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Genetically modified bacteria as a tool to detect microscopic solid tumor masses with triggered release of a recombinant biomarker

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Abstract

Current tomographic methods of cancer detection have limited sensitivity and are unable to detect malignant masses smaller than half a centimeter in diameter. Mortality from tumor recurrence and metastatic disease would be reduced if small lesions could be detected earlier. To overcome this limitation, we created a detection system that combines the specificity of tumor-targeting bacteria with the sensitivity of a synthetic biomarker. Bacteria, specifically *Salmonella*, preferentially accumulate in tumors and microscopic metastases as small as five cell layers thick. To create tumor detecting bacteria, an attenuated strain of *Salmonella* was engineered to express and release the fluorescent protein ZsGreen. A single-layer antibody method was developed to measure low concentrations of ZsGreen. Engineered bacteria were administered to a microfluidic tumor-on-a-chip device to measure protein production. In culture, half of produced ZsGreen was released by viable bacteria at a rate of $87.6 \text{ fg} \cdot \text{bacterium}^{-1} \cdot \text{h}^{-1}$. Single-layer antibody dots were able to detect bacterially produced ZsGreen at concentrations down to 4.5 ng/ml. Bacteria colonized in 0.12 mm^3 of tumor tissue in the microfluidic device released ZsGreen at a rate of $23.9 \text{ } \mu\text{g/h}$. This release demonstrates that ZsGreen readily diffuses through tissue and accumulates at detectable concentrations. Based on a mathematical pharmacokinetic model, the measured rate of release would enable detection of 0.043 mm^3 tumor masses, which is 2,600 times smaller than the current limit of tomographic techniques. Tumor-detecting bacteria would provide a sensitive, minimally invasive method to detect tumor recurrence, monitor treatment efficacy, and identify the onset of metastatic disease.

INTRODUCTION

Finding small malignant lesions is necessary to treat the primary causes of cancer mortality. Secondary metastatic tumors, for example, are often not discovered until pathological symptoms have manifested and the lesions are large.¹ Imaging techniques such as positron emission tomography (PET), magnetic resonance imaging (MRI), and computed tomography (CT) are good at identifying macroscopic tumors but are limited in their ability

to detect microscopic lesions.^{2,3} These techniques lack the spatial resolution needed to detect tumors and metastases less than 6–8 millimeters in diameter.^{3–10} Endogenous biomarkers can be used in conjunction with imaging techniques to identify cancer, but they are sub-type specific and highly variable.^{11,12} The biomarker concentration threshold, at which a reading is considered abnormal, is difficult to determine because expression from normal tissue can vary from patient to patient.^{13,14} Detecting small cancer masses would improve patient survival by identifying recurrence earlier and enabling more effective treatment.

Bacteria would be a useful tool to detect malignant lesions. Therapeutic bacteria possess unique properties that would make them excellent tumor-targeting probes. Facultative anaerobic bacteria, such as *Salmonella*, selectively accumulate and replicate in tumors.¹⁵ The specific tropism of *Salmonella* to malignant tissue has been demonstrated repeatedly.^{16–20} After systemic administration to tumor-bearing mice, *Salmonella* colonize tumors at densities 10,000 times greater than healthy organs.^{15,21,22} In addition to this high specificity to malignant tissue, therapeutic bacteria accumulate in metastases as little as five cell layers thick.^{15,23,24} *Salmonella* can be rendered nonpathogenic by partial deletion of the *msbB* gene, which diminishes the TNF immune response to bacterial lipopolysaccharides and prevents septic shock.¹⁷ In mice, the virulence (LD₅₀) of *msbB*[−] *Salmonella* is 10,000-fold less than wild-type *Salmonella*.^{20,25} In pre-clinical trials, these attenuated *Salmonella* have been administered systemically into mice and dogs without toxic side effects.^{26,27} In human trials with metastatic melanoma patients, attenuated *Salmonella* have been safely administered.²⁸

Several strategies have been described using bacteria for tumor detection. *Escherichia coli* expressing ferritin enhance magnetic resonance imaging (MRI) by increasing iron uptake and improving signal to background ratio.²⁹ *Escherichia coli* have also been used to enhance positron emission tomography (PET) through innate uptake of FDG.³⁰ Combined with the native uptake of malignant tissue, these bacteria amplified FDG uptake producing a higher radiologic signal. *Escherichia coli*, *Salmonella typhimurium*, *Vibrio cholera*, and *Listeria monocytogenes* have also been used to visualize bacterial colonization of different tumor models via expression of bioluminescent proteins.^{23,31–33} Bioluminescence performs well in small animals but translation to the clinic is difficult because light cannot penetrate through tissue. Bioluminescent signal decreases approximately 10-fold for every 1 cm of tissue depth.³⁴ Using bacteria with PET and MRI would still be limited by the resolution of tomographic techniques.

Combining the sensitivity of biomarker detection with the specificity of tumor-targeting bacteria has the potential to detect microscopic tumors smaller than the current resolution of tomography. Figure 1 describes a concept of how bacteria could be used to detect cancerous lesions. Bacteria would be injected systemically and preferentially accumulate in tumors (*step 1*). After growth in tumor tissue (*step 2*) and clearance from the rest of the body, expression of a biomarker would be triggered by a small inducing molecule (*step 3*). A protein biomarker would be released by the bacteria (*step 4*) and diffuse through tumor interstitium into the blood stream (*step 5*) where it could be detected (*step 6*). The presence of biomarker molecules in the blood would indicate bacterial colonization of malignant

tissue. For this conceptual strategy to be possible, bacteria must produce the biomarker at sufficient rates. This production is dependent on the rate of protein expression, the efficiency of secretion, the bacterial density, and the limit of detection.

To create a bacteria-based detection system and quantify the limiting mechanisms, we engineered *Salmonella* to produce and release a biomarker. We hypothesized that, when colonized in tumor cell masses *in vitro*, *Salmonella* release ZsGreen at rates sufficient to detect small tumors. To test this hypothesis *Salmonella* were engineered to express ZsGreen, a fluorescent protein, under control of the L-arabinose inducible promoter. ZsGreen release from bacteria was measured by fluorescence spectroscopy. Protein production was measured from bacteria colonized in tumor cell masses in a microfluidic tumor-on-a-chip device that mimics tumor tissue surrounding blood vessels (Figure 1).³⁵ Released ZsGreen was quantified using an antibody binding technique, which had improved detection over bulk fluid fluorescent measurements. Developing a bacteria-based biomarker detection system has the potential to identify microscopic lesions, smaller than current detection methods.

RESULTS

Salmonella secreted ZsGreen in liquid culture

Salmonella released ZsGreen, which was detectable when suspended in blood (Figure 2). A plasmid that expressed ZsGreen, pDF02, was transformed into *Salmonella* and grown in liquid culture. Although the plasmid contained resistance to ampicillin, antibiotic was not needed to maintain expression for up to 58 hours (Figure S2). ZsGreen expression increased for 24 hours after induction with L-arabinose (Figure 2A). Over the first 10 hours, a majority of the ZsGreen, 83%, was found in the intracellular fraction of the culture. Between 10 and 41 hours, ZsGreen levels increased in the extracellular fraction ($P<0.05$), while intracellular ZsGreen decreased ($P<0.05$). These changes in concentration indicate that ZsGreen was released from the bacteria. At 41 hours, over half of the protein was released, which was greater than the intracellular ZsGreen concentration ($P<0.05$). The release rate of ZsGreen was $87.6 \text{ fg-bacterium}^{-1}\cdot\text{h}^{-1}$.

To determine the threshold for fluorescence detection in blood, ZsGreen from liquid culture was diluted in whole blood and plasma (Figure 2B). Extracellular ZsGreen, at a concentration of $528 \mu\text{g/ml}$, was diluted at ratios of 1:1, 1:10, 1:100 and 1:1000. In whole blood, ZsGreen fluorescence was detected down to a 1:10 fold dilution ($52.8 \mu\text{g/ml}$), 3.2 times brighter than the background fluorescence of the blood ($P<0.05$). Erythrocytes and leukocytes have unique optical properties which can interfere with fluorescence molecule detection in blood.³⁶ Removal of these red and white blood cells by centrifugation increased detection sensitivity of extracellular ZsGreen. Identical concentrations of recombinant ZsGreen in plasma enabled detection down to a ratio of 1:1000 ($0.528 \mu\text{g/ml}$). At this dilution (100-fold less), fluorescence from ZsGreen was 1.8 times brighter than the background fluorescence of the plasma ($P<0.05$, Figure 2B).

ZsGreen release from viable *Salmonella*

Recombinant ZsGreen was released from living *Salmonella*, but was not released from *E. coli* (Figure 3). ZsGreen fluorescence intensity was measured in the intracellular and extracellular fractions of *Salmonella* and *E. coli* liquid cultures at 26 hours (Figure 3A). Bacteria density was described in colony forming units (CFU). *Salmonella* utilize type I, type III, type VI, sec and twin-arginine (Tat) secretion systems for protein export, whereas *E. coli* primarily utilize type I, type II, Sec and Tat mediated secretion systems.³⁷ *Salmonella* expressed 3 times more ZsGreen (2.6 mg/ml; $P<0.05$) and released 40 times more (1.4 mg/ml; $P<0.05$) than *E. coli*. Intracellular ZsGreen intensities in both species were approximately equal (Figure 3A).

ZsGreen was primarily secreted from living *Salmonella* indicating that an active secretion mechanism was responsible for its release (Figure 3). Transformed *Salmonella* were grown in LB and transferred to phosphate buffered saline (PBS) to prevent replication and observe a single bacterial generation (Figure 3B). The PBS-suspended bacteria were labeled with 4', 6-diamidino-2-phenylindole (DAPI) to identify all bacteria (blue) and ethidium homodimer-I to identify dead bacteria (red). Images of these bacteria were acquired for 11 hours to determine changes in ZsGreen content (green) and viability (Figure 3B). Overlay of fluorescent images enabled classification of individual bacterium as live or dead (Figure 3B,C). The ZsGreen content and cell viability of 255 individual bacteria were measured (Figure 3C). Cells were classified as live or dead based on a red intensity threshold determined from a dead cell control stained with ethidium homodimer-I (solid vertical line; Figure 3C). Suspension in nutrient-free PBS halted growth. For the 11 hours of observation, the number of bacteria did not increase and cell viability remained above 85% (Figure 3D). On average, live cells contained more intracellular ZsGreen compared to dead cells ($P<0.05$; Figure 3E). Over time, both live and dead *Salmonella* lost intracellular ZsGreen, indicating protein release. Living *Salmonella* released ZsGreen 1.475 times faster than dead bacteria ($P<0.05$, Figure 3E). Integration of ZsGreen release from viable and dead cells over time showed that 91% of released ZsGreen was from live cells (Figure 4F). Faster ZsGreen release from live cells than dead cells indicates that active secretion was the dominant mechanism of release and that ZsGreen was not released after cell lysis.

Detection of ZsGreen with single-layer antibody dots

ZsGreen measurement with a single-layer antibody technique improved sensitivity over direct fluorescent spectroscopy almost 1000 fold (Figure 4). To measure ZsGreen fluorescence, nitrocellulose membranes were cut into 1.5 mm diameter circles and coated with anti-ZsGreen antibody (Figure 4A). ZsGreen was applied to antibody dots in 96-well plates and measured by epifluorescent microscopy. The ability to measure extended fluorescent exposure of a surface with a CCD camera provided a greater range of detection compared to spectroscopy with a microtiter plate reader (Figures 4B,C). For each measurement method, the fluorescence signal intensity was normalized to a value of one at the lowest detectable ZsGreen concentration. This detection limit was defined as the concentration that produced a signal that was statistically greater than background fluorescence. The lowest detectable concentration of ZsGreen with single-layer antibody dots (4.5 ng/ml) was significantly lower than the limit with direct spectroscopy (4,500

ng/ml; $P < 0.05$; Figure 4C). To improve dynamic range, exposure times were increased for lower concentrations (Figure 4B,C). Increasing exposure time of the CCD camera enabled detection of lower concentrations of ZsGreen (Figure 4B). The dot fluorescence intensity from a 100 ms exposure equaled 10 and 2.5 times the intensities produced by 1000 and 400 ms exposure times, respectively. Fluorescence intensity on dots had a linear relationship with ZsGreen concentrations between 4.5 and 10,000 ng/ml (Figure 4C). At concentrations greater than 10,000 ng/ml, little change in fluorescence intensity was observed indicating saturation of antibody binding sites on the surface of the dots (Figure 4C). Fluorescence intensity of spectroscopic measurements was less sensitive than measurement on antibody dots, with a linear range of detectable concentrations between 1,000 and 1×10^6 ng/ml (Figure 4C).

Released ZsGreen was detected from small *in vitro* tumor cell masses

Tumor detecting *Salmonella* produced ZsGreen in microscopic tumor masses and was released into the flow channel of a microfluidic device (Figure 5). The device mimics tumor tissue adjacent to a blood vessel (Figure 5A). Ten chambers containing tumor cell masses were analyzed across three replicate experiments (A: $n=3$, B: $n=4$, C: $n=3$). In each run, the chambers are connected in series. The total volume for each run (A, B, and C) was the sum of the volumes of each cell mass in the device chambers. The average volume was $0.12 \pm 0.02 \text{ mm}^3$ ($n=3$; Figure 5B). Empty tumor-on-a-chip devices were used as negative controls ($n=3$), because they mimic healthy organs where bacteria are cleared by the immune system. Chambers filled with normal cells accumulate bacteria and would not be appropriate controls. Tumor detecting *Salmonella* were administered to the devices for one hour and flushed from flow channels with an additional eight hours of media flow. After the eight hours, ZsGreen expression was induced with the addition of L-arabinose (time zero, Figure 5C). Eight hours after induction, fluorescence in the tumor cell masses quickly increased (Figure 5C). By 20 hours, more than 90% of the cell masses contained over 100 $\mu\text{g/ml}$ ZsGreen ($n=10$; Figure 5B, C). Effluent samples were averaged over three runs to determine the release of ZsGreen. ZsGreen concentration was determined with antibody dots (Figure 5D). Little ZsGreen was seen in the controls. At 12 hours after induction, the concentration of ZsGreen in the effluent stream was greater than in controls ($P < 0.05$, Figure 5E). Measurements ceased at 24 hours, when bacteria flooded both systems. After 12 hours, ZsGreen was steadily released at an average rate of $23.9 \pm 2.5 \mu\text{g/h}$, which is equivalent to $200. \pm 21 \mu\text{g/h per mm}^{-3}$ of tissue (Figure 5E). Based on measurements of bacterial growth in tumor cell masses,³⁸ (Figure S1), the *per-bacterium* ZsGreen production rate was $1.48 \times 10^{-5} \mu\text{g} \cdot \text{CFU}^{-1} \cdot \text{h}^{-1}$.

DISCUSSION

A bacterial cancer detection system has been created that could identify microscopic tumors by releasing a fluorescent reporter protein. It has been shown previously that *Salmonella* target tumor tissue with high specificity and replicate within the tumor microenvironment.^{15,38–41} In this study, we engineered an attenuated strain of *Salmonella*, VNP20009, to express and release a fluorescent reporter protein after induction with an external inducer molecule (Figure 2). Protein release was found to be predominantly from

living *Salmonella* (Figure 3), which is essential for continual production and release of the biomarker from tumors. A method was developed to detect the released protein that was able to measure concentrations in the ng/ml range (Figure 4). The engineered bacteria colonized microscopic tumor cell masses in a microfluidic device (Figure 5). After induction, they produced and released the reporter protein, which diffused into the flow channel, where it was detectable. In the cell masses, bacteria released ZsGreen at a rate of $8.5 \mu\text{g}\cdot\text{ml}^{-1}\cdot\text{h}^{-1}$ per 0.12 mm^3 of tumor tissue. Detecting microscopic lesions would enable earlier treatment, which could reduce patient mortality rates.

Release of ZsGreen *in vitro* indicates that bacteria could detect small tumors in humans (Figure 6). Incorporating the ZsGreen production rate in the microfluidic device (Figure 5E) into a two compartment pharmacokinetic model (Figure 6A) predicts how blood concentration would depend on tumor size (Figure 6B). In this model, ZsGreen accumulation is equal to the amount produced in tumor tissue minus the amount cleared from the plasma. Based on this model, ZsGreen from a 0.043 mm^3 tumor would be detectable after 24 hours, at a clearance half-life of 24 h (Figure 6B). At 24 h, the ZsGreen in the blood would be greater than the detectable limit with antibody dots (4.5 ng/ml; Figure 4C). This volume is 2,600 to 6,200 times smaller than the current limits of tomographic imaging (6–8 mm in diameter).^{3,4,6} A larger tumor would have a higher rate of ZsGreen production and would be detectable earlier (Figure 6B). A larger tumor would support a greater number of bacteria, which would, in turn, produce more ZsGreen.

Waiting longer to measure the concentration would allow more time for ZsGreen to accumulate in the bloodstream and enable detection of smaller tumors (Figure 6C). If ZsGreen was measured at 72 hours and the clearance half-life was 24 h, the minimum detectable volume would be 0.016 mm^3 . This benefit is dependent on the clearance rate. For fast rates, e.g. $t_{1/2}=1 \text{ h}$, waiting would not affect detectable tumor size (Figure 6C). At times greater than 24 h, the elimination rate and the production rate equalize and the minimum detectable volume limit becomes time independent (Figure 6C). For slow clearance rates, e.g. $t_{1/2}=72 \text{ h}$, the minimum detectable tumor volume at 72 h would be 0.010 mm^3 .

The efficacy of detecting microscopic tumor lesions in the clinic will depend upon 1) the rate of biomarker production, 2) the number of bacteria colonizing neoplastic sites, 3) the tumor volume, and 4) the clearance rate of the biomarker reporter. Increasing the ZsGreen production rate or the maximum bacterial density would enable detection of smaller tumors (Figure 6D). Increasing either of these 10 fold would decrease the detectable tumor volume from 0.016 to 0.0015 mm^3 at a measurement time of 72 hours (Figure 6D). The rate of ZsGreen expression and release could be increased by increasing plasmid copy number or promoter strength. Eliminating rare codons in the ZsGreen DNA sequence would also increase production rates. To increase bacterial density, lipid A could be concurrently administered with the bacteria. Lipid A increases bacterial dispersion throughout tumor tissue by causing a temporary inflammatory response.⁴²

To be tested in animal models or used in humans, detecting bacteria would have to stably express ZsGreen. For long studies in animals, stable expression could be maintained by continual administration of ampicillin.^{43,44} Similarly, L-arabinose has been administered to

animals to induce protein expression from injected bacteria.^{30,45} For short studies, ZsGreen from cultures without ampicillin would maintain the same expression as cultures with ampicillin (Figure S2). In humans, antibiotics could not be used, and the ZsGreen construct would have to be maintained with a balanced lethal system or be incorporated into the *Salmonella* genome.^{46,47}

Active release of ZsGreen by *Salmonella* will greatly enhance the sensitivity of tumor detection. Continuous production would cause ZsGreen to build up in the circulation system until the eliminate rate balances the production rate (Figure 6C; Eq. 1). Waiting longer to measure the blood concentration would enable detection of smaller lesions (Figure 6C, D). In culture, over 90% of ZsGreen was actively secreted by living *Salmonella* (Figure 3). Release from dead bacteria would not be continuous and would limit efficacy. If bacteria produce ZsGreen in a single batch after injection, only a pulse of ZsGreen would appear in the blood and it would be more difficult to detect.

The difference in release rates between *Salmonella* and *E. coli* (Figure 3A), indicates that *Salmonella* actively secrete ZsGreen. Some of the major differences between these two organisms are their secretion mechanisms. Gram negative bacteria have seven classes of secretion systems that are activated in different environments to transport proteins across the cell envelope.^{48–52} *Salmonella* and *E. coli* contain many of the same secretion genes, although not all are functional.⁵³ For example, *Salmonella* and K-12 *E. coli* both activate type III secretion systems when quorum sensing is activated.^{54,55} However, DH5 α *E. coli* (a K-12 substrain), have a quorum sensing deficiency and are unable to synthesize autoinducer-2.⁵⁶ As a result, DH5 α *E. coli* cannot activate quorum-sensing-related secretion machinery.^{54,57,58} This difference suggests that type III secretion, or another quorum-sensing-related mechanism, is used by *Salmonella* to secrete ZsGreen. This difference in active secretion also shows that *Salmonella* would be superior to DH5 α *E. coli* for tumor detection.

Efficient molecular transport through tumor tissue is necessary for effective intravenous detection (Figure 1). For the biomarker to be present in the blood or the device effluent, it must diffuse through tissue and not be sequestered by cancer cells. Similarly, if the gene inducer does not diffuse through tissue, gene expression would not be activated. Appearance of ZsGreen in the effluent of the microfluidic device (Figure 5E) shows that both ZsGreen and L-arabinose diffuse through tumor cell mass. It also shows that *Salmonella* can both produce and release the protein when colonized in a tumor mass. The increase of ZsGreen throughout the chambers (Figure 5B–C) shows that L-arabinose was rapidly delivered. We have previously shown with another system that L-arabinose can activate gene expression throughout tumor tissue.⁴⁶ In this system, over 90% of the cell mass in the device contained at least 100 $\mu\text{g/ml}$ of ZsGreen after 24 hours (Figure 5C).

The inclusion of a triggered gene expression system is essential for bacterial detection. Systemic administration of the inducer molecule (L-arabinose) is necessary to activate protein production after bacteria have cleared from normal tissue. It is critical that tumor-detecting bacteria are not making the protein biomarker when they are injected into the body. After systemic injection of *Salmonella*, it takes approximately three days for them to

clear from healthy organs.²⁰ If a biomarker was constitutive produced, bacteria in healthy organs would express it during this period and obscure detection of malignant lesions. To prevent expression in normal tissue, the tightly regulated *pBAD* promoter would not express the biomarker until administration of L-arabinose.

Using these engineered bacteria after resection of a primary tumor could provide a fast and easy method to detect tumor recurrence or the onset of metastatic disease. Detecting tumors with bacteria would be minimally invasive. Measurement would require injection of bacteria followed by a single blood draw three days later. How the system is used will depend on whether it can be administered repeatedly. An immune response that produces anti-ZsGreen antibodies could induce adverse side effects and could be detrimental to the single-layer dot technique. In mice, it takes at least one week to develop antibodies, suggesting that a single administration would be possible.⁵⁹ Patients could receive a screening dose at a set period of time after surgery to detect recurrence or metastatic spread.

If repeated doses are possible, the utility of the technique would be greater. We envision that after surgery to remove a primary tumor, patients would receive an initial dose of tumor-detecting bacteria to establish a baseline plasma level of ZsGreen. Patients would then receive periodic screenings with bacteria. Signal increases would indicate recurrence or formation of secondary cancers. This system could also be used to determine the aggressiveness of a malignant mass or monitor tumor growth in response to treatment. The mathematical model predicts that the magnitude of ZsGreen signal is indicative of the volume of a malignant cell mass (Figure 6B). If a tumor were to grow, the number of bacteria would increase proportionally. Similarly, if a tumor shrinks in response to therapy, the number of colonized bacteria would decrease. In this way, monitoring tumor growth with bacteria could determine if a therapy is effective.

CONCLUSIONS

We have developed a bacterial diagnostic tool to detect microscopic tumor masses. Genetically modified tumor-targeting bacteria were engineered to release a reporter protein for detection. An *in vitro* tumor model was used to test tumor targeting and production of the reporter molecule in 3D tumor cell masses. Production of ZsGreen from tumor-targeting *Salmonella* detected microscopic 0.12 mm³ tumor masses. Mathematical analysis of these results predicts detection of tumor masses as small as 0.043 mm³ in humans, more than 2,600 fold smaller than the current limits of tomography. These results demonstrate the feasibility of a bacterial detection system for production of an exogenous biomarker from tumor tissue. This method has the potential to non-invasively monitor treatment, detect tumor masses that are invisible to current techniques, and increase life expectancy.

MATERIALS AND METHODS

Plasmids and bacterial strains

Bacteria were grown in LB broth at 37°C and 225 rpm. Two bacterial strains were used: attenuated *Salmonella enterica* serovar Typhimurium, strain VNP20009 (*purI*⁻, *msb*⁻, and *xly*⁻), and *Escherichia coli*, strain DH5α, (Invitrogen, Carlsbad, CA). ZsGreen expression

was controlled by transformation with plasmid pDF02, which contains the *ZsGreen* gene under control of the L-arabinose inducible promoter, P_{BAD} .⁴⁶ This plasmid contains the puc19 origin of replication and ampicillin resistance. To produce purified ZsGreen, a 6x histidine tag was added to the n-terminus of *ZsGreen* in pDF02, creating plasmid pZsG-His, which was transformed into *E. coli* DH5 α . All transformed cultures were supplemented with 100 μ g/ml ampicillin. ZsGreen expression was induced with 0.2% w/v (2 mg/ml) L-arabinose.

ZsGreen release

ZsGreen release was determined by measuring the concentration in the extracellular and intracellular fractions of liquid cultures. *Salmonella* were inoculated into LB from single colonies, grown overnight at 37°C, subcultured, and grown to an optical density (at 600 nm) of 0.4. ZsGreen expression was induced with 0.2% L-arabinose, and fluorescence was measured for 40 hours. Bacterial density was measured turbidimetrically at 600 nm (BioTek Instruments, Winooski, VT) and converted with factor of 5×10^8 CFU·ml⁻¹·OD₆₀₀⁻¹. Extracellular samples were acquired by centrifuging 1 ml of culture and collecting 200 μ l of supernatant. Intracellular fractions were acquired by resuspending cell pellets in 1 ml of fresh LB broth to equalize volumes with supernatant samples. Fluorescence of 200 μ l samples was measured in a microtiter plate reader with 460 nm excitation and 505 nm emission filters (Molecular Devices, Sunnyvale, CA). Background fluorescence of LB broth was subtracted from all measurements.

Fluorescence measurements were converted to concentration units by determining the fluorescence of pure ZsGreen. Cultures of *E. coli* containing pZsG-His were induced with 0.2% w/v L-arabinose and grown overnight. Bacteria were lysed with lysozyme and vortexed on ice with glass beads for 20 minutes. ZsGreen was purified from the lysate by immobilized-metal-ion (nickel) affinity chromatography. The ZsGreen concentration in the eluent was determined by colorimetric Bradford assay. Fluorescence of the eluent was determined by spectrometry as above. Serial dilutions were used to determine a conversion factor between fluorescence units and concentration. The average rate of ZsGreen release was determined by linear fitting to the measurements from 0 to 40 hrs.

ZsGreen detection in blood

ZsGreen was diluted in bovine blood to determine the detectable limit of the fluorescence signal. Cultures of *Salmonella* with pDF02 were induced at 0.4 OD₆₀₀ with 0.2% L-arabinose, and grown for 24 hours. Supernatant fractions from these cultures were sterile filtered through a 0.22 μ m filter and serially diluted in bovine blood or plasma (Hemostat Laboratories, Dixon, CA) in ten-fold increments. Plasma was isolated from whole blood by centrifugation for 10 min. Fluorescence was measured in a plate reader with 460 nm excitation and 505 nm emission filters. Intensities were normalized to the auto-fluorescence of blood and plasma, respectively.

Viability of ZsGreen-releasing bacteria

Bacterial viability was measured to determine the relationship between ZsGreen release and cell integrity. *Salmonella* and *E. coli* were induced with 0.2% L-arabinose at an optical

density OD₆₀₀ of 0.4 and grown for 24 hours, as described above. Cultures were centrifuged. The fluorescence of the supernatant and cell pellet, at equalized volumes, was determined and normalized by the density.

To determine the ratio of ZsGreen released by live and dead bacteria, ZsGreen-expressing bacteria were grown for 24 hours in LB and induced with 0.2% L-arabinose. At 24 hours, 1 ml of culture was centrifuged and resuspended in an equal volume of PBS. Suspension in PBS halted growth, because PBS contains only salts and no carbon source. Preventing growth enabled determination of release from one generation of bacteria. Resuspended bacteria were stained by adding an equal volume of a PBS solution containing 20 μ M ethidium homodimer-1 (ThermoFisher, Waltham, MA) and 25 μ M 4',6-diamidino-2-phenylindole (DAPI). Ethidium homodimer-1 stains dead cells and DAPI stains cell nuclei and identifies all bacteria. Ten μ l of the stained cell suspension was applied to glass slides and covered with a coverslip to prevent evaporation. For 11 hours, fluorescence from the bacteria on the slide was observed by microscopy (Olympus, Center Valley, PA) at 10X magnification. Three fluorescence filter sets (Chroma, Rockingham, VT) were used: DAPI (EX, 358 nm; EM, 461nm), ZsGreen (EX, 496 nm; EM, 506 nm), and ethidium homodimer-1 (EX, 528 nm; EM, 617 nm). Acquired images were overlaid to determine the location of individual bacteria from the DAPI stain. Red and green fluorescence intensities were determined for each bacterium. Background fluorescence was subtracted from these intensities. The viability threshold was determined by starving bacteria of all nutrients for 48 hours and staining with ethidium homodimer-1. ZsGreen fluorescence intensity was normalized by the brightest bacterium in all images. The fraction released from viable and dead bacteria was determined by comparing the integral of green fluorescence (ZsGreen content) over the 11 hours of observation.

Single-layer antibody dots

An antibody-based method was developed to improve detection of ZsGreen. Small circles of nitrocellulose membrane were cut using a 1.5 mm diameter biopsy punch. Antibody dots were prepared by coating with a pan-polyclonal antibody against α -reef coral fluorescent proteins (RCFP; Clontech, Madison, WI) that was diluted 1:10 in PBS, and stirring overnight at 4°C. To test sensitivity, supernatant was isolated from a 24-hour culture of ZsGreen-producing *Salmonella*. The supernatant was serially diluted in PBS at ratios of 1:1; 1:10; 1:100; 1:1,000; 1:10,000; 1:10⁵; 1:10⁶; 1:10⁷; 1:10⁸ and 1:10⁹. These solutions (200 μ l) were added to antibody dots in 96 well plates, which were incubated and rocked overnight at 4°C. The fluorescence of the antibody dots was acquired by microscopy (Olympus) and the fluorescence of diluted solutions was measured using a spectroscopic plate reader (Molecular Devices). The fluorescence intensity of each dot was quantified by averaging the intensity over the entire area. Images were acquired at three exposure times, 100, 400, and 1000 ms, to accommodate the range of the CCD camera (Hamamatsu, Shizuoka pref, Japan). The background fluorescence of antibody dots treated with PBS was subtracted from all measured intensities. The detection limit was defined as the intensity at which fluorescence measurements did not change with decreasing concentration. All antibody-dot fluorescence measurements values were normalized by this value. Spectroscopy measurements were background subtracted and similarly normalized.

ZsGreen release from bacteria colonized in tumor masses *in vitro*

A microfluidic tumor-on-a-chip device was used to determine the release rate of ZsGreen release from bacteria colonized in solid tumor masses. Devices were fabricated using soft lithography techniques described previously.^{35,38} The device pattern consisted of a flow channel bordering six chambers with filters at the rear end to trap injected spheroids (Figure 5A). Molds of the microfluidic pattern were formed on silicon wafers coated with SU-8 polymer by photolithography. A 1:10 mixture of Sylgard 184 polydimethylsiloxane (PDMS; Dow Corning, Midland MI), was cast onto the molds, vacuum pumped to remove bubbles, and heated overnight at 55°C. Holes were punched for the inlets and outlet channels using a 1.5 mm diameter biopsy punch. PDMS reliefs were cleaned with ethanol and treated with oxygen plasma to adhere to glass slides. Prior to inserting cells, devices were sterilized and degassed by flushing with 70% ethanol and PBS.

LS174T human colon adenocarcinoma cells were grown in Dulbecco's modified Eagles medium (DMEM) with 10 % fetal bovine serum (FBS), and incubated at 37 °C and 5% CO₂. To form spheroids, cells were suspended in flasks coated with poly(2-hydroxyethyl methacrylate), and fed every two days for three weeks. Spheroids were inserted into device chambers through flow channels, and were incubated at 37°C in an enclosed environment on the microscope stage. The medium flow rate through the device was 3.0 µl/min and pH was maintained with 25 mM HEPES.

ZsGreen-expressing *Salmonella* at were administered to the microfluidic devices for one hour at a density of 2×10^6 CFU/ml in DMEM with 10% FBS, 25 mM HEPES and 100 µg/ml ampicillin. Bacteria were administered to devices with empty chambers to serve as tumor-free negative controls. Chambers filled with normal cells would not have been suitable controls because they would have accumulated bacteria. Three-dimensional tissue grown from normal cells contains necrotic tissue, which would promote colonization, and has no immune system to clear bacteria. An empty chamber, where there is no environment for growth, was more representative of bacterial accumulation in normal tissue *in vivo*. After bacterial addition, devices were supplied with bacteria-free medium for eight hours to mimic systemic clearance. After this clearance period, medium containing 0.2% L-arabinose was administered to induce ZsGreen expression. Effluent from the devices was collected every four hours, immediately placed on ice, and sterile filtered to remove bacteria.

Images of tumor cell masses were acquired throughout by fluorescence microscopy (Olympus). To capture an entire chamber, two images were tiled together using a macro in IPLab (*BD Bioscience*, Rockville, MD). Transmitted light and fluorescence images were captured at 1 hour intervals and analyzed with ImageJ (NIH Research Services Branch). Fluorescence intensities were calibrated by flowing a 279.5 µg/ml solution of ZsGreen in PBS through the device. For each chamber, the total tissue area and the area with a ZsGreen concentration greater than 100 µg/ml was determined from transmitted and fluorescence images, respectively. The ZsGreen concentration in the effluent medium was determined by incubating 200 µl with antibody dots in 96-well plates overnight as described above. The fluorescence intensity of each dot was measured at an exposure of 400 ms and the background fluorescence of dots treated with fresh medium was subtracted.

The ZsGreen production rate was determined from a mass balance around the device.

$$FC_Z = K_P C_B V_T \quad (1)$$

Here, F is the flow rate in the channel, C_Z is the concentration of ZsGreen, K_P is the per-bacterium production rate, C_B is the bacterial density, and the V_T is the volume of the cell mass. The per-tissue ZsGreen production rate is given by $K_P \cdot C_B$. Bacterial density in the device was estimated from previously measured profiles of bacterial growth).³⁸ A Gompertz expression⁶⁰ was used to model bacterial growth in devices based on previous measurements³⁸.

$$C_B = C_{B,max} \exp \left[-\exp \left(\frac{\mu_m \exp(1)}{C_{B,max}} (\lambda - t) + 1 \right) \right] \quad (2)$$

Two Gompertz parameters were calculated by least squares regression analysis: the maximum growth rate, μ_m (CFU·ml⁻¹·h⁻¹); and the lag time, λ (h). Maximum bacterial density, $C_{B,max}$ (CFU·ml⁻¹) was determined by simultaneously solving equations (1) and (2) for $C_{B,max}$ and production rate, K_P , using least squares regression to the experimental ZsGreen production data.

Mathematical prediction of minimum detectable tumor volume

A two-compartment pharmacokinetic model was used to predict the detection limits *in vivo*. In this model, ZsGreen accumulation is equal to the amount produced in tumor tissue minus the amount cleared from the plasma.

$$V_p \frac{dC_Z}{dt} = K_P C_B V_T - K_e V_p C_Z \quad (3)$$

The concentration of ZsGreen, C_Z (µg·ml⁻¹), depends upon the bacterial production rate, K_P (µg·CFU⁻¹·h⁻¹); the bacterial density, C_B (CFU·ml⁻¹); the tumor volume, V_T (ml); the elimination rate, K_e (h⁻¹); and the plasma volume, V_p (ml). This ordinary differential equation was solved using a 4th order Runge-Kutta approximation. The rate of ZsGreen production, K_P , was determined from the microfluidic experiments and bacterial density, C_B , was determined from previous measurements in the microfluidic device.³⁸ The minimum detectable tumor volume was determined by calculating the volume that would produce the minimum detectable ZsGreen concentration with the antibody dot technique. The effects of clearance and measurement time on detection was determined by calculating the tumor volume that would produce the minimum detectable ZsGreen concentration over a 72 hour period for four half-lives. Clearance rate is inversely proportional to half-life: $K_e = \ln(2)/t_{1/2}$. The effect of increasing bacterial density or ZsGreen production was determined by increasing the production term, $K_P \cdot C_B$, in Eq. 3 and determining the minimum detectable volume as a function of time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Insight, innovation, integration

We developed a method to detect microscopic tumor masses. This technique couples the specificity of tumor-targeting bacteria with the sensitivity of an exogenous biomarker. With a microfluidic tumor on-a-chip device, we show that tumor-targeting *Salmonella*, equipped to express and release an exogenous fluorescent protein, have the potential to detect microscopic tumors. This system could provide a sensitive, minimally invasive method to detect tumor recurrence, monitor treatment efficacy, and identify the onset of metastatic disease.

Figure 1. Concept of bacterial tumor detection

This illustration shows how bacterial detection would function *in vivo*. The method combines biomarker detection with bacterial selectivity for tumors. After intravenous delivery, engineered *Salmonella* would preferentially accumulate in tumor tissue (1) and proliferate (2). Expression of a biomarker (stars, here ZsGreen) would be triggered by a small inducing molecule (3). The biomarker would be released from the bacteria (4) and diffuse into the blood stream (5). Biomarker molecules would be measured using specific antibodies (6) and the concentration would indicate the presence and size of tumor masses. These mechanisms were quantified by administering tumor-detecting bacteria to a microfluidic tumor-on-a-chip device that mimics tissue surrounding blood vessels in tumors.

Figure 2. ZsGreen was released by *Salmonella* and was detectable when suspended in blood
A) After induction (time 0), transformed *Salmonella* produced ZsGreen. At 10 h, the intracellular concentration was greater than the extracellular concentration (*, $P < 0.05$; $n = 3$). At 40 h, the extracellular concentration was greater (*, $P < 0.05$). The total concentration is the sum of the intracellular and extracellular concentrations. **B)** ZsGreen was isolated from liquid culture supernatant and serially diluted in bovine blood and plasma. The fluorescence intensity was greater than background fluorescence at concentrations of 0.528 μg/ml in plasma and 52.8 μg/ml in whole blood (*, $P < 0.05$). Intensity values were normalized by the autofluorescence of whole blood.

Figure 3. ZsGreen was released from viable *Salmonella*.

A) In liquid culture, *Salmonella* expressed (total) and released (extracellular) more ZsGreen than *E. coli* (*, $P < 0.05$). **B)** *Salmonella* were stained with DAPI (blue) and ethidium homodimer (EtHd-1, red) to identify viable (blue only) and dead (both red and blue) bacteria. In the merged image, most ZsGreen expressing bacteria (green) are viable (not red). **C)** Fluorescence from ZsGreen and EtHd-1 (cell death) of individual *Salmonella* in microscopy images (**B**). Bacteria were grouped into viable and dead (solid vertical line) based on control measurements. ZsGreen fluorescence was normalized by the greatest intensity measured in an individual bacterium and multiplied by 100. Cell death was normalized by the fluorescence of positive-control bacteria. **D)** Bacterial viability was greater than 85% for 11 hours of incubation in PBS. **E)** Over time, ZsGreen fluorescence decreased in all bacteria. This rate of ZsGreen release was greater from viable compared to dead cells ($P < 0.05$). **F)** More ZsGreen was released by viable cells (*, $P < 0.05$).

Figure 4. Single-layer antibody dots

A) Antibody dots were formed by binding anti-ZsGreen antibodies to 1.5 mm diameter circles of nitrocellulose membrane. ZsGreen concentrations were measured by incubating solutions overnight and measuring dot fluorescence. **B)** The fluorescence of antibody dots decreased with decreasing ZsGreen concentration. Higher camera exposure times were used to detect lower concentrations of ZsGreen. **C)** Fluorescence increased for increasing concentrations of ZsGreen. Intensities were normalized by the lowest detectable value. Dot measurements greater than 4.5 ng/ml and spectroscopic measurements greater than 1,000 ng/ml were significantly greater than auto-fluorescent values (*, $P < 0.05$).

Figure 6. Mathematical predictions of detection efficacy

A) Two compartment model of ZsGreen production and clearance. ZsGreen is produced at rate K_P by bacteria at density C_B in a tumor of volume V_T . The concentration of ZsGreen C_Z in the plasma, with volume V_P , decreases by clearance rate K_e . **B)** Predictions of ZsGreen concentrations in human plasma using parameters derived from device measurements and the two compartment pharmacokinetic model in (A). A 0.043 mm³ tumor, with a nominal clearance half-life of 24 h, would produce the limiting ZsGreen concentration by 24 h. Larger tumors, 0.087 and 0.174 mm³, would produce more ZsGreen and be detectable by 14.2 and 17.2 h. The lag in ZsGreen production is caused by the predicted delay in bacterial growth ($\lambda=10.3$ h; Figure S1). **C)** The volume of the smallest detectable tumor decreases as the time of measurement is increased and the when the clearance half-life ($t_{1/2}$) is longer. **D)** Increasing the production rate of ZsGreen (K_P) or the bacterial density in tumors (C_B) would reduce the volume of the minimum detectable tumor.