

**Engineered bacteria detect spatial profiles in glucose concentration within solid  
tumor cell masses<sup>†</sup>**

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## ABSTRACT

Tumor heterogeneity makes cancer difficult to treat. Many small molecule cancer drugs target rapidly dividing cells on the periphery of tumors but have difficulty in penetrating deep into tumors and are ineffective at treating entire tumors. Targeting both rapidly dividing and slower growing regions of tumors is essential to effectively treat cancer. A cancer drug carrier that penetrates deep into tumors and identifies metabolically activity could supply treatment to those areas based on the local microenvironment. We hypothesized that glucose sensing bacteria could identify sugar gradients in solid tumors. To test this hypothesis, a genetic circuit was designed to trigger expression of a green fluorescent protein (GFP) reporter through the chemotaxis-osmoporin fusion protein, Trz1, a receptor for sensing glucose and ribose sugars. *E. coli* equipped with the Trz1-GFP expression system, were administered to an *in vitro* model of a continuously perfused tumor tissue that mimics systemic delivery and clearance of bacteria through a blood vessel adjacent to a solid tumor. The level of GFP expressed, per bacterium, was time independent and indicated the glucose concentration as a function of penetration depth within the microfluidic tumors. The measured glucose concentration, correlated (P-value =  $2.6 \times 10^{-5}$ ) with tumor cell viability as a function of depth. Mathematical analysis predicted drug delivery by glucose-sensing bacteria would eliminate a higher percentage of the viable tumor cell population than a systemically administered drug. Glucose-sensing bacteria could deliver cancer therapies with increased drug penetration and nutrient-dependent dosing to continuously treat viable regions of cancer tissue that have a higher prevalence for metastatic dissemination. This article is protected by copyright. All rights reserved

**Key Words:** Cancer, *E. coli*, bacterial cancer therapy, biosensor, Trz1

## INTRODUCTION

Effectively treating all cancer cells in a tumor is necessary to reduce the likelihood of metastatic disease. Tumor heterogeneity is the main cause of transport limitations that hinder nutrient and drug uptake. This heterogeneity leads to changes in cell growth and metabolism that impede effective treatment of all malignant cells (Chary and Jain, 1989; Jain, 1988; Jain, 1994; Jain, 1999; Sutherland and Durand, 1984; Trédan et al., 2007). Most cancer drugs are small molecules that rely on passive diffusion to treat rapidly dividing cells through mechanisms of action that target cell division. Due to transport limitations, these drugs do not penetrate tumors well and are ineffective at treating all viable tumor cells (Jain, 1987). Glucose availability affects tumor growth and metabolic activity (Gullino et al., 1967) and correlates with increased probability of metastatic dissemination (Tubiana et al., 1989). A cancer drug carrier able to 1) penetrate tumor tissue and 2) identify glucose concentration could target viable regions of cancer cells throughout tumors and improve treatment outcomes.

Chemotactic bacteria, like *E. coli*, naturally overcome many of the transport limitations in tumors because they actively swim and penetrate deep into tumor tissue. Gram-negative bacteria colonize tumor tissue with high specificity at ratios as high as 10,000-fold over healthy organs (Forbes et al., 2003; Ganai et al., 2011; Zhao et al., 2005) and proliferate selectively throughout tumor tissue (Li et al., 2003; Low et al., 2004; Pawelek et al., 1997; Yu et al., 2004; Zhao et al., 2005). Chemotaxis toward glucose can affect bacterial localization within solid tumor cell masses (Kasinskas and Forbes, 2007). Reprogramming the chemotaxis pathway to link a genetic output to sensing receptors could identify glucose concentrations gradients within the tumor microenvironment.

A hybrid receptor has previously been created to sense glucose concentration and trigger recombinant protein expression. The fusion protein, Trz1, combines the periplasmic domain of chemotactic receptor, Trg, to the cytoplasmic domain of osmoporin sensor, EnvZ, to induce expression through the osmoporin promoter  $P_{OmpC}$  (Fig. 1; Baumgartner et al., 1994). The endogenous Trg chemotaxis receptor senses ribose, glucose, and galactose concentration through respective periplasmic

binding proteins and triggers protein phosphorylation events that result in bacteria motility (Barnakov et al., 1998; DeFranco et al., 1979; Park and Hazelbauer, 1986). The endogenous EnvZ osmoporin senses solute concentration and regulates formation of membrane pores through activation of promoter,  $P_{OmpC}$  (Mizuno et al., 1983). Differences in solute concentration cause conformational changes in the histidine kinase tail of EnvZ leading to phosphorylation of OmpR, a cytoplasmic phosphotransferase, which activates transcription of *ompC* (Forst et al., 1989; Slauch et al., 1988). In the hybrid Trz1 receptor, the ligand binds to its respective binding proteins and interacts with the with Trg periplasmic receptor domain of Trz1. This binding causes conformational changes in the cytoplasmic tail (EnvZ) leading to OmpR phosphorylation and activation of transcription in the *ompC* promoter,  $P_{OmpC}$  (Baumgartner et al., 1994). Tumor-targeting bacteria equipped with the Trz1 receptor could directly sense glucose gradients within the tumor microenvironment by expression of a fluorescent reporter.

Combining the glucose sensing capabilities of the Trz1 receptor with the tumor-targeting ability of bacteria has the potential to improve treatment by increasing drug penetration and providing continuous nutrient-dependent method for drug delivery to tumors. We hypothesized that Trz1-mediated glucose sensing could measure glucose gradients in solid tumors and that glucose concentration would reflect the viability of tumor cells. To test this hypothesis, *E. coli* were equipped with Trz1 regulating expression of GFP (Fig. 1). The bacterial ability to sense glucose was measured in liquid culture and in microfluidic tumor cell masses. Bacteria were administered to solid tumors in tumor-on-a-chip microfluidic devices that were designed to mimic nutrient and oxygen gradients in tumor tissue adjacent to a blood vessel (Walsh et al., 2009). Glucose concentration gradients in the microfluidic tumor cell masses were quantified by GFP expression and compared to previously measured cell viability profiles (Kasinskas et al., 2014). Developing a drug carrier with increased tumor penetration and the ability to sense tumor metabolic activity could improve treatment by delivery of recombinant protein drugs directly to different regions of tumors based on local microenvironment.

## MATERIALS AND METHODS

**Plasmid constructions.** Plasmid pOmpC-GFP was generated by insertion of promoter,  $P_{OmpC}$ , from pMY150 (Mizuno et al., 1983) into plasmid pFVP25 (Valdivia and Falkow, 1996) to control expression of green fluorescent protein (GFP), GFPmut3. Plasmid pTrz1a was generated by inserting *Trz1*, from plasmid pRB020 (Baumgartner et al., 1994), downstream of pOmpC-GFP, under control of the  $P_{tac}$  promoter, separated by the t1 terminator (BBa\_B0010, Registry of Standard Biological Parts).  $P_{tac}$  is a derivative of the *lac* promoter, which in the presence of the repressor *lacIq*, is inducible with the addition of isopropyl beta-D-thiogalactoside (IPTG; de Boer et al., 1983). Plasmid pTrz1 was generated by replacing the  $P_{tac}$  promoter of pTrz1a with the  $P_{BAD}$  promoter. Plasmid pTrz1-red was created by adding a third operon in pTrz1 containing  $P_{lac}$  mediated DsRed-Express2 red fluorescent protein (RFP) expression. All plasmids and strains used for this work are listed in Table 1.

**Strains and growth conditions.** For cloning purposes, DH5 $\alpha$  *E. coli* (New England Biolabs Inc., Ipswich, MA) were grown in LB, supplemented with 100  $\mu$ g/ml ampicillin or 34  $\mu$ g/ml chloramphenicol. Salt sensitivity of the *ompC* promoter,  $P_{OmpC}$ , was measured in DH5 $\alpha$ , which contains the endogenous *EnvZ* osmosensor. *E. coli*, HB3521, is a derivative of *E. coli* MH225 (CP919), which contains the ribose binding protein, *RbsB* on plasmid pAI12 and a genomic deletion of *Envz*,  $\Delta EnvZ::Km^r$  (Baumgartner et al., 1994). TOP10F' *E. coli* (Life Technologies) were used to study the effect of Trz1 receptor abundance on signal transduction in plasmid pTrz1a. TOP10F' *E. coli* contain the *lacIq* repressor and endogenous *EnvZ*. IPTG inducibility was used to control Trz1 expression and evaluate the effect of receptor over expression on GFP production.

**In vitro sensing in aqueous solutions.** Salt sensitivity of the OmpC promoter,  $P_{OmpC}$ , was measured by increasing salt concentration of liquid cultures of *E. coli* in the presence or absence of endogenous *EnvZ* osmosensor. Plasmid pOmpC-GFP was transformed into DH5 $\alpha$  (*EnvZ*) or HB3521 ( $\Delta EnvZ$ ) and grown in overnight liquid cultures of LB broth supplemented with 0.17 M or 0.27 M NaCl. To determine the relationship between Trz1 receptor abundance and signal transduction, plasmid

pTrz1a was transformed into TOP10F' *E. coli*, containing the *lacIq* repressor and grown in LB (10g/L NaCl and 1g/L glucose) supplemented with IPTG. To determine if  $P_{BAD}$  regulation of Trz1 was sufficient for sugar sensing, bacteria, pTrz1-HB3521, pTrz1a-HB3521, and pOmpC-GFP-HB3521, were grown in M9 minimal media with 0.5% casamino acids and 1.1 M glucose. To test the sensitivity of the Trz1 receptor, bacteria pTrz1-red-HB3521, were grown in M9 minimal media with the increasing glucose concentrations. Expression of GFP was measured in a microtiter plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA), with 495 nm excitation and 511 nm emission. Bacterial density was measured turbidimetrically at 600 nm (BioTek Instruments, Winooski, VT), with an OD of 1 equal to  $5 \times 10^8$  CFU/ml. GFP and optical density measurements were background subtracted and the GFP intensity was normalized to 1.0 at the minimum.

**Tumor cell line and spheroid formation.** Human LS174T colon adenocarcinoma cells (ATCC, Manassas, VA) were grown in high glucose Dulbecco's modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). Spheroids were made by plating single cell suspensions of  $2.5 \times 10^4$  cells per ml on non-adherent poly(2-hydroxethyl methacrylate)-coated flasks (Toley et al., 2013).

**Bacteria pTrz1-red sensing of glucose concentration in solid tumor cell masses in vitro.** The generation of 3D microfluidic devices has been described previously (Walsh et al., 2009). Bacteria, pTrz1-red-HB3521, were grown in overnight cultures of M9 minimal media supplemented with 0.5% casamino acids, and without glucose, to prevent premature GFP expression. At time zero, bacteria were administered to the flow channel of the devices at a density of  $1 \times 10^6$  colony forming units per milliliter ( $\text{CFU} \cdot \text{ml}^{-1}$ ) in PBS solution at a flow rate of  $3 \mu\text{l}/\text{min}$ . After 1 hour, bacterial infusion was replaced with growth medium (high glucose DMEM +10% FBS supplemented with 25mM HEPES, 100 $\mu\text{g}/\text{ml}$  ampicillin and 34 $\mu\text{g}/\text{ml}$  chloramphenicol) was administered to devices at  $3 \mu\text{l} \cdot \text{min}^{-1}$ .

Images of tumor cell masses were acquired by fluorescence microscopy (Olympus, Waltham, Massachusetts). Transmitted light and fluorescence images were captured at 20, 26, 32, 38 and 44

hours after bacterial administration and analyzed using ImageJ (NIH Research Services Branch). RFP and GFP intensities were background subtracted by the minimum pixel intensity of an empty chamber and normalized by the intensity of LS174T tissue autofluorescence. RFP intensity was calibrated to bacterial density by administration pTrz1-red-HB3521 at three densities,  $3 \times 10^8$ ,  $1.5 \times 10^9$ , and  $3 \times 10^9$  CFU·ml<sup>-1</sup> to tumor-on-a-chip microfluidic devices.

***Bacterial measurement of glucose concentration in LS174T microfluidic tumor cell masses.***

Glucose concentration in tumor-cell masses was calculated from the amount of GFP expressed per bacteria as a function of depth. To calculate the glucose concentration within tumor cell masses, the GFP and RFP intensities were divided to generate relative intensity vs. distance plots at each time point. The average ratio of GFP to RFP was assumed to be directly proportional to the glucose concentration within the microfluidic tumor cell masses. The maximum GFP/RFP ratio, at the edge of the tissues, was equal to the concentration of glucose in growth media, 5500 μM, and scaled linearly with the intensity ratio.

***Glucose consumption rate in LS174T tumor cell masses.*** The consumption rate of glucose by LS174T cells was determined from the glucose concentration profiles. At steady state, the rate of diffusion was equivalent to the glucose consumption rate in a rectangular slab of tumor tissue.

$$D_g \frac{\partial^2 C_g}{\partial x^2} - k_g C_g = 0 \quad (1)$$

In this balance,  $C_g$  is the concentration of glucose,  $t$  is time,  $D_g$  is the diffusion coefficient for glucose in tumor tissue,  $x$  is the depth into the tumor, and  $k_g$  is the first-order consumption rate by LS174T tumor cells. The solution to Eq. 1 is

$$C_g = C_{g0} e^{-\sqrt{\frac{k_g}{D_g}} x} \quad (2)$$

At the channel boundary, the glucose concentration,  $C_{g0}$ , was 5500 μM. Deep in the tissue the concentration was zero. The effective diffusivity of glucose,  $D_g$ , in multicellular tumor spheroids was

$3.9 \times 10^{-7} \text{ cm}^2/\text{s}$  (Casciari et al., 1988). The consumption rate of glucose,  $k_g$ , ( $\text{s}^{-1}$ ), was found by least-squares regression of Equation 2 to the measured glucose concentration profile.

**Measurement of caspase-3 activity, apoptosis and tumor cell viability.** Caspase-3 activity in LS174T tumor cell masses has previously been measured and is an indicator of apoptosis (Kasinskas et al., 2014). The fraction of apoptotic cells as a function of penetration depth in tumor cell masses was assumed to directly reflect tumor cell viability. The average value reported is a hyperbolic tangent function fit by least squares regression to the data and was used for the initial condition of tissue viability for the mathematical analysis.

**Simulation of drug delivery and cancer cell death in solid tumors.** A mathematical model of Trz1-mediated bacterial drug delivery was compared to a model of systemically delivered free drug to predict efficacy of tumor treatment. Treatment of tumor was described by tumor cell viability over space and time. Tumor tissue cell death was a function of drug concentration,  $C$ , and the maximum death rate,  $\mu_{max}$ , and the saturation constant,  $K_m$  (Toley et al., 2013).

$$\frac{\partial l}{\partial t} = \left( \frac{-\mu_{max}C}{K_m + C} \right) l \quad (3)$$

The live cell fraction,  $l$ , for the model system was initially set to the average profile for cell viability previously measured in LS174T tumor cell masses (Kasinskas et al., 2014).

The concentration of systemically delivered drugs within a tumor was described by the transient diffusion equation:

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} \quad (4)$$

In this equation,  $C$  is the concentration of drug,  $D$  is the effective diffusivity in tumor tissue,  $x$  is distance from the tumor edge,  $C_0$  is the initial concentration in the blood,  $k$  is the systemic clearance rate of the drug, and  $t$  is time. At the channel boundary, the concentration decreased exponentially,  $C = C_0 e^{-kt}$ . Deep in the tissue, the concentration was zero. Initially, the concentration was zero throughout. The drug parameters used for mathematical analysis were:  $D = 0.05 \text{ mm}^2 \cdot \text{h}^{-1}$ ,  $C_0 = 1 \mu\text{M}$ ,



$k=0.2\text{h}^{-1}$ ,  $\mu_{max}= 20 \text{ h}^{-1}$ , and  $k_m=0.2\mu\text{M}$ . The solutions reported are dimensionless drug concentration and the resulting cell viability.

The concentration of drug delivered by sugar sensing Trz1 bacteria was described by transient diffusion with a drug generation term represented by the experimentally measured rate of Trz1-mediated GFP production:

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} + \frac{\alpha}{2} \left( 1 - \tanh \left( \frac{x-p}{2w} \right) \right) \quad (5)$$

At the tissue boundary, the concentration was  $\frac{\alpha}{2} \left( 1 - \tanh \left( \frac{-p}{2w} \right) \right)$ . Deep in the tissue and initially the concentration was zero. The production rate of recombinant protein drug was described as a hyperbolic tangent function, where  $\alpha$  is maximum production rate,  $p$  is the mid-point for transition between high and low expression and  $w$  is the slope of the transition. These parameters were found by least squares regression of the hyperbolic tangent function to the GFP production rate measured in tumor-on-a-chip microfluidic device, after dimensionless normalization in time and space, where  $\alpha = 1.3533 \text{ concentration} \cdot \text{time}^{-1}$ , dimensionless  $p = 0.6712$  and  $w = 0.1567$ .

To simulate drug treatment using a constitutive bacterial delivery system, the rate of RFP production was used to estimate drug production as a function of position in tumor tissue. Drug production was linearly proportional to the RFP production rate,  $r_B$ , with drug production constant,  $\beta$ .

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} + \beta \cdot r_B \quad (6)$$

At the tissue edge, the rate of production was  $\beta \cdot r_B(0)$ . The constant  $\beta$  was fixed so that the maximum drug production rate was equal to the maximum calculated for Trz1-mediated drug expression. The RFP production rate was determined by fitting a polynomial to the normalized RFP intensity using least squares regression.

The solutions to partial differential equations for systemic drug treatment (Equations 3 and 4) and Trz1-mediated drug treatment (Equations 3 and 5 or 3 and 6) were solved using MATLAB (Mathworks, UK) PDEPE subroutine which uses an adaptive step Runge-Kutta ODE solver.

**Statistical analysis.** Statistical analysis was performed using Student's t-test, with  $P < 0.05$  considered significant. The linear correlation between cell viability (X) and glucose concentration (Y) was calculated using Pearson's correlation coefficient,  $r$ , by considering all possible combinations of error,  $(X \pm \sigma_x, Y \pm \sigma_y)$ . A hypothesis test between the difference of the means of the x-intercepts for the linear range of viability and glucose concentration as a function of depth in tumors were compared to determine if cell viability and glucose concentration gradients occurred at the same position within tissue.

## RESULTS

### Construction of the Trz1 sugar sensing circuit

A Trz1-mediated sugar sensing reporter system was constructed to regulate *GFP* expression in the presence sugars (Fig. 1,2). The sugar sensing system was made up of the *ompC* promoter regulating expression of *GFP* and an operon controlling expression the fusion receptor, *Trz1* (Fig. 2A). Each plasmid construct (Fig. 2A) was tested for salt and sugar sensitivity. The Trz1 receptor was also examined for the effect of receptor abundance on signal transduction by changing the strength of its associated promoter.

Deletion of the osmosensor, *EnvZ*, was necessary to eliminate salt sensitivity for Trz1-mediated sugar sensing (Fig. 2B). The *ompC* promoter,  $P_{OmpC}$ , was activated by salt concentration in the presence of endogenous *EnvZ* (Fig. 2B). Increasing salt concentration, from 0.17 M to 0.27 M NaCl in liquid cultures of pOmpC-GFP-DH5 $\alpha$ , increased GFP expression per bacteria 2.1-fold (Fig. 2B). Deletion of *EnvZ* (*E. coli* strain HB3521  $\Delta EnvZ$ ), prevented GFP expression in response to salt and was equivalent to a negative control (Fig. 2B).

Trz1 receptor abundance affected signal transduction (Fig. 2C). Construct pTrz1a was transformed into Top10F<sup>+</sup> *E. coli* with the *lacIq* repressor to control Trz1 expression by IPTG induction.

In the presence glucose, increasing IPTG concentration decreased GFP expression per bacteria, indicating that low level expression of *Trz1* is required for adequate sensing ( $P < 0.05$ ; Fig. 2C). Expression of Trz1 under the  $P_{BAD}$  promoter produced a sugar sensitive GFP signal response (Fig. 2D). This construct did not contain the AraC repressor necessary for sugar-responsive activation, but relied on the low basal expression of  $P_{BAD}$  to maintain low levels of Trz1. Constructs pOmpC-GFP, pTrz1a, and pTrz1, were transformed into HB3521 ( $\Delta EnvZ$ ) *E. coli* and grown in liquid cultures with 1.1 M glucose. GFP expression was greater (22.8-fold) under the  $P_{BAD}$  promoter (pTrz1) than  $P_{tac}$  promoter (pTrz1a;  $P < 0.05$ ; Fig. 2D).  $P_{tac}$ -mediated Trz1 expression was not different from the negative control, pOmpC-GFP (Fig. 2D). This indicates that the constitutive leakiness of the  $P_{BAD}$  promoter is much weaker than the  $P_{tac}$  promoter and is sufficient for expression of *Trz1* at low levels suitable for sensing and signal transduction.

The Trz1 receptor's sensitivity to ribose and glucose was measured in liquid cultures of pTrz1-HB3521 bacteria (Fig. 2E). The Trz1 receptor was more sensitive to ribose than to glucose, with significant increase in GFP expression observed at ribose concentrations between 10- 1000  $\mu$ M and glucose concentrations between 100- 10,000  $\mu$ M ( $P < 0.05$ ; Fig. 2E). Physiologically, sensitivity to glucose is more relevant because glucose gradients are more likely to be present in tumors and ribose is not present in blood. Bacterial density increased significantly at glucose concentrations greater than 1000  $\mu$ M ( $P < 0.05$ ; Fig. 2F).

### **Sugar gradients were detected in microfluidic tumor cell masses**

Sugar sensing bacteria, pTrz1-red HB3521 were used to visualize temporal and spatial gradients in bacterial accumulation and sugar concentration within microfluidic tumor cell masses (Fig. 3). Tumor-on-a-chip microfluidic devices mimic tumor tissue adjacent to a blood vessel (Fig. 3A).

Bacteria and media were administered to the adjacent flow channel, simulating systemic delivery to the tumor cell masses (Walsh et al., 2009; Fig. 3A). In this study, tumors were analyzed for bacterial density (RFP expression) and Trz1-mediated GFP expression over time, at 20, 26, 32, 38, and 44 hours post bacterial injection, and as a function of depth in tumors (Fig. 3B). RFP intensity was linearly related to bacterial density (Fig. 3C).

Bacteria colonized throughout the tumor cell masses and penetrated more than 600 $\mu$ m into the tissue (Fig. 3D). Between 20 and 44 hours after bacterial administration, the average bacterial density increased from  $1.4 \times 10^9$  CFU $\cdot$ ml $^{-1}$  to  $20.0 \times 10^9$  CFU $\cdot$ ml $^{-1}$  with an average growth rate of  $7.7 \times 10^8$  CFU $\cdot$ ml $^{-1} \cdot$ h $^{-1}$  ( $P < 0.05$ ; Fig. 3E,G). The average growth rate was relatively constant in time (Fig. 3E), however; growth rate as a function of position varied with depth in the tumor (Fig. 3F). Bacterial growth at distances within 250 $\mu$ m from the tissue edge was  $6.8 \times 10^8$  CFU $\cdot$ ml $^{-1} \cdot$ h $^{-1}$ . At distances between 300 to 400  $\mu$ m, growth rate was 2.2 times faster and at distances beyond 475  $\mu$ m growth rate was 1.8 slower than the in the edge ( $P < 0.05$ ; Fig. 3F).

Trz1-mediated GFP expression decreased with penetration depth into tissue (Fig. 3G). The average amount of GFP expressed across tumors increased from 20 to 44 hours at a constant rate (Fig. 3H) but varied with position (Fig. 3G). GFP expression rate was the greatest within 200  $\mu$ m of the tissue edge ( $P < 0.05$ ; Fig. 3I). Beyond 200  $\mu$ m, the production rate dropped significantly every 150 $\mu$ m (\*\*,  $P < 0.05$ ; Fig. 3I). At distances greater than 400  $\mu$ m, the GFP production rate per bacterium plateaued to a non-zero minimum (Fig. 3I).

### **Bacterial measured glucose concentration correlated with tumor cell viability**

The amount of GFP expressed per bacterium was equivalent to the glucose concentration profile in tumors and correlated with tumor cell viability (Fig. 4). At five different time points, 20, 26, 32, 38, and 44 h, the amount of GFP expressed per bacterium was equal and decreased with location in the tumor (Fig. 4A). The glucose concentration within microfluidic tumors was estimated from the average GFP per bacteria profile (Fig. 4B). The GFP/RFP profile decreased exponentially with depth

into tissue (Figure 4A,B). This profile mimics previous measurements of glucose concentration in tumor cylindroids, in which concentration also dropped exponentially with depth into tissue (Kasinskas et al., 2014).

The glucose consumption rate of LS174T human colon carcinoma cells was calculated from the glucose concentration gradient (Fig. 4B). The glucose gradient in tumor cell masses was dependent upon the bulk media concentration, diffusion in tumor interstitium, and cellular consumption by cancer cells. The first order glucose consumption rate in multicellular LS174T tumor cell masses was  $1.66 \times 10^{-3} \text{ s}^{-1}$  (red line, Fig. 4B).

Glucose concentration correlated with tumor cell viability (Fig. 4C,D). The health of the tumor cell population as a function of distance was based on previously measured apoptosis activity in tumor spheroids (Kasinskas et al., 2014). Cell viability was highest in the periphery of tumors and decreased with distance into the tissue (Fig. 4C). The relationship between depth in the tumor, glucose concentration, and cell viability was measured by calculating the error in the x-intercept in the linear portions of the profiles (Figure 4B,C) within 400  $\mu\text{m}$  of the edge of the tumors. The changes in glucose concentration and tumor cell viability occurred at the same positions within the tumor cell masses with no statistical difference observed between the x-intercepts (P value = 0.921). Glucose concentration had a linear relationship with tumor cell viability with a Pearson's correlation coefficient of  $r = 0.949$  and a P value =  $2.6 \times 10^{-5}$  (Fig. 4D).

## DISCUSSION

In this study, we investigated whether bacterial Trz1-mediated sensing detects glucose gradients in solid tumors and if the identified glucose concentration reflects the local cell viability. Our results show that ratio of Trz1-mediated GFP expression by *E. coli* can quantitatively identify glucose concentration in LS174T tumor cell masses and correlates strongly with tumor cell viability. Gram negative bacteria have previously been shown to target tumor tissue with high specificity and penetrate

and grow within the tumor microenvironment (Brader et al., 2008; St Jean et al., 2014; Thamm et al., 2005; Toley and Forbes, 2012; Weibel et al., 2008; Yu et al., 2008). In this study, a K-12 strain of *E.coli*, HB3521, were engineered with a hybrid receptor system to sense glucose and produce GFP (Fig. 1, 2). Glucose dependent GFP expression was observed in aqueous solution (Fig. 2) as well as in solid tumor cell masses (Fig. 3). The ratio of GFP to RFP expression was found to be independent of bacterial density and time and was used to estimate the glucose concentration within solid LS174T tumor cell masses (Fig. 4). The glucose concentration within solid tumors correlated strongly with previously measured apoptosis activity (Fig. 4) indicating that Trz1-mediated glucose sensing could be used to identify local tumor cell viability.

Bacterial penetration throughout tumor tissue is essential to sense sugar gradients. Bacteria, *E.coli* HB3521, accumulate throughout the tumor cell masses and grow over time (Fig. 3D,E). The distribution of bacteria across the tumors showed different densities and growth rates with position in the tissue (Fig. 3D,F). Bacterial density did not affect rate of GFP expression across the tissue but did change the magnitude of expression (Fig. 3D,G). Across several tissues and at different times, the amount of GFP expressed per bacteria remained constant (Fig. 4A) even though bacterial growth increased (Figure 3E). This uniformity indicates that glucose sensing is independent of bacterial magnitude and time (Fig. 4B) and suggests that Trz1-mediated glucose sensing could be used to detect sugar gradients across all tumors.

Tumor type and bacterial-tumor interaction can affect bacterial penetration, tumor metabolism, and the sensing of glucose concentration. Comprehensive studies of bacterial accumulation in metabolically and mechanically different tumor types still need to be investigated. Previous studies of gram negative bacterial accumulation in murine tumor models, show heterogeneous accumulation in tumors (Forbes et al., 2003). More recent studies, however, indicate that inducing inflammation at the time of bacterial administration can improve bacterial distribution in solid tumors (Zhang et al., 2014).

The bacteria themselves can also affect local tumor microenvironment. Changes in tumor cell

metabolism and tumor stiffness have been shown to affect tumor growth, metastasis, and response to therapy (Cox and Erler, 2011; Tung et al., 2015; Yano et al., 2014a). *Salmonella* have been shown to change the cell cycle of quiescent cells in tumors (Yano et al., 2014b), which could result in changes in local cancer metabolism and affect glucose measurement within tumors. To investigate this further, solid tumor-cell masses could be grown in Gelfoam® histoculture, which has been shown to mimic the cell cycle phase distribution within *in vivo* tumors (Yano et al., 2015), and these masses could be incorporated into tumor-on-a-chip microfluidic devices (Walsh et al., 2009). In addition, the bacteria are metabolically active, which will have an effect on the local glucose concentration. This activity, however, will not limit the ability to detect regions of tissue with low and high glucose concentrations. The penetration capabilities of HB3521 *E.coli in vitro*, suggest that it could be excellent platform for tumor biosensing; however, accumulation, glucose sensing, and toxicity still need to be investigated *in vivo*.

Trz1 glucose sensitivity is within the relevant range for detection of cancer glucose levels. The range of glucose sensitivity for pTrz1-red-HB3521 bacteria spans four orders of magnitude, between 10 $\mu$ M and 10 mM (Fig. 2E). The average biologically relevant glucose concentration for metastatic and non-metastatic cancers are between 0.3 - 2.4 mM (Walenta et al., 2000; Walenta et al., 2003). On average, normal blood glucose levels are 5.1 times as large with concentrations ranging between 6.1-7.8 mM (Anon, 2001). This causes concern for extra-tumoral drug production if pTrz1-red-HB35212 bacteria were used in the clinic. Higher glucose levels in healthy tissues will produce more recombinant drug per bacterium in these regions. The targeting capabilities of gram negative bacteria, which have been shown to colonize tumors at densities 10,000-fold higher than healthy organs (Forbes et al., 2003; Ganai et al., 2011; Zhao et al., 2005), would offset the higher glucose concentration and result in 2,000-fold lower drug concentrations in healthy organs. Bacterial specificity to tumors would reduce systemic toxicity and Trz1-sensing could provide glucose dependent dosing within tumors.

Trz1-mediated glucose sensing could be used to improve treatment and diagnostics of cancer by identifying and targeting therapeutics to metabolically distinct regions in tumors. The tight correlation between glucose concentration (GFP/RFP) and tumor viability ( $r = 0.949$ ; Fig. 4D), indicates that sugar sensing bacteria could identify viable regions of tumors (Fig. 5). Targeting therapy and diagnostic strategies to viable region would provide a useful tool in the treatment of cancer.

To evaluate efficacy of a live bacterial therapy, the Trz1-mediated protein production rate (Fig. 3I) and the cell viability as a function of position in tumors (Fig. 4C) were incorporated into a mathematical model that describes spatial-temporal drug concentration (Equations 4,5) and the resulting effect on the tumor cell viability (Equation 3). Cell viability profiles, obtained in the absence of bacteria, enable direct comparison between the locations of systemic and bacterial delivery. This model predicted better treatment via Trz1-activated drug delivery than systemic delivery or constitutively produced bacterial delivery (Fig. 5). Many small molecule drugs are ineffective at penetrating tumors and diffusing deep into the tumor sites (Heldin et al., 2004; Minchinton and Tannock, 2006; Tannock, 2001). Systemically administered drugs have high concentrations closest to vasculature,  $x = 0$ , but due to systemic elimination and poor diffusivity, these drugs are unable to penetrate deep into tissue to effectively treat all viable tumor cells (Fig. 5A,B). For a slow diffusing drug that is rapidly cleared, over 29.2% of the starting cell population would remain viable ( $t=0.5$ , Fig. 5B). Trz1-activated bacterial delivery, however, would increase drug penetration and continue to dose the tumor over time (Fig. 5C). With bacterial delivery, drug concentration was initially low, but as bacteria grew, more recombinant drug was produced, increasing the concentration in the tissue (Fig. 5C). From estimates of GFP production (Fig. 3I), the drug concentration was predicted to be greater in regions close to vasculature (Fig. 5C) where tumor tissue is more viable (Fig. 4C) and glucose availability was higher (Fig. 4B). Trz1-bacterial drug delivery predicted that, for the same drug, Trz1-bacterial delivery would have treated 99.2 % of cells across all tumor regions (Fig. 5D), not just those in the proliferating edge (Fig. 5B).



Glucose-mediated drug delivery was predicted to be superior to constitutive delivery because it focused treatment onto viable tissue. A constitutively delivered drug eliminated regions of tumors that systemically administered drugs failed to treat, but was ineffective at treating the periphery of tumors (Fig. 5E,F). At the same maximum rate of expression, Trz1-mediated drug delivery treated more of the tumor (Fig. 5D) than constitutive expression (Fig. 5F), which did not eliminate 5% of the tumor cells. These models demonstrate how a drug carrier that penetrates tumors and identifies local cancer metabolic activity could significantly improve treatment efficacy by directly targeting viable cancer tissue.

## CONCLUSIONS

We have developed a bacterial biosensor to detect nutrient availability in tumor masses. Genetically modified tumor-targeting bacteria were engineered to sense glucose concentration and trigger recombinant protein expression within tumors. An *in vitro* model was used to test tumor targeting and sensing capabilities within 3D tumor cell masses. Glucose sensing strongly correlated with cell viability as a function of position in the tumors. A mathematical model predicts more effective treatment of cancer cells by sugar sensing bacteria over systemic drug delivery. These results demonstrate the capabilities of bacteria to sense metabolic activity and growth characteristics of tumors and their potential to improve cancer treatment by directly targeting viable regions of tumors.

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**Table 1. Plasmids and strains used in this study**

| Plasmid             | Relevant Characteristics                                                                                                                                           | Source/Reference                              |
|---------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------|
| pMY150              | Source of OmpC promoter, $P_{ompC}$ , Amp <sup>R</sup>                                                                                                             | Gift from Inouye Lab (Mizuno et al., 1983)    |
| pFVP25              | <i>GFPmut3</i> , ColEI-ori, Amp <sup>R</sup>                                                                                                                       | Addgene.org/20667, (Raphael Valdiva, 1985)    |
| pRB020              | Source of <i>Trz1</i> fusion receptor, $P_{tac}/Trz1$ , ColEI-ori, Amp <sup>R</sup>                                                                                | Gift from Hazelbauer Lab, (Iida et al., 1985) |
| pAI12               | Expresses Ribose Binding Protein (RBP), <i>rbsB</i> and <i>rbsK</i> , p15a-ori, Cm <sup>R</sup>                                                                    | Gift from Hazelbauer Lab, (Iida et al., 1985) |
| pOmpC-GFP           | $P_{OmpC}/GFPmut3$ , ColEI-ori, Amp <sup>R</sup>                                                                                                                   | This study                                    |
| pTrz1a              | $P_{OmpC}/GFPmut3$ , <i>t1-term</i> , $P_{tac}/Trz1$ , ColEI-ori, Amp <sup>R</sup>                                                                                 | This study                                    |
| pTrz1               | $P_{OmpC}/GFPmut3$ , <i>t1-term</i> , $P_{BAD}/Trz1$ , ColEI-ori, Amp <sup>R</sup>                                                                                 | This study                                    |
| DsRed Express2      | Source of <i>DsRed</i> , red fluorescent protein                                                                                                                   | Clontech                                      |
| pTrz1               | pTrz1 with downstream, $P_{lac}/DsRed$                                                                                                                             | This study                                    |
| Strain              |                                                                                                                                                                    |                                               |
| HB3521              | <i>E. coli</i> strain CP919 { <i>rbsB</i> lO2:: <i>Tn10</i> , $\phi$ ( <i>ompC-lacZ</i> )10-25 $\Delta$ <i>EnvZ</i> :: <i>KmR</i> },containg plasmid pAI12         | Gift from Hazelbauer Lab, (Iida et al., 1985) |
| DH5 $\alpha$        | <i>E. coli</i> cloning strain used for all plasmid construction                                                                                                    | New England BioLabs                           |
| One-Shot-Top-Ten F' | <i>E. coli</i> containing F' episome which carries the <i>lacI</i> <sup>q</sup> repressor for inducible expression from <i>tac</i> promoter, $P_{tac}$ , with IPTG | LifeTechnologies                              |



## Figures:

**Figure 1. Trz1-mediated gene expression pathway.** The Trz1 fusion receptor combines the ribose/glucose/galactose sensing domain of Trg chemotaxis receptor with the EnvZ osmoporin signal transduction domain to transduce sugar sensing into genetic output. Ribose and glucose sugar molecules, freely diffuse across the outer membrane (OM) of *E. coli* and bind to their respective periplasmic (PP) binding proteins, the ribose binding protein (RBP) or the Glucose/Galactose binding protein (GBP). In the ligand bound form, the ligand-binding protein complex then interacts with the periplasmic portion of the Trz1 receptor, a fusion between chemotaxis receptor, Trg, and the osmoporin receptor, EnvZ. This interaction causes conformational change in Trz1 across the inner-membrane (IM) leading to phosphotransfer to cytoplasmic osmoporin regulator, OmpR. In its phosphorylated state, OmpR then acts as a transcriptional activator on the osmoporin promoter,  $P_{OmpC}$ , activating expression of green fluorescent protein (GFP).

**Figure 2. Construction of sugar sensing Trz1-GFP expression plasmid system** **A)** Plasmid constructs used in this study. **B)** In liquid cultures, the osmoporin promoter,  $P_{OmpC}$ , triggers GFP expression with the increasing salt concentration in DH5 $\alpha$  *E. coli* bearing plasmid pOmpC-GFP. Deletion of the osmosensor,  $\Delta EnvZ$  (pOmpC-GFP-HB3521), eliminated salt response. **C)** IPTG-inducible expression of Trz1 (pTrz1a), in Top 10F<sup>+</sup> *E. coli*, containing the lacIq repressor, decreases GFP expression with the addition of IPTG (\*,  $P < 0.05$ ). Low level expression of Trz1, in the fully repressed state ([IPTG]=0), permitted Trz1 signal transduction through GFP expression. **D)** Liquid culture of pOmpC-GFP, pTrz1a, or pTrz1 transformed into *E. coli* HB3521 ( $\Delta EnvZ$ ) with 1.1 M glucose. Expression of Trz1 under the weak PBAD promoter triggers GFP expression in the EnvZ deficient *E. coli* (\*,  $P < 0.05$ ). **E)** Sugar sensitivity of pTrz1-red-HB3521 in defined liquid culture. Increasing ribose and glucose concentration increased GFP expression (\*,  $P < 0.05$ ). The pTrz1 system is 10-fold more sensitive to ribose than glucose (\*,  $P < 0.05$ ). **F)** Glucose concentration increases bacterial growth (\*,  $P < 0.05$ ).

**Figure 3. Bacteria sense sugar gradients in tumor-on-a-chip microfluidic device.** **A)** A tumor-on-a-chip microfluidic device was used to monitor bacterial accumulation and sugar response via microscopy. **B)** Tumors were monitored for bacterial colonization (red) and sugar sensing (green) over the course of 44 hours,  $n=4$  (scale bar = 100  $\mu m$ ). **C)** RFP intensity was converted to bacterial density by administration of known bacterial densities to the microfluidic device. **D)** Bacteria colonized throughout the tissue with an increase in growth over time ( $n=4$ ). **E)** The average growth of bacteria across tumors was constant. **F)** Bacterial growth varied with position, as determined by RFP expression rate, with the highest rate between 300-400  $\mu m$  in depth and lowest value beyond this point (\*,  $p < 0.05$ ). The average RFP expression rate was normalized to 1 at its maximum (solid red line) and was used to estimate the constitutive expression rate for mathematical analysis of tumor treatment. **G)** GFP expression increased over time but decreased as a function of penetration depth, indicating a drop in sugar concentration with depth in tissue ( $n=4$ ). **H)** The average level of GFP expressed over time increased at a constant rate (\*,  $P < 0.05$ ). **I)** The highest rate of GFP expression was within the leading edge of the tumor-cell masses (0-200  $\mu m$ ; \*,  $P < 0.05$ ). At depths greater than 200  $\mu m$ , The GFP production rate decreased significantly every 150  $\mu m$  into the tumor cell masses and plateaued to non-zero value at distances greater than 500  $\mu m$  (\*\*,  $P < 0.05$ ). GFP-production rate was used to estimate drug production rate (solid red-line) for mathematical analysis of tumor treatment.

**Figure 4. Sugar sensing bacteria measure glucose gradient in tumor cell masses which correlates with local tumor cell viability.** **A)** The ratio of GFP/RFP expressed across tumor cell masses was time independent and decreased with depth in the tissues. **B)** The average GFP/RFP ratio was used to estimate glucose concentration within the tumor cell masses as a function of penetration depth. The average glucose concentration profile, red line, was used to estimate the consumption rate of glucose by the cancer cells. **C)** Tumor tissue viability as a function of tissue depth as measured by caspase-3 activity (data from Kasinskas et al., 2014). The average value was fit to continuous function for mathematical analysis of tumor treatment. **D)** The relationship between glucose concentration and local tumor cell viability (caspase-3 activity) as a function of position, shows that glucose concentration correlates strongly with local tumor cell viability ( $r = 0.949$ ,  $P < 0.01$ ).

**Figure 5. Predicted treatment of tumor tissue via Trz1-mediated drug delivery.** **A)** Small molecule chemotherapeutic drug administered to tumor will diffuse into tissue and washout quickly. **B)** Systemic administration of drug is unable to eliminate all viable cells, leaving 29.2% of the starting cell population alive. **C)** The same drug produced by sugar-sensing, tumor-targeting bacteria, persists and increases in magnitude with time. **D)** Trz1-bacterial delivered drug eliminates the majority of viable tissue within the same time frame, with only 0.8% of the initial tumor cells remaining alive. **E)** Drug concentration produced by a constitutive expression system would increase based on bacterial growth, **F)** and reduce tumor viability in the difficult to treat quiescent and necrotic regions of tumors, but would leave a fraction (5%) of the tumor periphery untreated.



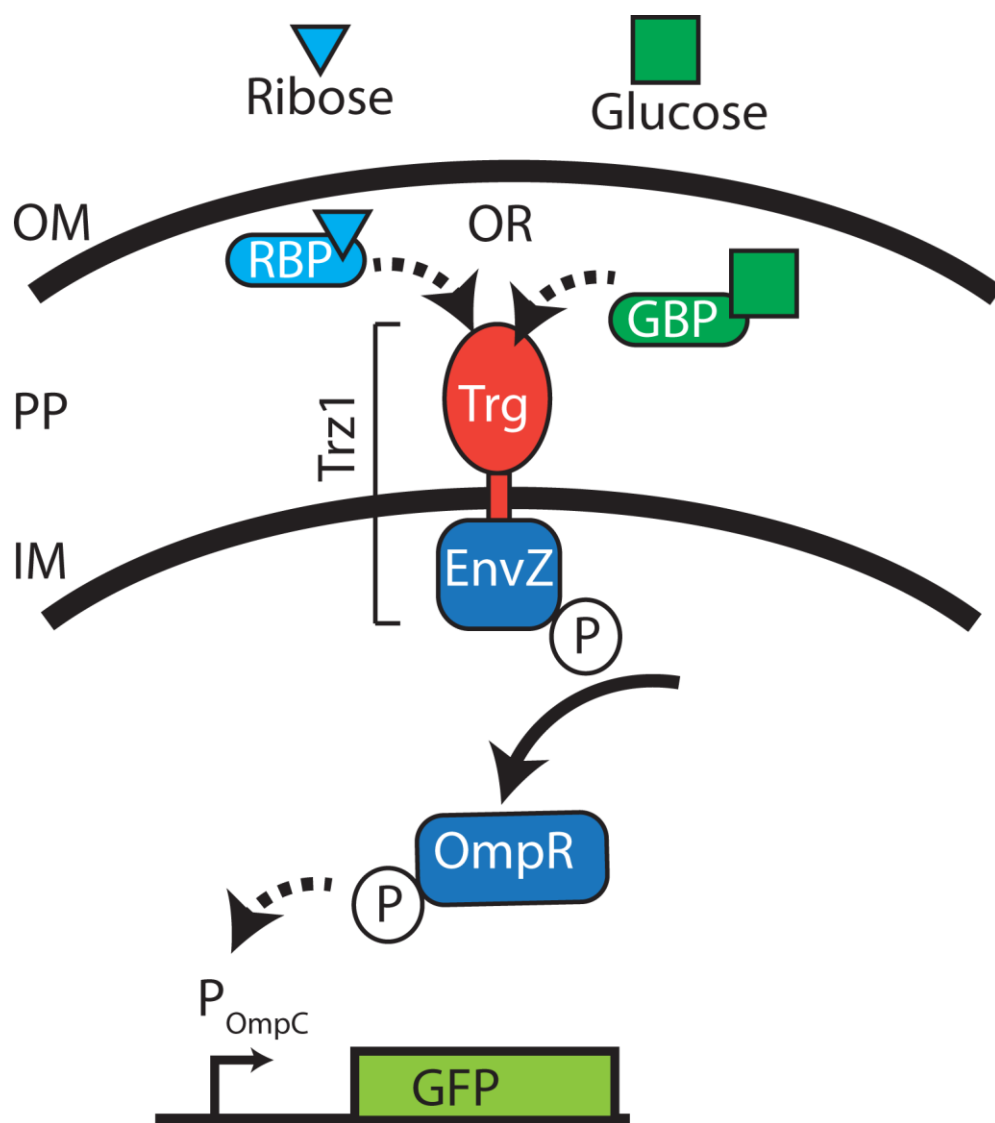
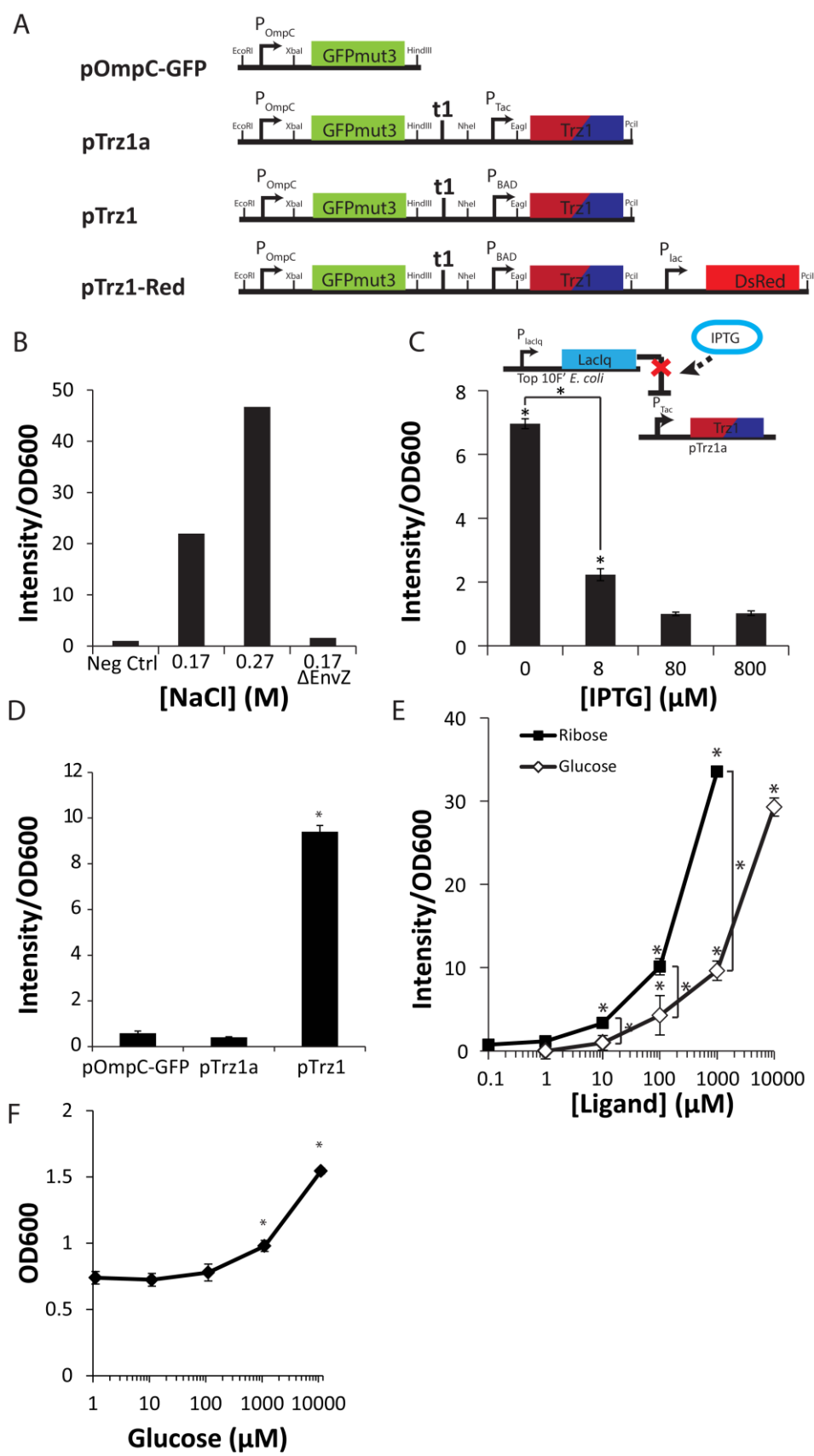


Figure 1



**Figure 2**

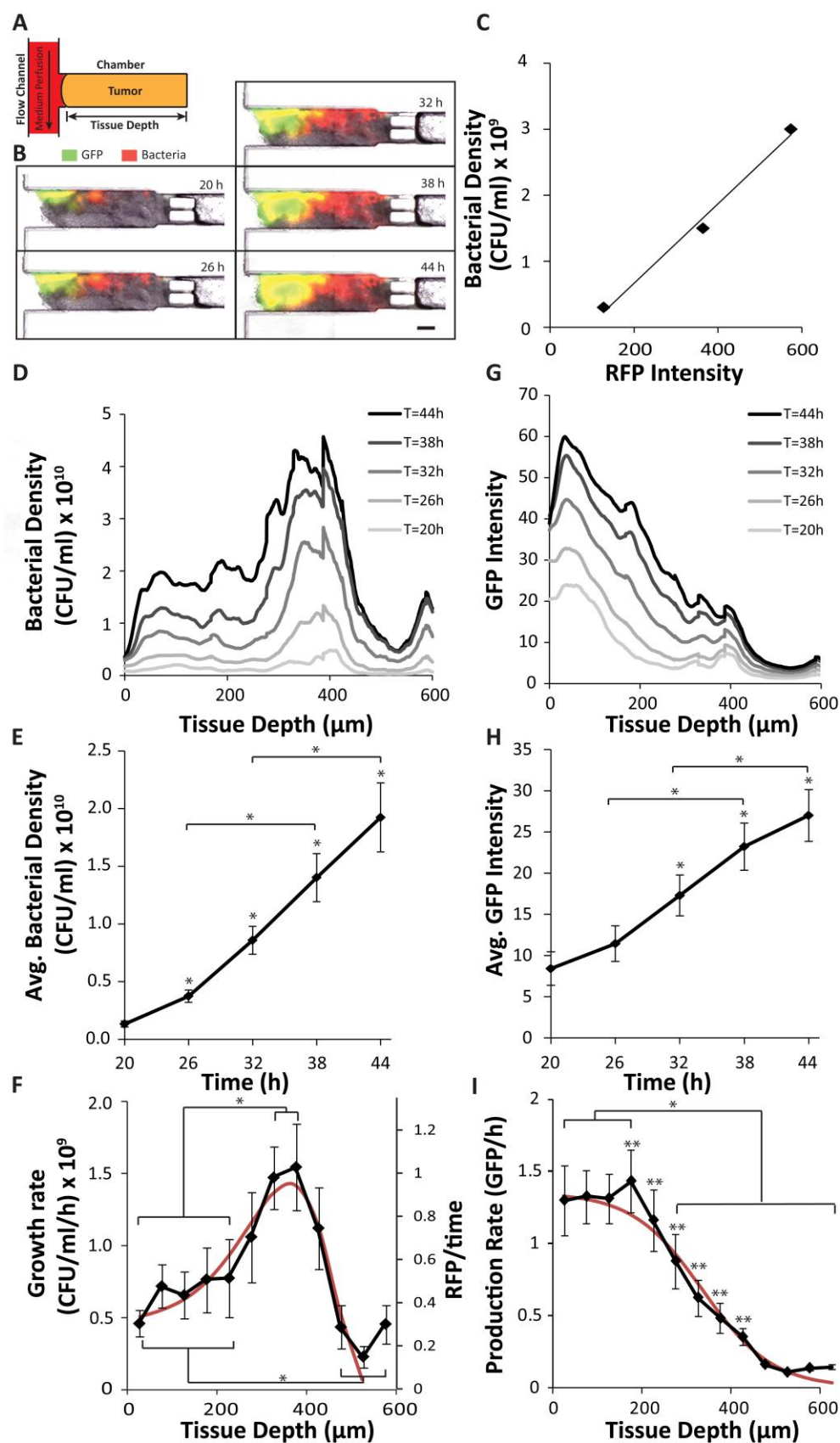


Figure 3

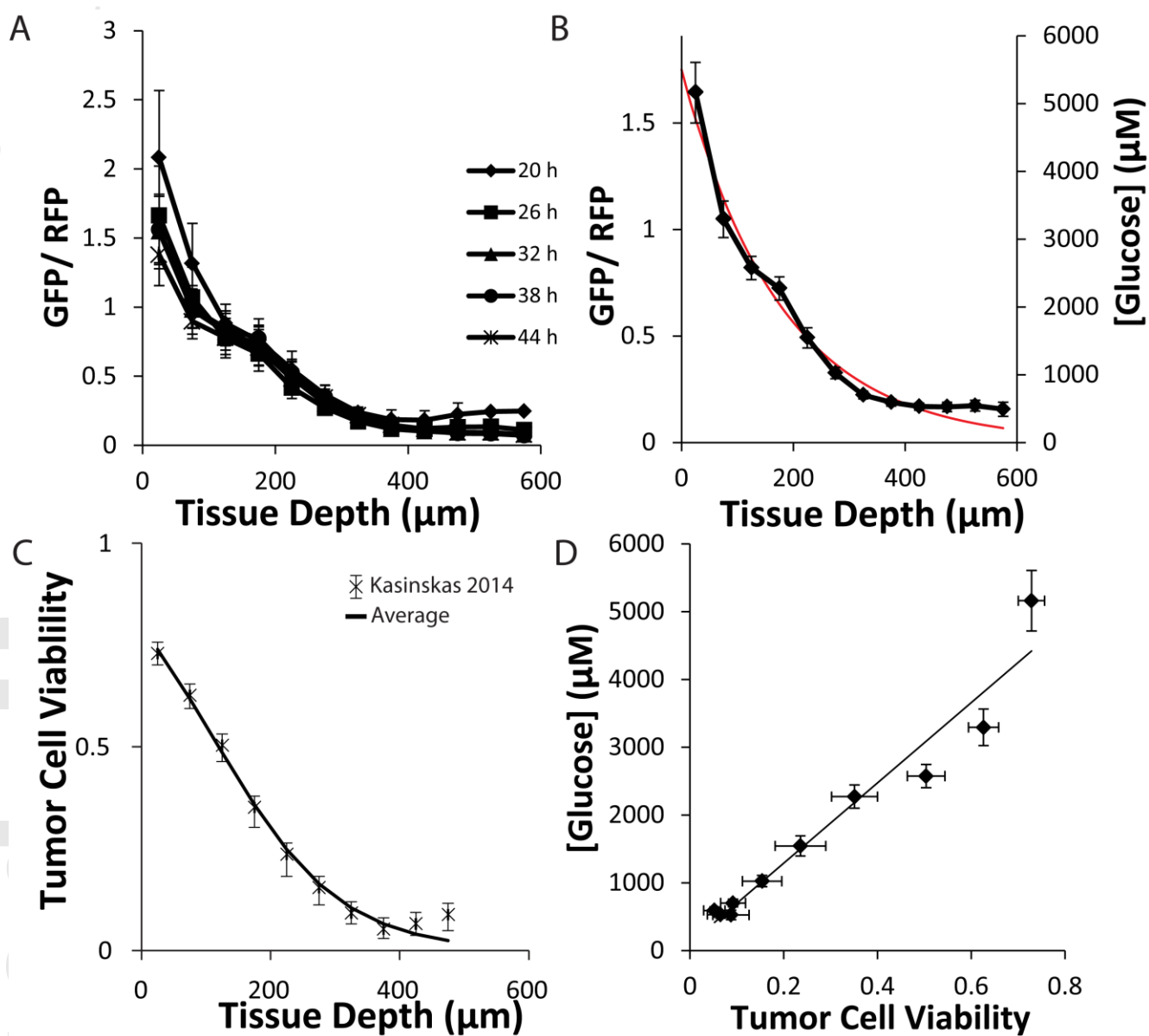
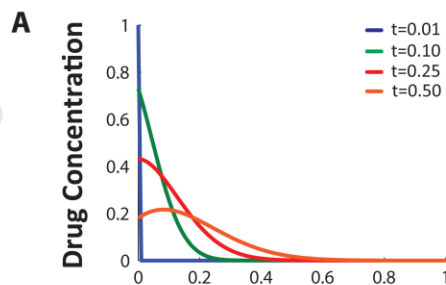
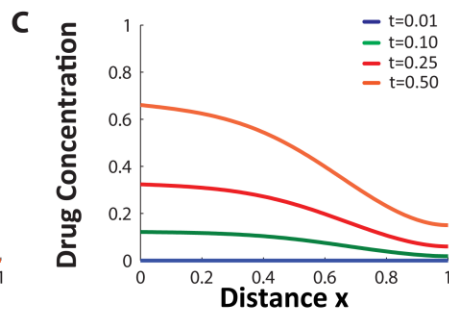


Figure 4

### Systemic Delivery



### Trz1 Bacterial Delivery



### Constitutive Expression

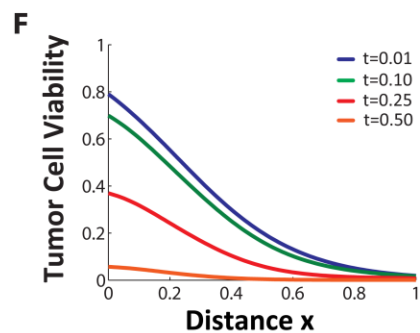
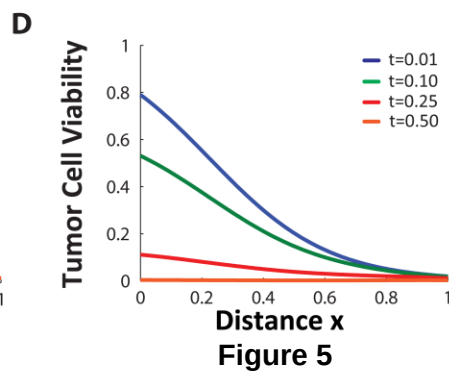
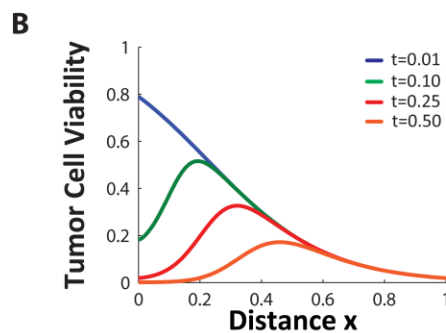
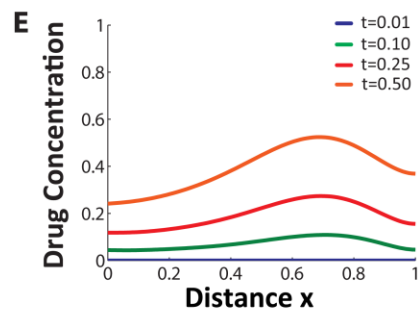


Figure 5