

## In Vivo Gene Expression Dynamics of Tumor-Targeted Bacteria

Tal Danino,<sup>†</sup> Justin Lo,<sup>†</sup> Arthur Prindle,<sup>‡</sup> Jeff Hasty,<sup>‡,§,||</sup> and Sangeeta N. Bhatia<sup>\*,†,⊥,#,□</sup>

<sup>†</sup>Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States

<sup>‡</sup>Department of Bioengineering, <sup>§</sup>Biocircuits Institute, and <sup>||</sup>Molecular Biology Section, Division of Biological Science, University of California, San Diego, La Jolla, California 92093, United States

<sup>⊥</sup>Broad Institute of Harvard and MIT, Cambridge, Massachusetts 02142, United States

<sup>||</sup>Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02115, United States

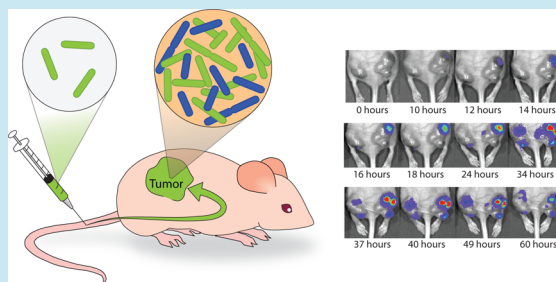
<sup>#</sup>Electrical Engineering and Computer Science and David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States

<sup>□</sup>Howard Hughes Medical Institute, Chevy Chase, Maryland 20815, United States

### S Supporting Information

**ABSTRACT:** The engineering of bacteria to controllably deliver therapeutics is an attractive application for synthetic biology. While most synthetic gene networks have been explored within microbes, there is a need for further characterization of *in vivo* circuit behavior in the context of applications where the host microbes are actively being investigated for efficacy and safety, such as tumor drug delivery. One major hurdle is that culture-based selective pressures are absent *in vivo*, leading to strain-dependent instability of plasmid-based networks over time. Here, we experimentally characterize the dynamics of *in vivo* plasmid instability using attenuated strains of *S. typhimurium* and real-time monitoring of luminescent reporters. Computational modeling described the effects of growth rate and dosage on live-imaging signals generated by internal bacterial populations. This understanding will allow us to harness the transient nature of plasmid-based networks to create tunable temporal release profiles that reduce dosage requirements and increase the safety of bacterial therapies.

**KEYWORDS:** synthetic biology, *S. typhimurium*, bacterial cancer therapy, plasmid-loss dynamics



Over the past century, the observation that bacteria accumulate preferentially in tumors has prompted the investigation of the use of a number of strains for cancer therapy, including *C. novyi*, *E. coli*, *V. cholerae*, *B. longum*, and *S. typhimurium*.<sup>3,13,2,27,17,24</sup> Attenuated strains of *S. typhimurium* have generated particular interest as they can innately home to and colonize tumors of a variety of sizes and have exhibited safety and tolerance in human clinical trials.<sup>25,5,22,8</sup> *S. typhimurium* were initially shown to mediate antitumor effects through recruitment of the host immune system and by competition with cancer cells for nutrients. Subsequently, engineered production of therapeutic cargo was added through simple genetic modifications. While these studies represent important advances in the use of bacteria for tumor therapies, the majority of existing efforts have relied on constitutive, “always on” cargo production<sup>9,26,16,6</sup> that typically results in the delivery of high dosages, off-target effects, and development of host resistance.

As a next step, synthetic biology seeks to add controlled and dynamic production of cargo by utilizing computationally designed “circuits” that have sophisticated sensing and delivery capabilities.<sup>7,4,19,1</sup> These circuits can be designed to act as delivery systems that sense tumor-specific stimuli and self-regulate cargo production as necessary. Since plasmids are the

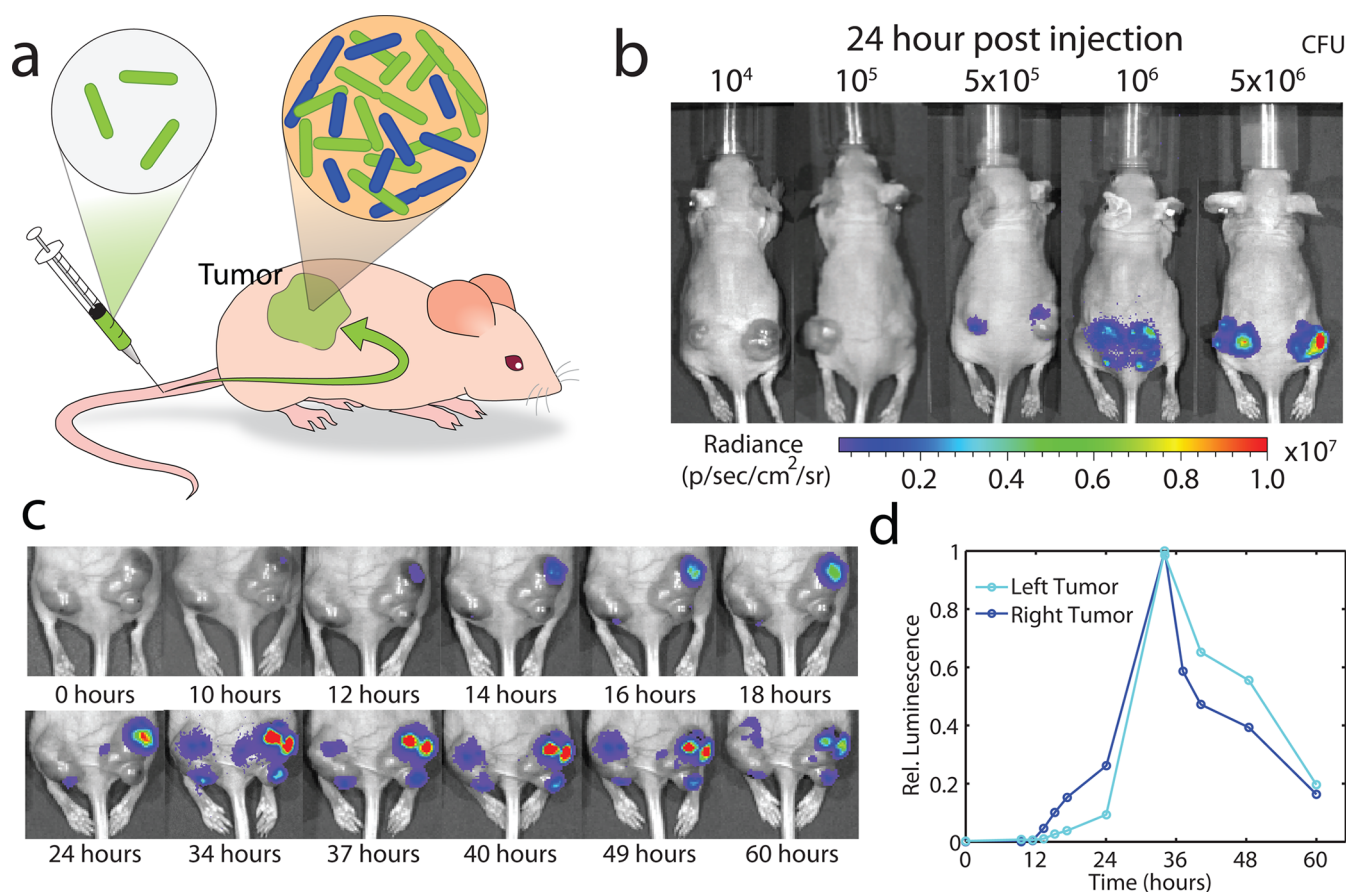
common framework for synthetic circuits, we begin by characterizing the dynamics of plasmid-based gene expression in an *in vivo* mouse model by utilizing real-time luminescence imaging, quantitative biodistribution measurement, and computational modeling. Together, these approaches provide a framework for exploiting the inherent instability of plasmid-based networks, which will facilitate the generation of specific temporal release profiles directly within the tumor environment.

We began by transforming two different attenuated strains of *S. typhimurium* with a constitutively expressed luciferase plasmid (luxCDABE genes on a pBR322/colE1 high-copy without partitioning machinery) to allow for real-time monitoring of luminescence with an *in vivo* imaging system (IVIS).<sup>13</sup> Strain A (*ELH430:SL1344 phoPQ-*) is attenuated for the PhoPQ regulon, which is known to activate a number of genes related to virulence, while Strain B (*ELH1301:SL1344 phoPQ- aroA-*) contains an additional aromatic amino acid synthesis mutation that effectively allows it to grow only in

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**Figure 1.** Tumor homing bacteria and dosage variation. (a) *S. typhimurium* are injected via tail vein into nude mice and localize to subcutaneous tumors where they replicate. (b) Dosages between  $10^4$  and  $5 \times 10^6$  bacteria (Strain A) were injected into mice, and IVIS images were taken after 24 h. Higher initial dosages show an increasing signal and a minimum value of  $5 \times 10^5$  bacteria required to visualize tumor colonization at 24 h. (c) Sequence of IVIS images for strain Strain A at  $10^6$  dosage over the course of 60 h. (d) Total flux of left (light blue) and right (dark blue) tumors as a function of time normalized to the maximum value across the trajectory. The IVIS signal rises due to rapid bacterial growth and then decays due to plasmid loss and luciferase instability.

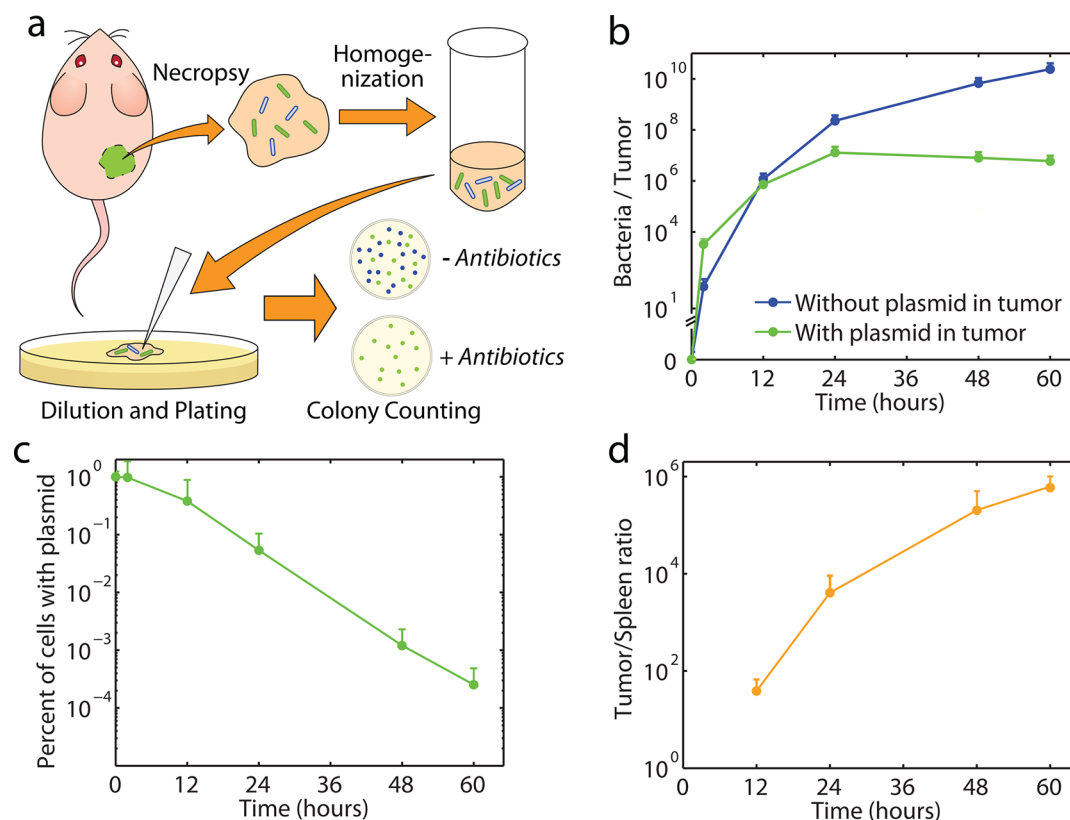
nutrient-rich environments. Importantly, while these strains are derived from the same parent (SL1344), their growth rates and therefore plasmid-loss dynamics differ significantly. To investigate the *in vivo* gene expression dynamics of these strains, we generated model xenograft tumors in mice by subcutaneous injection of a human ovarian cancer cell line (OVCAR-8). After measurable tumors were established, bacterial strains were injected intravenously via tail vein (Figure 1a) with dosages varying from  $10^4$  to  $5 \times 10^6$  bacteria. Once injected, bacteria specifically colonized tumors at a rate proportional to the dosage administered, as measured by IVIS signal at 24 h post-injection (Figure 1b).

We then monitored the tumor site for Strain A-derived signal over the course of 60 h using time-lapse IVIS imaging (Figure 1c,d). These trajectories followed the specific pattern of an initial steep increase followed by a gradual decrease back to baseline (Figure 1d). We hypothesized that the shape of this waveform was the result of the initial exponential growth of plasmid-containing bacteria followed by increasing rates of plasmid loss in the absence of antibiotic selection. Eventually, the rate of luciferase production by the remaining plasmid-containing bacteria is overtaken by luciferase decay, and the total signal begins to decline.

To test this hypothesis, we counted the number of plasmid-containing and non-plasmid-containing bacteria in tumors over time by observing bacterial growth on selective media (Strain A,

$10^6$  bacteria injected). Each measurement was compared to counts taken in the spleen, a control tissue where there a stable subpopulation is present, as the bacteria initially accumulate but do not grow or die in this site. At each time point, organs were excised from the mouse and then homogenized and plated with or without antibiotic selection (Figure 2a,  $n = 3-5$  tumors). Colony counting on these plates yielded an accurate measure of the plasmid state of the bacterial population over time (Figure 2b). After 2 h, roughly  $3 \times 10^3$  plasmid-containing bacteria reside in the tumor, or about 0.3% of the injected dose. After 12 h, plasmid-containing bacteria grow to a level of  $10^6$ , and the number of non-plasmid-containing bacteria reaches a similar level. This accumulation corresponds to a doubling time of approximately 75 min. Growth rate declined further over time, presumably due to nutrient limitation, ultimately resulting in a 300 min doubling time for non-plasmid-containing bacteria (Figure 2b).

While the total population of bacteria grew throughout the course of the experiment (60 h), the number of plasmid-containing bacteria reaches a maximum at 24 h (Figure 2b). By taking the ratio of these populations, we can calculate the percentage of plasmid-containing bacteria over time (Figure 2c). After 12 h, roughly 50% of the population retains the plasmid, a fraction that drops to 10% after 24 h (Figure 2c). The slope of this line remains constant throughout the 60-h



**Figure 2.** Population dynamics of bacteria inside tumor environments. (a) Schematic showing tumor extraction, homogenization, and plating to count internal populations of bacteria. Plating on antibiotics reveals the number of plasmid-containing bacteria, while plating without antibiotics gives the total number of bacteria. (b) Measured number of plasmid-containing and non-plasmid-containing bacteria per organ calculated for plasmid-containing bacteria (green) and non-plasmid-containing bacteria (blue). Bacteria with the plasmid reach a steady state around 24 h, while the non-plasmid bacteria continue to grow. (c) Percentage of cells containing the plasmid as a function of time showing a constant loss rate over the course of 72 h. (d) Ratio for total number of bacteria in the tumor versus the spleen. This number is typically reported as a measure of specificity of the tumor-targeting strain. Strain A at a dosage of  $10^6$  cells was used for all panels. Lines indicate a piecewise linear fit of the data.

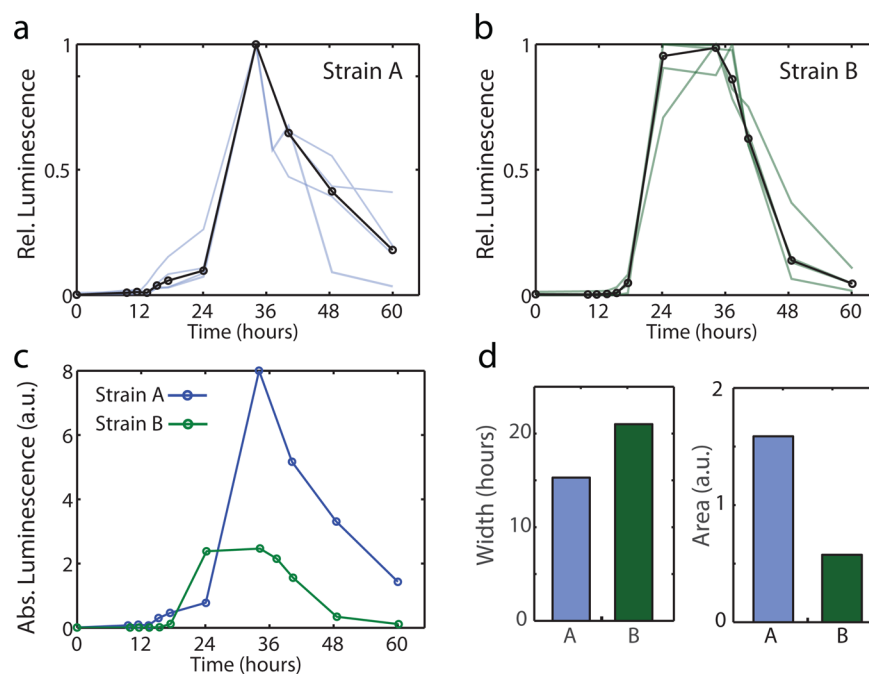
experiment and represents the rate of plasmid loss in the tumor environment.

The tumor-spleen ratio is commonly reported as a characteristic measure of specificity and tumor-homing ability for a given strain. Bacteria accumulate in the spleen from the initial dosing yet do not subsequently grow and divide. Given that we observed essentially no increase in the bacterial count in the spleen throughout the duration of our experiments, the tumor-spleen ratio increased over time (Figure 2d). Since this ratio is typically reported as a fixed number in the literature, its time-dependence may help to explain the wide range of reported values.<sup>14,23</sup>

To explore how bacterial growth rate affects the dynamics of plasmid instability over time, we injected two groups of mice with Strain A and B (at a dosage of  $10^6$ ) and monitored their signal over the course of 60 h. The two strains displayed markedly different profiles, with Strain A peaking and decaying sharply and the slower growing Strain B peaking broadly over a longer period of time before decaying (Figure 3a,b). We plot the average trajectories for Strains A and B on an absolute luminescence scale in Figure 3c for comparison. To quantify these differences, we measured the width at half-maximum and total area under each curve for the average trajectories (Figure 3d). These measurements illustrate that Strain A produces more luminescence quickly while Strain B produces less luminescence over a longer period of time (Figure 3d). Additionally, to confirm that signal intensity is a representative

measure of the population of plasmid-containing bacteria, we compared counts of antibiotic resistant bacteria with absolute IVIS values at the 72-h time point and found them to be highly correlated ( $R = 0.832$ , Supporting Information).

Developing a fully tunable dynamic expression platform will require a more complete understanding of the underlying processes. Plasmid-loss dynamics have been well described in a variety of *in vitro* and *in vivo* contexts;<sup>20,12,15</sup> however, modeling of population or gene-expression dynamics has not yet been studied for *in vivo* tumor environments. Specifically, we hope to learn how expression dynamics are dictated by the rates of growth and plasmid-loss for a given strain. To accomplish this, we developed an ordinary differential equation (ODE) model describing internal plasmid and non-plasmid-containing bacteria and their respective expression of luciferase signal (Figure 4a). Initially,  $N_0$  bacteria are injected. These plasmid-containing bacteria replicate and lose their plasmids at rate  $\tau$ , resulting in populations of plasmid ( $N^+$ ) and non-plasmid ( $N^-$ ) containing bacteria that continue to grow at rates  $\mu^+$  and  $\mu^-$ , respectively (Figure 4a). Both populations grow exponentially for 24 h until available nutrients become limiting, a process modeled by including a finite quantity of tumor substrate that is consumed according to Michaelis–Menten kinetics<sup>21</sup>. The tumor environment is also spatially restrictive of bacterial growth, with bacteria in the center consuming nutrients more slowly than bacteria on the rapidly growing periphery. Thus, despite a nearly constant population of plasmid-containing bacteria, IVIS



**Figure 3.** Characterization of Strain A and Strain B IVIS profiles. (a, b) Time-course trajectories for Strain A (a) and Strain B (b) over the course of 60 h normalized to their maximum value across the trajectory. Colored lines indicate individual trajectories, and the solid black line indicates the average trajectories. (c) Time-course trajectories for Strain A and Strain B on an absolute scale. Average trajectories from (a) and (b) were scaled by the average absolute value of the individual trajectories. (d) (left) Full-width at half-maximum and the area (right) under the curve for the average trajectory of both strains. These parameters characterize the dosage and duration of transient gene-expression.

signal fails to increase after 24 h since most of these bacteria reside in the non-growing center of the colony. We accounted for this behavior by limiting the amount of bacteria that can consume the tumor substrate, which effectively limits plasmid-containing bacterial growth and allows luciferase decay to dominate.

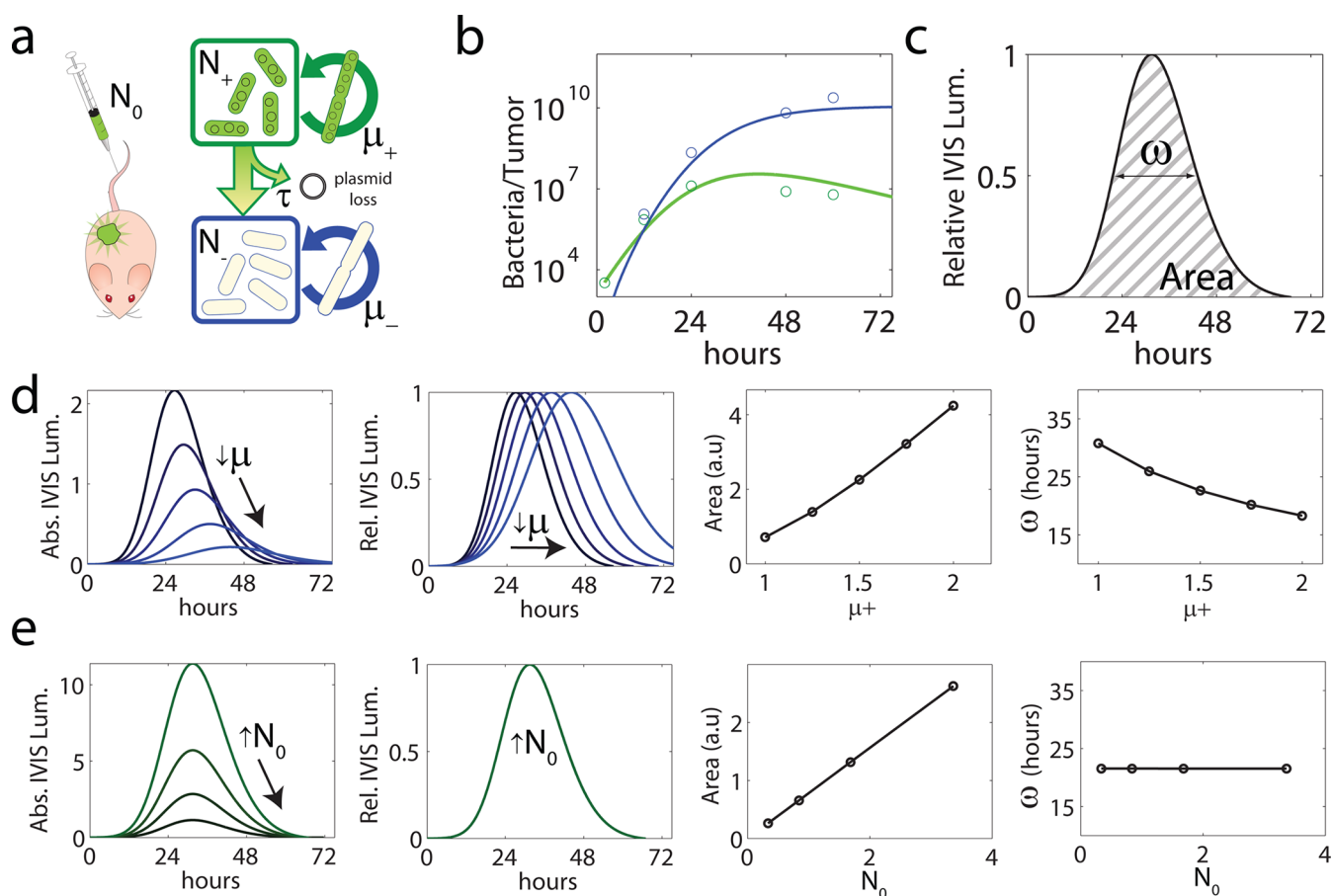
Our ODE model produced dynamics that were consistent with our experimental observations, where IVIS signal is taken to be proportional to the plasmid-containing population (Figure 4b). We define the full-width at half-maximum,  $\omega$ , and area under the curve as important parameters that characterize the duration and magnitude of dosage, respectively (Figure 4c). To understand how to tune *in vivo* expression profiles according to these parameters, we varied growth rate and dosage level and modeled the effects on IVIS signal in each case (Figure 4d,e). Lower growth rates yield IVIS curves that are shifted toward later times with broader widths and lower areas (Figure 4d). In contrast, larger initial dosages result in a linear increase of IVIS signal that increases area but does not alter the width (Figure 4e). The latter linear increase in area as a function of dosage is reflective of doses much lower than the carrying capacity of the system. Finally, decreasing the plasmid-loss rate resulted in an increase in area under the curve as well as a slight shift in the width and time to peak of the gene expression profile (Supporting Information).

These effects correlate with experimental observations that can be explained based on differences in strain growth rate. Since plasmids are lost during cell division, the faster a cell replicates, the more frequently it loses plasmid. Thus, the faster growing Strain A accumulates luciferase quickly but loses a comparatively larger fraction of plasmids per day, resulting in higher IVIS values that peak at earlier time points than Strain B (Figure 3a,b). In contrast, Strain B grows more slowly, producing less luciferase but maintaining its plasmids much

longer, yielding a broader expression profile compared to Strain A (Figure 3a,b).

In the context of drug delivery, a critical parameter is the rate at which a device releases drug into the surrounding environment. For instance, materials have been investigated that generate “burst”, “delayed”, or “sustained” release characteristics.<sup>11</sup> The transient plasmid-based system we have developed here can generate a similar variety of expression dynamics. For instance, Strain A produces an expression profile analogous to burst release due to its fast growth rate and high rate of plasmid loss. In contrast, Strain B yields a sustained release profile owing to its slow growth rate and moderate rate of plasmid loss. Bacteria are unique in the context of drug-delivery vehicles in that they produce their own cargo, in contrast to other devices that are preloaded and depleted. This difference allows them to deliver a time-varying concentration of cargo in a designed profile directly on site. In the future, this work will enable a variety of drug-release profiles from engineered bacteria for therapeutic applications.

Developing both experimental and computational techniques in concert will be critical to engineering *in vivo* genetic circuits.<sup>7</sup> Computational modeling can rapidly probe system parameters to explore potential outputs but must remain closely tied to experimental results to remain relevant. On the other hand, *in vivo* experiments present the most direct application of engineered circuits, but involve long time scales and the results are often difficult to interpret. Here, we have utilized plasmid instability to generate transient expression profiles in tumor environments. In our computational model, we can predict how dosage, strain growth rate, and plasmid loss rate combine to yield differing expression dynamics. Subsequently, these designs can be implemented experimentally by varying plasmid type, copy number, and maintenance system<sup>18</sup> or by modifying the strain growth rate. Building on this platform, future applications



**Figure 4.** Modeling bacterial dynamics inside of tumor environments. (a) Schematic of mathematical model. Bacteria containing plasmids are injected at a dosage  $N_0$ . Plasmids are lost from cells at a rate  $\tau$ , and growth of resulting plasmid-containing and non-containing cells continues at  $\mu_+$  and  $\mu_-$ , respectively. (b) Modeling results (solid lines) for the number of plasmid-containing (blue) and non-plasmid-containing (green) bacterial population data from Figure 2b. Open circles indicate experimental points. (c) Typical time course of an IVIS trajectory indicating parameters  $\omega$  and area for characterizing transient gene expression. The relative IVIS trajectory is typically measured experimentally due to mouse-to-mouse variability, while the absolute IVIS trajectories are used to calculate  $\omega$  and area. (d) The effect of growth rate on transient gene expression. Decreasing growth rate shifts the absolute IVIS signals to a lower value and longer peak times, therefore increasing  $\omega$  and decreasing the area under the curve. (e) The effect of initial dosage on transient gene expression. Increasing the dosage shifts the absolute IVIS signals to a higher level, increasing the area under the curve, but does not change the relative IVIS trajectory shape or  $\omega$  value.

will include engineered gene circuits that further extend the range of expression dynamics, sensing tumor-specific stimuli and self-regulating cargo production.

## METHODS

*S. typhimurium* strains Strain A (SL1344 PhoPQ<sup>-</sup>) and Strain B (SL1344 PhoPQ<sup>-</sup> aroA<sup>-</sup>) were provided by Elizabeth Hohmann (MGH).<sup>10</sup> The constitutive plasmid bearing luxCDABE genes was received as a gift from the Weiss lab.<sup>13</sup> On the day of injection, bacteria containing plasmids were diluted 1/1000 into fresh LB media (Difco, 0.22  $\mu\text{m}$  filtered) with antibiotics (Ampicillin 100  $\mu\text{g}/\text{mL}$ ) and grown up to  $\text{OD}_{600} = 0.4\text{--}0.6$ . Cells were then prepared by washing 4 times with PBS (0.22  $\mu\text{m}$  filtered) and measured for  $\text{OD}_{600}$ . Colony counts were performed on the preparation as a calibration and cells were prepared at various concentrations for 100  $\mu\text{L}$  injections.

Subcutaneous human xenograft tumors were generated by injecting  $5 \times 10^6$  OVCAR-8 cells (NCI DCTD Tumor Repository, Frederick, MD) bilaterally into the hind flanks of 4-week-old female Ncr/Nu mice. Cells were grown to 80–100% confluency in RPMI 1640 media supplemented with 10% fetal bovine serum and antibiotics (100  $\mu\text{g}/\text{mL}$  penicillin and 100

$\mu\text{g}/\text{mL}$  streptomycin) before injection. Cells were pelleted and resuspended in phenol red-free DMEM with 15% reduced growth factor Matrigel (BD Biosciences). Tumors were allowed to grow for 10–20 days until tumor masses of 200–500 mg were reached.

Colony counts were measured by dissecting tumors and organs from mice, homogenizing using a Tissue-Tearor (BioSpec), and plating serial dilutions on LB and LB Ampicillin plates. Prior to imaging, mice were anesthetized with 2–3% isoflurane. IVIS signals were measured using the IVIS Spectrum imaging system (Caliper Life Sciences) with 1–60 s exposure times, and Living Image software (Caliper Life Sciences) was used for analysis. Data where the tumor had ulcerated or had low signal (maximum of trajectory did not reach above  $10^6$  radiance, or approximately  $<5\times$  initial background) were not included. Error bars drawn are standard error.

## ASSOCIATED CONTENT

### Supporting Information

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## AUTHOR INFORMATION

### Corresponding Author

\*E-mail: sbhatia@mit.edu.

### Notes

The authors declare no competing financial interest.

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