. One of its drawbacks was the relatively high background bioluminescence emitted from cells transduced with luciferase reporters driven by pGB; however, it responded best to activation of the T cells with a 10-fold gain in signal. This strong response is most likely due to the presence of transcription factor binding sites for CREB (cyclic AMP-response element-binding protein) and NF-kB in CMVe (30, 31). Both of these transcription factors are upregulated during T-cell activation (32, 33). One concern may be that the addition of CMVe to a tissue-specific promoter might alter the promoter’s properties such as its kinetics of activation and cell specificity. In our hands, addition of CMVe did not alter activation kinetics of pGB-driven gene expression: peak gene expression by both the hybrid promoter and endogenous pGB occurred at 72 hours. Surprisingly, the reporter gene expression was detected not only in CD8+ T cells but also in CD4+ T cells. This could be due to lack of regulatory sequences that are located further upstream of the minimal promoter (34). However, cytotoxic CD4+ T cells have been described before, and Hanson et al. (35) also observed Granzyme B mRNA in some CD4+ T cell clones isolated from their transgenic mice. Granzyme B mRNA was also found in CD4+ T cells by other groups (36–38) including regulatory T (Treg) cells (39). Others have shown that the use of CMVe to increase weak promoter activity retains cell specificity (40, 41). Therefore, detection of reporter gene activity in CD4+ T cells could be due to expression from the minimal pGB and not due to the addition of CMVe.

As proof of principle, we used purified CD8+ T cells from OT1 transgenic mice to detect T-cell activation *in vivo* as measured by luciferase expression driven by pGB. Granzyme B promoter activity peaked 72 hours post T-cell transfer in the target tumor (E.G7), even when tumors were implanted 6 days after adoptive transfer of T cells (data not shown). This correlated with our *in vitro* measurements of Granzyme B protein expression as assessed by Western blot. In addition, the peak signal for pGBeLT-transduced OT1 T cells coincided with tumor regression. A small signal could be detected in the parental, nontarget tumor (EL4) and we were able to detect OT1 T cells in these tumors by flow cytometry. However, the signal from pGBeLT transduced OT1 T cells in the EL4 tumors did not increase significantly over the course of the study and the loss of CFSE label in these intratumoral T cells could be due to activated/proliferating T cells in the circulation that were trapped in the tumor at the time of harvest (42). This is supported by the observation that OT1 T cells in the spleen and in EL4 tumors had undergone the same number of cell divisions.

Other groups have reported visualization of T-cell activation in living subjects (25, 43, 44). Ponomarev et al. (43) used a
synthetic promoter consisting of the multiple repeats of the binding site for the transcription factor nuclear factor of activated T cells (NFAT), which is a commonly found sequence in many promoters that are triggered during T-cell activation. This promoter was used to drive the expression of Herpes Simplex Virus Type 1 Thymidine Kinase (HSV1-tk) for micro positron emission tomography imaging (µPET). This study enabled noninvasive imaging of T-cell activation using tumors derived from transduced Jurkat T cells and direct activation through the use of stimulatory antibodies against CD3 and CD28. However, the authors did not proceed to further noninvasively detect T-cell activation in response to an actual tumor or infection. Shu et al. (25) visualized T-cell activation by following the proliferative response of T cells to actual tumors using reporter genes under the control of the constitutively expressed Ubiquitin C promoter (pUbi). As TCR-dependent T-cell activation leads to proliferation (26, 45), we wanted to ensure that the signal increase from pGBeLT-transduced T cells in the target tumor was in fact due to T-cell effector function and not to a simple increase in cell number. For this, we determined if the kinetics of pUbiLG and pGBeLT transfected T cells in the target tumor hold true to T-cell response dynamics. It is not clear if there is a significant amount of proliferation prior to T cells developing effector function. In our study, both signals peaked at similar times in the E.G7 tumors. Other studies using the OT1 mouse model have shown that only cells that had lost CFSE label (cells that had undergone several rounds of proliferation) in the E.G7 tumors expressed Granzyme B (42, 46). Therefore, in the target tumor, the kinetics may be linked. On the single cell level, the events of TCR-dependent activation and proliferation occur within the first 24 hours of antigen recognition (47); but in our study, proliferation as measured by an increase in signal from pUbiLG transfected T cells was not visualized completely until 48 hours postadoptive transfer. This is most likely due to the limit of detection of BLI (48), in which a critical number of cells must be focalized to be detected by the CCD (charge coupled device) camera. These initial events of T-cell dynamics occur in the draining lymph nodes (26); however, we were not able to detect signal in lymph nodes due to the limit of detection of BLI and at later time points due to overlapping signal from the tumors. However, we were able to observe signal from the draining lymph nodes ex vivo and we were able to detect OT1 T cells by flow cytometry. Twenty-four hours postadoptive therapy, we were able to detect activated OT1 T cells by flow cytometry in the draining lymph nodes of E.G7 tumors; however, we did not observe signal from pGBeLT transduced T cells in these lymph nodes until day 3. This finding is supported by the fact that Granzyme B is a marker for T-cell effector function (28) and in a previously published study, Granzyme B–expressing T cells were also only detected in lymph node T cells that had undergone several rounds of division (46). The spleen is normally not a site where T cells acquire effector function and in our model should not show an increase in signal from pGBeLT-transduced T cells and therefore serves as a control for visualizing T cells as they acquire effector function. We did not observe any statistical increase in signal from pGBeLT-transduced T cells in tumor-challenged mice and OT1 T cells isolated from the spleens did not show expression of CD69. As the spleen is a lymphoid filtration organ, we did observe an accumulation of OT1 T cells transduced with pUbiLG and as these cells did have a loss of CFSE label, they could be proliferating cells that had migrated from the draining lymph nodes and entered the circulation (42, 46). This spatially and temporally defined bioluminescence signal, together with our flow cytometry data showing CD69 expression on the tumor-infiltrating T cells, indicates that we were most likely visualizing T cells as they acquired effector function.

In summary, we have created a hybrid promoter on the basis of CMV and pGB that significantly increases reporter gene expression over pGB alone and also correlates with endogenous Granzyme B protein production. Using a bioluminescent reporter, the level of transcriptional activity from this hybrid promoter upon T-cell stimulation in vivo is sufficient to be detected in small living animals. We believe that this method will be applicable over a broad range of animal models that benefit from functional, noninvasive visualization of CTL function such as immune response to infection and adoptive immunotherapy. By replacing pGB with other promoters turned on during T-cell activation, such as the IL-2 promoter, IFN-γ promoter, or NFAT synthetic promoter (44), it should be feasible to visualize different stages of T-cell function and differentiation noninvasively in vivo and in vitro; however, kinetics of activation are dependent on disease model (44). In addition, by replacing the bioluminescence reporter genes with reporter genes for PET imaging, it should be feasible to monitor T-cell activation in patients. Our laboratory has recently imaged T-cell localization using PET reporter gene imaging and a constitutive promoter in glioma patients undergoing T-cell therapy (49). Recently, Radu et al. developed a small-molecule PET probe capable of imaging lymphoid organs and immune activation (50) which may be useful to image inflammation (51) and leukemic cells, but is somewhat limited due to tumor uptake of the PET probe and cannot discern tumor from activated T cells. Therefore, imaging of tumor-targeted T-cell activation may eventually be possible in patients by utilizing a T-cell activation promoter.

Disclosure of Potential Conflicts of Interest

S.S. Gambhir is founder of CellSight Inc. which develops and markets strategies for imaging of cell trafficking. C.H. Contag has financial interest in Caliper Life Sciences, an in vivo imaging company. The other authors disclosed no potential conflicts of interest.

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