Video Article

Measuring Growth and Gene Expression Dynamics of Tumor-Targeted *S. Typhimurium* Bacteria

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Abstract

The goal of these experiments is to generate quantitative time-course data on the growth and gene expression dynamics of attenuated *S. typhimurium* bacterial colonies growing inside tumors.

We generated model xenograft tumors in mice by subcutaneous injection of a human ovarian cancer cell line, OVCAR-8 (NCI DCTD Tumor Repository, Frederick, MD).

We transformed attenuated strains of *S. typhimurium* bacteria (ELH430:SL1344 phoPQ- [1]) with a constitutively expressed luciferase (luxCDABE) plasmid for visualization[2]. These strains specifically colonize tumors while remaining essentially non-virulent to the mouse[1].

Once measurable tumors were established, bacteria were injected intravenously via the tail vein with varying dosage. Tumor-localized, bacterial gene expression was monitored in real time over the course of 60 hours using an *in vivo* imaging system (IVIS). At each time point, tumors were excised, homogenized, and plated to quantitate bacterial colonies for correlation with gene expression data.

Together, this data yields a quantitative measure of the *in vivo* growth and gene expression dynamics of bacteria growing inside tumors.

Video Link

The video component of this article can be found at http://www.jove.com/video/50540/

Introduction

Synthetic biology has progressed rapidly over the last decade and is now positioned to impact important problems in energy and health. However, expansion into the clinical arena has been slowed by safety concerns and an absence of developing design criteria for *in vivo* genetic circuits. Accelerating high impact medical applications will require utilizing methods that interface directly with medical infrastructure, genetic circuits that function outside of the controlled lab setting, and safe and clinically-accepted microbial hosts.

A number of strains have been investigated for cancer therapy due to their ability to grow preferentially in tumors. These have included *C. novyi, E. coli, V. cholorae, B. longum*, and *S. typhimurium*[3-8]. *S. typhimurium* has generated particular interest as they have exhibited safety and tolerance in a number of human clinical trials[9-12]. These bacteria were initially shown to create anti-tumor effects through stimulation of the host immune system and by depletion of nutrients required for cancer cell metabolism. Production of therapeutic cargo was later added through genetic modifications. While these studies represent important advances in the use of bacteria for tumor therapies, the majority of existing

efforts have relied on high-level expression that typically results in the delivery of high dosages, off-target effects, and development of host resistance[13-16].

Now, synthetic biology may add programmable cargo production by utilizing computationally-designed genetic circuits that can perform advanced sensing and delivery[17-20]. These circuits can be designed to act as delivery systems that sense tumor-specific stimuli and self-regulate cargo production as necessary. However, studying the function of these circuits *in vivo* has thus far been challenging.

Since plasmids are the common framework for synthetic circuits, we describe a method to characterize the dynamics of plasmid-based gene expression *in vivo* using a mouse model. These methods utilize time-lapse luminescence imaging and quantitative measurement of biodistribution. Together, these approaches provide a framework for studying plasmid-based networks *in vivo* for clinical applications.

Protocol

1. Cell Preparation

1. Passage cell lines using standard cell culture techniques. In this experiment, we used OVCAR-8 cells (NCI DCTD Tumor Repository, Frederick, MD). Grow cells to a target confluency of 80 - 100%.
2. Incubate cells with 5 ml trypsin for 5 min, then add 5 ml RPMI medium + FBS to inactivate trypsin.
3. Harvest and count cells on a hemocytometer. Resuspend in phenol red-free DMEM at a target concentration of 5 x 10^7 cells/ml, or about 200 µl per flask.
4. Add 15% reduced growth factor Matrigel (BD Biosciences) and keep the cell suspension on ice until implantation.

2. Tumor Implantation

1. Anesthetize 4 week old female Ncr/Nu mice using 3% isoflurane and wait roughly 5 min to ensure deep anesthesia. Use vet ointment on eyes to prevent dryness while under anesthesia.
2. Load the cells (100 µl per tumor) in a 1 ml syringe and attach a 27 1/2 gauge needle.
3. For each of two bilateral hind flank injection sites, lift the skin gently with forceps to make a tent and inject cells at the base. Take care not to penetrate too deep, producing blood. Use tweezers to gently remove syringe without spatter.
4. Monitor tumor growth daily for 10 - 20 days until a tumor diameter of 2 - 4 mm is reached.

3. Bacteria Preparation

1. Start an overnight culture of bacteria in 3 ml LB media + Ampicillin from an -80 C freezer stock or refrigerated plate. In this experiment, we used *S. typhimurium* strain ELH430 (SL1344 PhoPQ- aroA-).
2. In the morning, dilute the culture 1:100 into filtered LB and grow to OD600 0.4 - 0.6.
3. Spin down and wash 3 times in phosphate buffered saline (PBS) and resuspend at a final OD600 of 0.1 (about 1 x 10^7 cells/ml).

4. Bacteria Injection

1. Anesthetize the animal and position on its side with the tail pointing toward your dominant hand. Use vet ointment on eyes to prevent dryness while under anesthesia.
2. Dilate the tail vein using warm water or a heat lamp.
3. Load the bacteria (100 µl per mouse) in a 1 ml syringe and bend the tip just less than 90 degrees.
4. Align the syringe tip with the tail vein, penetrate at a shallow depth (should feel no resistance), and inject bacteria. Observe bloodflow displacement for a successful injection.

5. Mouse Imaging

1. Anesthetize animals in the induction chamber then place each mouse on the imaging platform. Use vet ointment on eyes to prevent dryness while under anesthesia. Maintain precise positioning to ensure quantitative results between time points.
2. Image animals using the IVIS Spectrum imaging system (Caliper Life Sciences). If imaging more than 1 animal, place light barriers between them to prevent cross-illumination. Animals should be imaged first on the ventral side to confirm localization of bacteria.
3. Image animals dorsally using IVIS every 2 hr for the duration of the experiment (36 hours). Generate a time course for a given ROI.

6. Quantifying Bacterial Biodistribution

1. Euthanize animals using CO_2 and place on the back on an absorbent diaper.
2. Turn the animal to expose the two hind flank subcutaneous tumors. Cut the surrounding skin tissue to separate and remove the tumors. Holding the tumor with tweezers, remove the skin using a reversed scissor motion. When the majority of the skin has been separated, fully remove the tumor using tweezers.
3. Place the organs in pre-weighed microcentrifuge tubes. Since the mass of these tubes can vary significantly it is important to pre-weigh them for quantitative results.

7. Quantifying Bacterial Biodistribution

1. For each time point, excise and weigh the tumors.
2. Add 500 µl PBS + 15% glycerol and homogenize using a Tissue-Tearor (BioSpec). Rinse the homogenizer 3 times with ethanol between samples.
3. Generate serial dilutions and plate for bacterial colony counting. To plate multiple dilutions on a single plate use pre-sectioned plates.

Representative Results

Using this protocol, we are able to generate data on the *in vivo* growth and gene expression dynamics of tumor targeted bacteria. The overall workflow is summarized in **Figure 1**.

In the first stage, we inject bacteria (green) and image the animal using IVIS to measure gene expression with luminescence (blue) as a reporter.

Then, in the second stage, we excise, homogenize, and plate tumors for colony counts to determine the number of bacteria growing in the tumor.

Bioluminescence generated by bacterial strains is visualized using IVIS (**Figure 2**). Signal increases with injected dosage, with a minimum colonizing dosage around 5 x 10^5 (**Figure 2A**). In all cases, attenuated bacteria are only able to either specifically colonize tumors or fail to grow in the mice. Signal is quantified using radiance units to normalize for exposure time, where typical values of 10^6 or greater are indicative of colonization.

For a given infection, signal increases with time over 24-48 hr (**Figure 2B**). The peak onset is around 36 hours after injection, but the timing may vary depending on the strain and plasmid being used. Be sure to position the mouse exactly the same way each time to ensure quantitative results.

In the next set of experiments, bacterial biodistrution is quantified to measure the growth of bacteria over time (**Figure 3**). Viable bacteria in tumors are quantified by excising and homogenizing the tumor and plating at serial dilutions (**Figure 3A**). Other organs can also be quantified similarly. Here, we have used the spleen, since the tumor-spleen ratio is a typical measure of bacterial specificity [21, 22].

These counts allow us to measure the bacterial population over time (**Figure 3B**). On the left, we can see that the number of bacteria grow steadily in the tumor throughout the duration of the experiment, with a doubling time of 1-3 hr, substantially slower than in batch culture experiments. This reduced rate is likely due to the poor nutrient conditions in the tumor environment. On the right, we have quantified the tumor-spleen ratio and observe that it increases throughout the experiment. This finding demonstrates specificity, in that the spleen counts are essentially constant while bacteria continue to grow in the tumor.

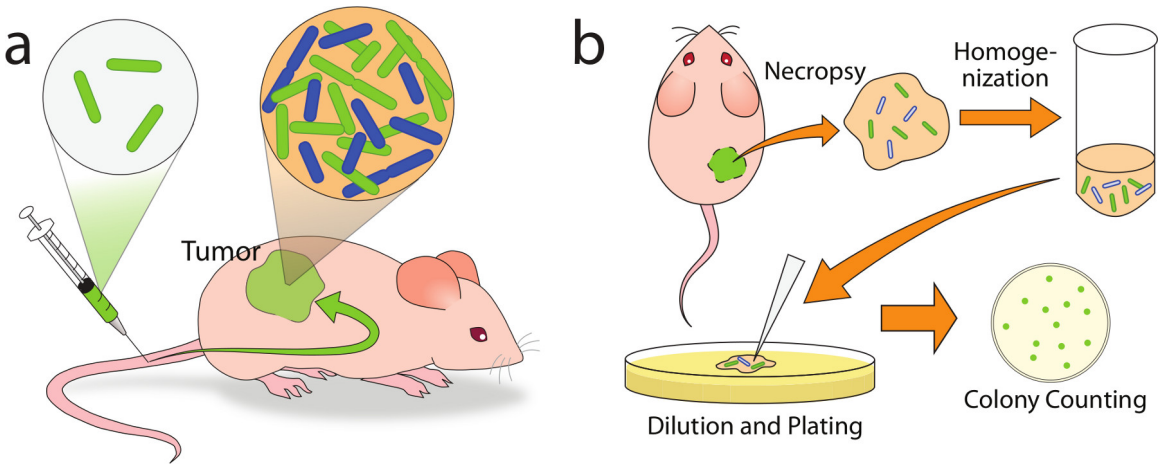

Figure 1. Schematic of the overall experimental process. (a) Bacteria are injected at the tail-vein and specifically colonize tumors. **(b)** At each time point, tumors are excised, homogenized, and plated for colony counts. Reprinted with permission[25]. Copyright 2012 American Chemical Society.

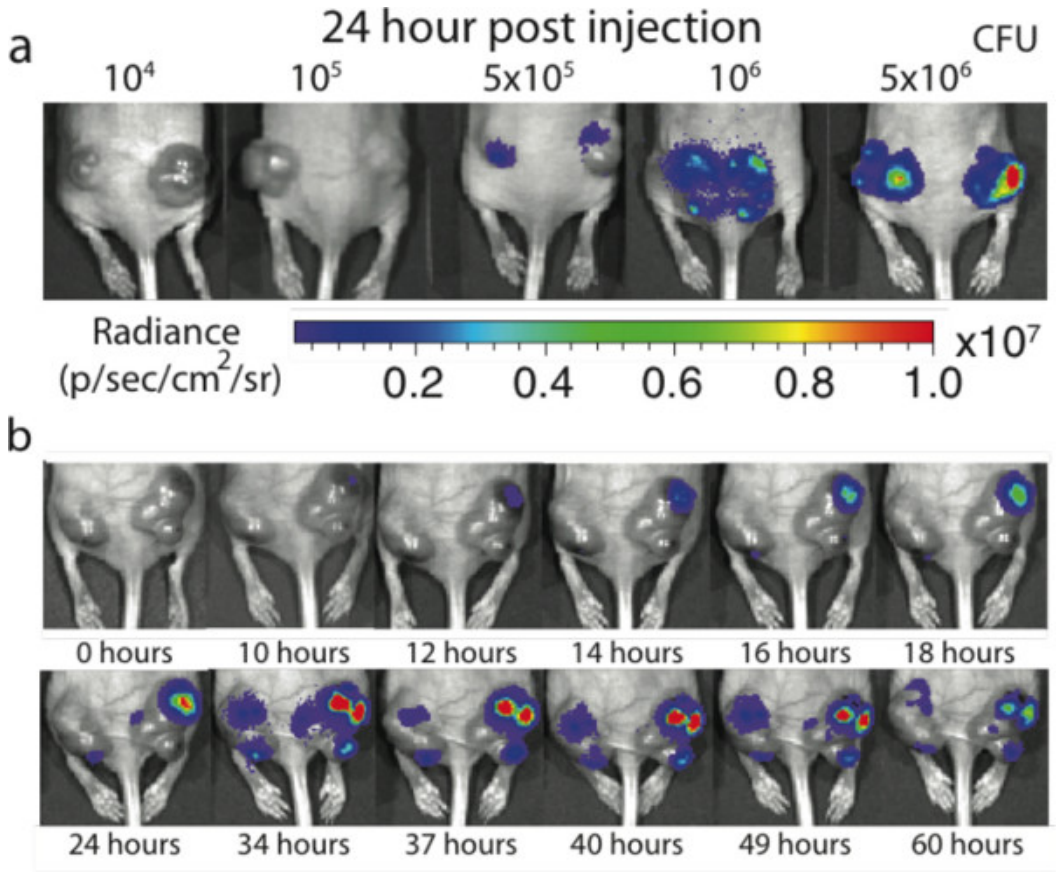

Figure 2. Whole-animal luminescence imaging using IVIS. (a) Signal increases with injection dosage and **(b)** time. Adapted with permission[25]. Copyright 2012 American Chemical Society.

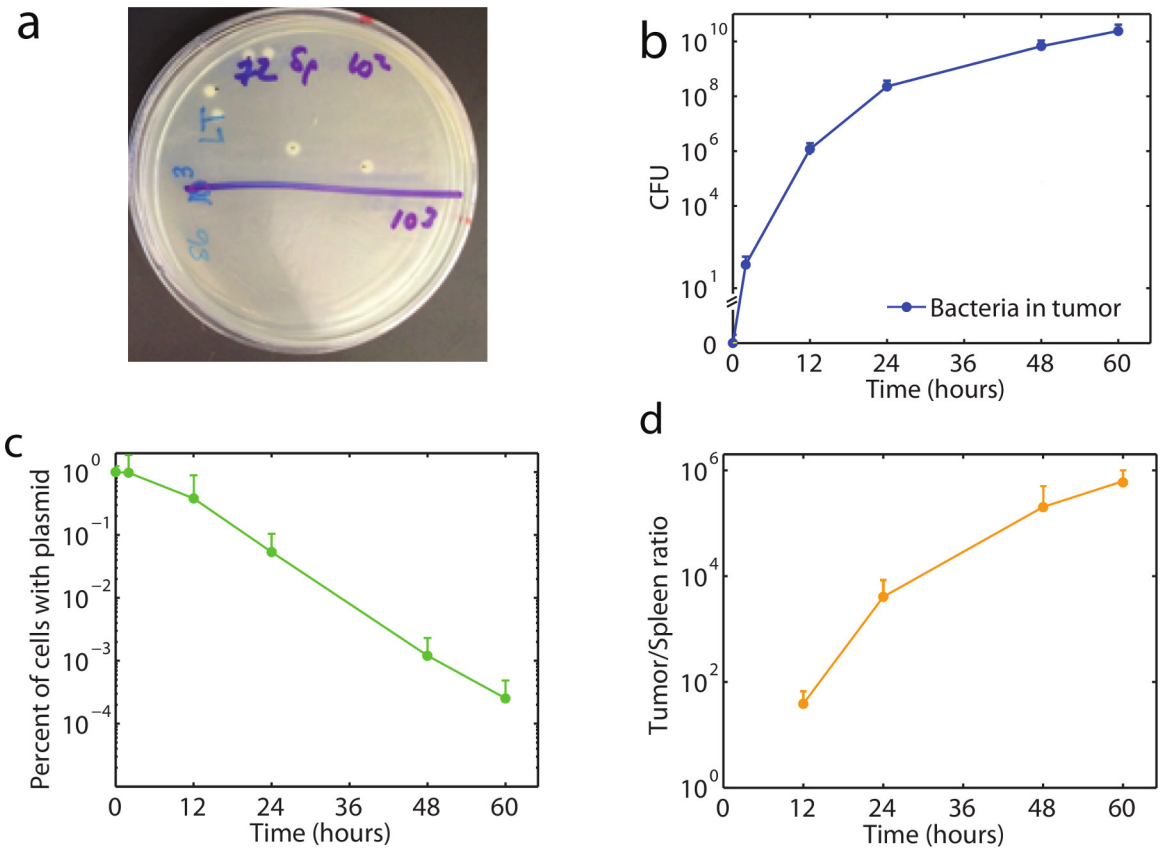

Figure 3. Plating and colony counts to measure growth dynamics. (a) Serial dilutions allow individual colonies to be counted for a representative spleen sample (bottom). **(b)** By following these counts, we can create *in vivo* population measures of bacteria inside the tumors (as in **b**) number of cells with and without the plasmid (as in **c**) and the tumor to spleen ratio (as in **d**). Adapted with permission [25]. Copyright 2012 American Chemical Society.

Discussion

Using this procedure, we are able to generate time courses for the growth and gene expression dynamics of bacteria colonizing tumors. While these measurements are routinely performed *in vitro* in batch culture or microfluidics devices, they are much more difficult to perform *in vivo*.

There are several modifications that can be applied to these methods. While we used the OVCAR-8 cell line to generate our mouse models, a number of other cell lines may be used equivalently. For example, luciferized cell lines may be used that allow for 3D colocalization of tumor and bacterial luciferase channels. Additionally, while we used a bilateral hind flank model, a different number and locations of injection sites may be used to generate models to study different phenomena. For example, if tumor size is an important variable, serial dilutions of cells can be prepared and injected to different sites simultaneously, generating smaller- and larger-sized tumors. Finally, while we used *S. typhimurium* strain ELH430, other strains of *S. typhimurium* or other bacteria such as *C. novyi, E. coli, V. cholorae, B. longum* may be used equivalently with these methods, though the bacterial counts for injection may need to be adjusted.

The most critical steps in this protocol involve the creation of the mouse cancer model, and the preparation and injection of bacteria. For the creation of the cancer model, precision in the cell count and concentration, injection volume, and injection site placement will greatly enhance the consistency of the tumors generated. We have found that equivalently sized, shaped, and placed tumors create the most quantitatively comparable data. For the preparation of bacteria, it is critical to completely wash them prior to injection and to control for the bacteria concentration, volume, and injection site carefully. These steps are required to avoid excessive immunological impact of the injection, and to maintain quantitative comparison between separate mouse models. Since the number of bacteria that enter the tumor is a small fraction of the injected dose, variations in the number of injected bacteria between mice can result in dramatic differences on colony progression. For additional information, see several additional references on bacterial preparation for quantitative *in vivo* studies [3, 4],

While these techniques are highly adaptable to a variety of specific studies, some limitations exist for extending to certain applications. For example, intravenous delivery of high bacterial counts may not be desirable for certain bacteria and/or mouse models due to immunological impact. One alternative route of administration is oral delivery (gavage), a technique which is described on JoVE[23]. Additionally, time-lapse imaging can be quite labor-intensive depending on the specific experiment length, time-resolution, and equipment available. Automating the imaging process would alleviate many of these burdens, but also introduces new technical challenges. Mouse vitals such as heart rate, breath rate, and temperature must be continuously monitored by additional equipment. Additionally, for longer-term anesthesia, eye ointments must be used to maintain adequate moisture. Finally, the level of anesthetic delivered to the mouse must be continuously adjusted to maintain the proper

depth of anesthesia. A closed-loop feedback control system that integrates mouse vital measurements with anesthetic delivery would greatly facilitate this process.

While synthetic biology has achieved much success[17, 19, 20], applying engineered gene circuits to *in vivo* applications will require quantitative, *in vivo* time courses to inform design principles. *S. typhimurium* is an excellent strain for clinical synthetic biology for cancer therapy since it is similar to *E. coli*, specifically colonizes tumors, and has been shown safe in human clinical trials[6, 12, 14, 22]. Additionally, recent studies have found that many published circuits function in *S. typhimurium* without modification[24]. Using the experimental platform described here, we recently described how dynamic *in vivo* expression profiles can be programmed using the inherent instability of plasmid vectors[25]. In addition, recent research has developed novel methods for stably maintaining plasmid vectors *in vivo*[23].

While luciferase is a common reporter used for *in vivo* imaging for its sensitivity[5], there are examples of excellent studies on quantitative *in vivo* gene expression using GFP[26]. Using GFP would allow the researcher to study the spatial-temporal dynamics of individual bacteria *in vivo*. Additionally, while we used a subcutaneous model, extending these studies toward more relevant models of cancer would further increase their predictive power[7]. Future studies like this may enable a next generation of *in vivo* synthetic biology for clinical applications.

Developing both experimental and computational techniques in concert will be critical to engineering *in vivo* genetic circuits. Computational modeling can rapidly probe system parameters to explore potential outputs but must remain closely tied to experimental results to remain relevant. Thus far, these results have been difficult to obtain quantitatively, due in part to a lack of methods for studying genetic circuits *in vivo*.

Building on this platform, future applications will include engineered gene circuits that further extend the range of expression dynamics, sensing tumor-specific stimuli and self-regulating cargo production as needed. As the sophistication of these *in vivo* circuits increases, the requirements on quantitative data required to tune them will also increase. We believe that the methods described here, along with new innovations in long-term mouse imaging, will make this process possible.

Disclosures

No conflicts of interest declared.

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