

Video Article

In vivo Dual Substrate Bioluminescent Imaging

Michael K. Wendt¹, Joseph Molter¹, Christopher A. Flask¹, William P. Schieman¹

¹Case Comprehensive Cancer Center, Case Western Reserve University

Correspondence to: William P. Schieman at william.schieman@case.edu

URL: <https://www.jove.com/video/3245>

DOI: [doi:10.3791/3245](https://doi.org/10.3791/3245)

Keywords: Medicine, Issue 56, firefly luciferase, Renilla Luciferase, breast cancer, metastasis, Smad

Date Published: 10/11/2011

Citation: Wendt, M.K., Molter, J., Flask, C.A., Schieman, W.P. *In vivo* Dual Substrate Bioluminescent Imaging. *J. Vis. Exp.* (56), e3245, doi:10.3791/3245 (2011).

Abstract

Our understanding of how and when breast cancer cells transit from established primary tumors to metastatic sites has increased at an exceptional rate since the advent of *in vivo* bioluminescent imaging technologies¹⁻³. Indeed, the ability to locate and quantify tumor growth longitudinally in a single cohort of animals to completion of the study as opposed to sacrificing individual groups of animals at specific assay times has revolutionized how researchers investigate breast cancer metastasis. Unfortunately, current methodologies preclude the real-time assessment of critical changes that transpire in cell signaling systems as breast cancer cells (i) evolve within primary tumors, (ii) disseminate throughout the body, and (iii) reinitiate proliferative programs at sites of a metastatic lesion. However, recent advancements in bioluminescent imaging now make it possible to simultaneously quantify specific spatiotemporal changes in gene expression as a function of tumor development and metastatic progression *via* the use of dual substrate luminescence reactions. To do so, researchers take advantage for two light-producing luciferase enzymes isolated from the firefly (*Photinus pyralis*) and sea pansy (*Renilla reniformis*), both of which react to mutually exclusive substrates that previously facilitated their wide-spread use in *in vitro* cell-based reporter gene assays⁴. Here we demonstrate the *in vivo* utility of these two enzymes such that one luminescence reaction specifically marks the size and location of a developing tumor, while the second luminescent reaction serves as a means to visualize the activation status of specific signaling systems during distinct stages of tumor and metastasis development. Thus, the objectives of this study are two-fold. First, we will describe the steps necessary to construct dual bioluminescent reporter cell lines, as well as those needed to facilitate their use in visualizing the spatiotemporal regulation of gene expression during specific steps of the metastatic cascade. Using the 4T1 model of breast cancer metastasis, we show that the *in vivo* activity of a synthetic Smad Binding Element (SBE) promoter was decreased dramatically in pulmonary metastasis as compared to that measured in the primary tumor⁴⁻⁶. Recently, breast cancer metastasis was shown to be regulated by changes within the primary tumor microenvironment and reactive stroma, including those occurring in fibroblasts and infiltrating immune cells⁷⁻⁹. Thus, our second objective will be to demonstrate the utility of dual bioluminescent techniques in monitoring the growth and localization of two unique cell populations harbored within a single animal during breast cancer growth and metastasis.

Video Link

The video component of this article can be found at <https://www.jove.com/video/3245/>

Protocol

1. Stable Expression of CMV-Driven Renilla Luciferase

1. Transfection and selection of a stable clonal population is the preferred method for expression of this renilla luciferase reporter. This approach yields a more consistent and uniform renilla luciferase expression following the subsequent introduction of additional secondary reporter constructs (e.g., firefly luciferase or fluorescent proteins).
2. Transfect malignant cells of interest with the expression vector encoding renilla luciferase, such as pcDNA3.1-Hygro or another plasmid harboring a selectable marker.
3. Following transfection, place the transfectants under an optimized antibiotic concentration for several days-to-weeks to facilitate the isolation of individual colonies, which are subsequently isolated individually and subcultured.
4. Select ≥10 individual renilla luciferase-expressing colonies to monitor the extent of renilla expression.
5. Colonies expressing high quantities of renilla luciferase are subsequently subjected to functional analyzes to ensure that the (i) pathophysiological properties of their parental counterparts are retained, and (ii) renilla expression values do not deviate or change over time. These steps are absolutely essential to avoid isolating and studying clonal variants/deviants, and to validate proper integration of the renilla construct into the genome.
6. Once a stable transfectant is isolated and verified, one can remove the selective pressure and be assured that the expression of renilla luciferase will remain constant over extended lengths of time both *in vitro* and *in vivo*.

2. Expression, Selection, and Functional Verification of Inducible Promoter-Driven Firefly Luciferase

1. Subclone the promoter of interest into a pGL4-luciferase reporter plasmid harboring a selectable marker (e.g., puromycin) that is distinct from that used to select for stable renilla expression (e.g., hygromycin).
2. Transfect malignant cells as in Step 1, and subsequently select for a stable polyclonal population of firefly luciferase-expressing cells. Because the location where a report gene integrates into the genome can elicit erroneous effects on its expression and regulation, it is strongly recommended to select for polyclonal populations of firefly luciferase-expressing cells as opposed to clonal populations to ensure that (i) reporter gene expression more accurately reflects that of the endogenous gene in parental cells; and (ii) integration effects on gene expression are averaged and diminished across the heterogeneous cell population.
3. Malignant cells engineered to stably express both renilla and firefly luciferases are collectively referred to as dual bioluminescent reporter cells, or **DBR** cells.
4. Using traditional *in vitro* cell-based luciferase reporter gene assays, verify that the DBR cells regulate firefly luciferase expression, either positively or negatively, in a manner concordant with that observed in parental cells transiently transfected with these vectors. Likewise, verify that CMV-driven expression of renilla is not regulated by various cell stimulations or treatment conditions.
5. To do so, culture DBR and parental cells in 24-well plates, and subsequently transiently co-transfect parental cells with the original CMV-renilla and promoter-firefly constructs used to generate DBR cells.
6. Afterward, treat DBR and transfected parental cells with factors or pharmacological agents known to regulate the promoter of interest, and subsequently quantitate firefly and renilla luminescence using the Promega Dual Luciferase Assay Kit.

3. Establishing 4T1 Primary Mammary Tumors

1. Metastatic 4T1 mammary carcinoma cells that were found to lack adventitial rodent pathogens were engineered to stably express a CMV-driven renilla luciferase (pcDNA3.1-CMV-renilla luciferase-hygro) and a SBE-driven firefly luciferase (pGL4.2-SBE-firefly luciferase-puro) as described above. Orthotopic engraftment of these breast cancer cells results in the formation of spontaneous metastases primarily in the lungs.
2. 4T1 cells should not be allowed to reach confluence during their propagation in traditional 2-dimensional tissue culture systems, and they should be passaged the day before their *in vivo* inoculation.
3. 4T1 cells should be dissociated by trypsinization, washed thoroughly in growth media, and diluted in PBS to a concentration of 2×10^5 cells/ml and immediately stored on ice.
4. Female Balb/C mice (4-6 weeks old) should be anesthetized with an induction dose of 3% isoflurane and maintained under anesthesia at a dose of 1% isoflurane. Prepare the injection site by swabbing with 70% isopropyl alcohol. Using a forceps, gently grasp and lift the 4th inguinal mammary pad. Carefully, place a 27.5 gauge needle bevel side up and inject into the mammary fat pad directly under the nipple, taking special care not push the needle into the abdominal cavity. Release the gland and slowly inject 50 μ l of the cell suspension (1×10^4 cells) into the mammary fat pad.

4. Initial Dual Luminescent Imaging

1. Immediately after engrafting 4T1 cells onto the mammary fat pad and while the mice are still anesthetized, inject 100 μ l of RediJect Coelenterazine into the lateral tail vein. This is the optimal substrate concentration recommended by the vendor and the best tolerated by the animal.
2. Immediately place the mouse into the isoflurane nose cone within the IVIS-200 imaging system and acquire a 0.5-1 minute luminescent image. Efficiency between injection and image acquisition is very important, as the signal for renilla luciferase drops precipitously ~30 seconds post-injection. Place the mouse back into its cage and allow it to recover for ≥ 1 hour, which ensures that any residual renilla luciferase signal has dissipated and that the mouse has fully recovered from the anesthesia.
3. Administer 150 mg/kg of D-luciferin potassium salt *via* I.P. injection and wait 5 minutes. This is the optimal concentration of D-luciferin, which provides a stable luminescent signal for 5-15 minutes following its injection into test animals. Anesthetize the mouse using isoflurane and replace in the IVIS-200, taking care to position the animal in a very similar position relative to the original renilla acquisition. The overall intensity of this firefly signal will depend on the activity of the promoter of interest, and as such, visualizing firefly luciferase activity may require extended acquisition times.
4. Using the IVIS Living Image software set to "photons" mode, establish values for both the renilla and firefly acquisitions as a means to establish baseline relative luminescence ratios (RLR).

5. Longitudinal Luminescent Imaging

1. 4T1 cells are highly aggressive, such that inoculation of 1×10^4 cells typically leads to palpable tumor formation within 1 week, and to the lethality of the animal within 4-5 weeks. 4T1 tumors have an inherent propensity ulcerate in week 4. The growth and maintenance of ulcerated tumors may require separate IACUC approval. The 4T1 tumor studies shown herein have been approved by the IACUC at Case Western Reserve University.
2. As described above, mice should be injected weekly with RediJect Coelenterazine to monitor general tumor growth and metastasis, as well as with D-luciferin to monitor pathway-specific signaling during various stages of tumor development. Take care each time to ensure the renilla luciferase signal has completely dissipated prior to acquisition of the firefly luciferase signal.
3. As 4T1 tumors develop and progress, renilla luciferase acquisitions should be normalized to the initial renilla values measured at the time of tumor cell inoculation as a means to normalize and track primary tumor growth. More importantly, calculating RLR values over time will establish the temporal regulation of individual signaling systems relative to tumor growth and progression.

- Overt pulmonary metastasis becomes apparent within 3-4 weeks following 4T1 cell engraftment onto the mammary fat pad. Comparing pulmonary RLR values *versus* those calculated for the primary tumor establishes the spatial regulation of individual signaling systems relative to tumor growth and metastasis.

6. Dual Bioluminescent Imaging of Two Unique Cell Types in a Single Animal

- Engineer one breast cancer cell line to stably expression CMV-driven renilla luciferase as described above. Repeat this engineering process using CMV-driven firefly luciferase on a second distinct breast cancer cell line.
- Herein we mixed renilla luciferase-expressing 4T1 cells with their nonmetastatic and isogenic firefly luciferase-expressing 4T07 counterparts^{10, 11}. Varying ratios of these mixed breast cancer cell populations are subsequently injected into the mammary fat pad as described above.
- Immediately acquire both firefly and renilla luciferase images to establish initial RLRs representative of a given inoculated cell mixture.
- Longitudinal dual bioluminescent imaging will visualize and track changes in the cellular composition within the primary tumor, as well as the spatiotemporal metastasis of each breast cancer derivative relative to tumor growth and progression.
- Alternatively, individual breast cancer cell populations can be inoculated into different locations in the mouse (*i.e.* the right and left abdominal mammary fat pads) to assess the systemic influences these two populations exhibit over one another during various stages of tumor growth and metastasis.

7. Representative Results:

A major strength of dual bioluminescent imaging lies in the fact that each image is internally consistent and controlled, such that the continued signals derived from the primary tumor function as a important metric to gauge the overall success or failure of each renilla and firefly acquisition. This aspect of the imaging procedure is particularly important during acquisitions of the activity of renilla luciferase, whose Coelenterazine substrate is highly sensitive to oxidation that results in its ability to auto-luminescence. Figure 1A shows an example of this side effect, which immediately manifests as nonspecific luminescent signals derived from the intestinal tract, not the established primary tumor. Typically, these nonspecific Coelenterazine signals transpire following failed intravenous injections of this renilla luciferase substrate. However, once robust primary tumor-derived renilla signals have been obtained (Figure 1B, *left image*), it is safe to proceed in capturing pathway-specific signaling measurements derived from imaging firefly luciferase (Figure 1B, *right panel*), whose D-luciferin substrate is highly stable upon I.P. injection and produces negligible levels of background auto-luminescence.

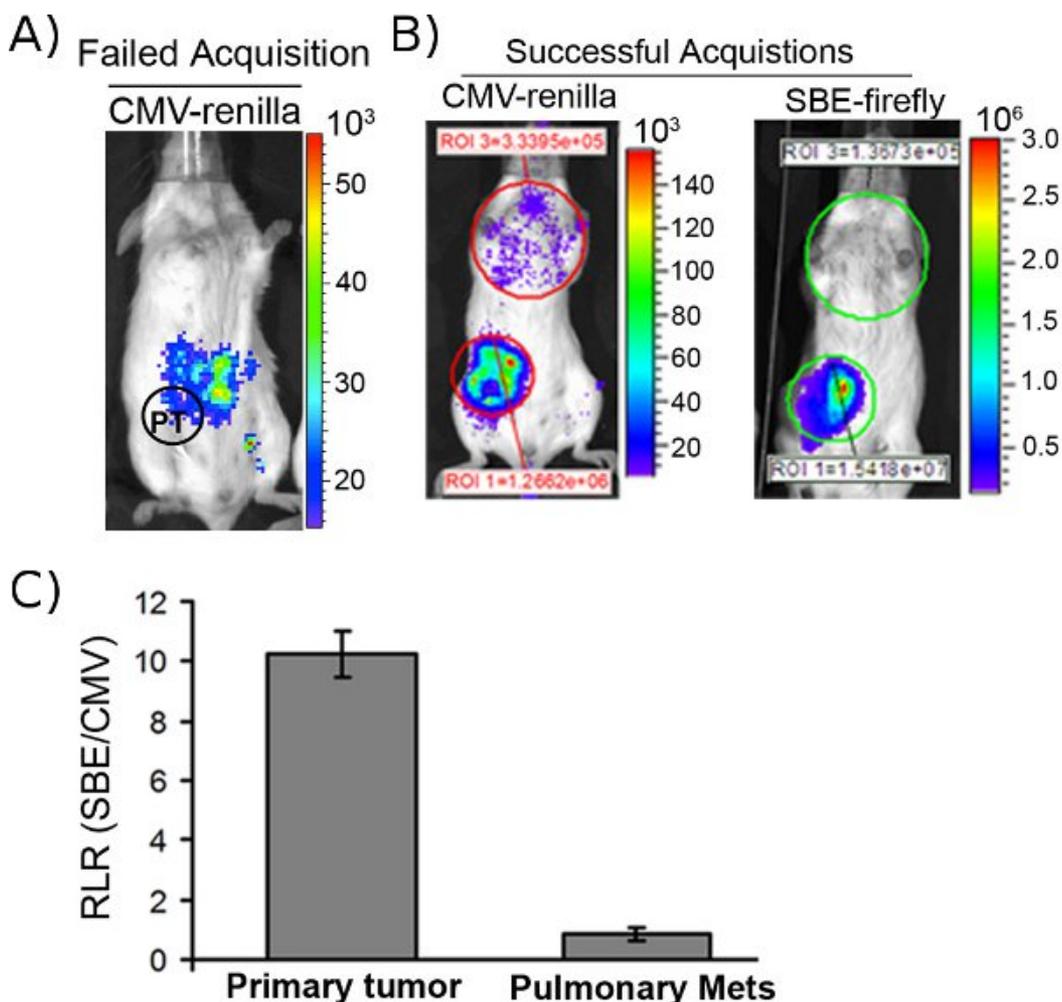


Figure 1. Acquiring renilla and firefly-derived bioluminescent images to calculate *in vivo* RLRs. **(A)** An example of a failed I.V. injection of Coelenterazine resulting in nonspecific signal from the intestinal tract. Circle and PT indicate the approximate location of the primary tumor. **(B)** A successful renilla acquisition of a mouse bearing a 4T1 primary tumor and pulmonary metastases (left panel; 4wk after fat pad engraftment). The corresponding firefly acquisition allows for calculation of RLRs from both the primary tumor and its pulmonary metastases. **(C)** Graphical representation of the primary tumor and pulmonary metastasis RLRs calculated from mice shown in panel B (n=4).

Discussion

The absolute power of bioluminescent imaging techniques lies in their ability to quantify tumor growth and metastasis in complex longitudinal studies, which herein involved the use of aggressive 4T1 breast cancer cells. Because these procedures rely on the stable integration of both luciferase reporter constructs, these techniques can be readily adapted and translated to other cancer cell lines of varying tumor latencies and metastatic capabilities. Similar to the results shown herein, calculating specific RLRs within slowly developing primary tumors and their eventual metastases allows for the real-time spatiotemporal identification of specific signaling events that transpire during the metastatic progression of tumors irrespective of their duration of latency. Upon identification of specific promoter regulatory events, it is important to excise both the primary tumor and its metastatic lesions for the performance of standard immunohistochemical and differential gene expression analyses to verify similar regulation of the endogenous gene and/or protein.

Technically speaking, the primary challenge of dual bioluminescent analyses lies in the relatively short duration of the renilla luciferase signals. As such, this imaging technique requires significant optimization of the tail vein injection procedure, as well as the immediate imaging of individually injected animals, a process that is time consuming and somewhat inefficient relative to imaging firefly luciferase. Recently, Promega introduced "Viviren," which represents a second generation luciferase substrate whose sites of oxidation are blocked by esterification until this molecule gains entry into cells, at which point the molecule is rapidly de-esterified. Collectively, this novel substrate effectively lowers auto-luminescence and nonspecific modification and/or degradation coupled to renilla-induced auto-luminescence¹². In doing so, this new renilla luciferase substrate produces brighter luminescent signals; however, the excessive costs associated with the acquisition and use of "Viviren" have provided relatively few studies necessary to evaluate the overall utility of this substrate in dual bioluminescent analyses. Finally, the sensitivity of any bioluminescent assay is critically dependent upon the CCD camera operant in acquiring individual light units. Indeed, as these technologies improve, we foresee a point in the future where our ability to visualize complex light-mediated bioluminescent reactions may transpire efficiently in free moving animals¹³.

Disclosures

No conflicts of interest declared.

Acknowledgements

W.P.S. was supported in part by grants from the National Institutes of Health (CA129359), the Susan G. Komen for the Cure Foundation (BCTR0706967), the Department of Defense (BC084561), and the Seidman Cancer Center, while M.K.W. was supported by a fellowship from the American Cancer Society (PF-09-120-01). Additional support for this work was provided by the Case Center for Imaging Research, the Case Comprehensive Cancer Center (P30 CA43703), and the Cystic Fibrosis Center (P30 DK027651).

References

1. Wetterwald, A., *et al.* Optical imaging of cancer metastasis to bone marrow: a mouse model of minimal residual disease. *Am. J. Pathol.* **160**, 1143-1153 (2002).
2. Wendt, M.K., Drury, L.J., Vongsa, R.A., & Dwinell, M.B. Constitutive CXCL12 Expression Induces Anoikis in Colorectal Carcinoma Cells. *Gastroenterology*. **135**, 508 (2008).
3. Wendt, M. & Schiemann, W. Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-beta signaling and metastasis. *Breast Cancer Research*. **11**, R68 (2009).
4. Korpai, M., *et al.* Imaging transforming growth factor-beta signaling dynamics and therapeutic response in breast cancer bone metastasis. *Nat Med.* (2009).
5. Giampieri, S., *et al.* Localized and reversible TGFbeta signalling switches breast cancer cells from cohesive to single cell motility. *Nat Cell Biol.* **11**, 1287-1296 (2009).
6. Giampieri, S., Pinner, S., & Sahai, E. Intravital imaging illuminates transforming growth factor beta signaling switches during metastasis. *Cancer Res.* **70**, 3435-3439 (2010).
7. DeNardo, D.G., *et al.* CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell.* **16**, 91-102 (2009).
8. Wyckoff, J. *et al.* A Paracrine Loop between Tumor Cells and Macrophages Is Required for Tumor Cell Migration in Mammary Tumors. *Cancer Res.* **64**, 7022-7029 (2004).
9. Kim, M.Y., *et al.* Tumor self-seeding by circulating cancer cells. *Cell.* **139**, 1315-1326 (2009).
10. Aslakson, C.J. & Miller, F.R. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Res.* **52**, 1399-1405 (1992).
11. Wendt, M.K., Smith, J.A., & Schiemann, W.P. Transforming growth factor-beta-induced epithelial-mesenchymal transition facilitates epidermal growth factor-dependent breast cancer progression. *Oncogene*. **29**, 6485-6498 (2010).
12. Hawkins, E., Unch, J., Murhpy, N., Vidugiriene, J., Scurria, M., Klaubert, D., & Wood, K. Bright Light, No Lysis. Measuring Renilla Luciferase Luminescence in Living Cells. http://www.promega.com/notes/90/12727_10/12727_10.pdf (2005).

13. Roncali, E., *et al.* New device for real-time bioluminescence imaging in moving rodents. *J. Biomed. Opt.* **13**, 054035 (2008).