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A rapid screening platform to coculture bacteria within tumor spheroids

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The prevalence of tumor-colonizing bacteria along with advances in synthetic biology are leading to a new generation of living microbial cancer therapies. Because many bacterial systems can be engineered to recombinantly produce therapeutics within tumors, simple and high-throughput experimental platforms are needed to screen the large collections of bacteria candidates and characterize their interactions with cancer cells. Here, we describe a protocol to selectively grow bacteria within the core of tumor spheroids, allowing for their continuous and parallel profiling in physiologically relevant conditions. Specifically, tumor spheroids are incubated with bacteria in a 96-well low-adhesion plate followed by a series of washing steps and an antibiotic selection protocol to confine bacterial growth within the hypoxic and necrotic core of tumor spheroids. This bacteria spheroid coculture (BSCC) system is stable for over 2 weeks, does not require specialized equipment and is compatible with time-lapse microscopy, commercial staining assays and histology that uniquely enable analysis of growth kinetics, viability and spatial distribution of both cellular populations, respectively. We show that the procedure is applicable to multiple tumor cell types and bacterial species by varying protocol parameters and is validated by using animal models. The BSCC platform will allow the study of bacteria–tumor interactions in a continuous manner and facilitate the rapid development of engineered microbial therapies.

Introduction

Recent microbiome studies of patients with cancer have revealed diverse bacteria species present in many solid tumors[1,2]. These observations of natural tumor tropism, in combination with mouse studies that have shown that bacteria selectively colonize immune-privileged tumor cores after systemic administration, have generated renewed interest in the use and modification of bacteria as cancer therapies[3,4]. Here, synthetic biology approaches have enabled increasingly sophisticated control of bacteria via synthetic gene circuits to sense and produce therapeutic agents, transforming microorganisms as next-generation therapeutic agents[5]. By locally releasing recombinant payloads along with their inherent immune-stimulating components, microorganisms can alter tumor progression, immune landscapes and drug metabolism[6–11].

Although several bacterial therapy candidates have progressed to clinical trials[12–17], a universal bottleneck for development is the lack of in vitro tools to test bacteria in tumor-like environments. The rapid proliferation rate of bacteria hinders continuous coculture with mammalian cells, and the technical complexity required for recapitulating tumor microbial colonization can often compromise practicality and throughput[18]. As a result, the vast majority of studies rely on a small number of animal models to study a particular bacteria strain, therapeutic payload and synthetic gene circuit, leading to slow incremental improvements[18,19]. Furthermore, spatiotemporal profiles of bacteria in tumors have remained elusive, because of the limitations of in vivo imaging technologies. Because living therapeutics are proliferative and motile, understanding their dynamics is critical for ensuring expected efficacy and safety.

In this protocol, we describe a bacteria spheroid coculture (BSCC) platform that allows for high-throughput and long-term growth of bacteria in the hypoxic and necrotic core of tumor spheroids in vitro (Fig. 1). Using this coculture technique, studies have tested tumor-sensing gene circuits, characterized bacteria-mediated tumorigenesis and screened antitumor payloads that recapitulate therapeutic outcomes in vivo[20–23]. The ability to stably coculture bacteria in multiple tumor spheroids in parallel at a scale unattainable with an in vivo system will serve to increase throughput and accessibility for the study of microbial therapies and tumor–microorganisms interactions.

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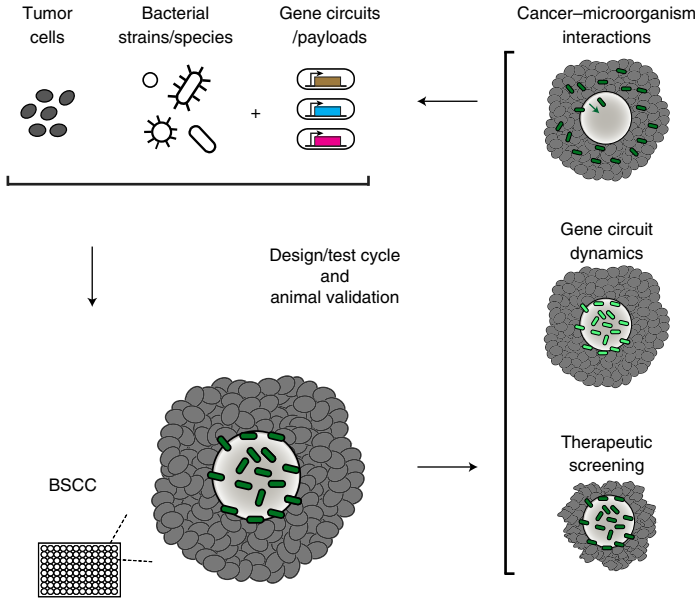

Fig. 1 | BSCC system to evaluate microbial cancer interactions and therapies. Various tumor and bacterial cell types, gene circuits and therapeutic payloads can be simultaneously tested in the 3D multicellular bacteria spheroid coculture (BSCC) system. In brief, tumor spheroids are inoculated with bacteria in 96-well low-adhesion plates, and subsequently a washing and antibiotic protocol enables selective bacteria growth within spheroids. The coculture is stable for ≥2 weeks and is compatible with downstream assays for kinetic studies of both cell populations. Bacterial candidates are rapidly iterated by using the BSCC platform and are tested in a mouse tumor model for validation.

Development of the protocol

To study the complex multicellular interactions between tumor cells and microorgnaisms and develop effective microbial therapies for cancer, an ideal coculture system must simultaneously meet several criteria: (i) stable coexistence of both populations that enables longitudinal studies to monitor cellular interactions and responses; (ii) recapitulation of the solid tumor microenvironment that reproduces bacterial performance and tumor responses in native conditions; and (iii) parallel culture format that allows comparisons with different bacterial strains and cancer types, which can facilitate accessibility and standardization across the research community. Although various coculture designs have been reported[10,24–38], it has been difficult to simultaneously satisfy the above features with existing methods (Table 1).

3D culture systems including tumor spheroids and organoids provide a way to recapitulate the solid tumor microenvironment. In these models, cancer cells are organized in geometry and stoichiometry that can be similar to native environments, creating a physiological distribution of nutrients, metabolites, pH and oxygen. Using 3D culture systems, several groups have cocultured bacteria with cancer cells, focusing on the use of multicellular tumor spheroids to evaluate bacteria infiltration into the tumor[39]. Although these 3D coculture systems recapitulate physiologically relevant bacteria–cancer interactions, inoculations of bacteria into the 3D culture system without mechanisms to contain bacterial growth in tumors leads to rapid microbial overgrowth in the surrounding media, which can cause problems for some downstream assays.

Our BSCC system takes advantages of the 3D tumor spheroids while also addressing the limitation caused by bacteria overgrowth. This is achieved by restricting bacterial growth to tumor necrotic cores by using a specific protocol built on the use of repeated wash steps and an addition of a poorly diffusible antibiotic, gentamicin, in the surrounding media (Fig. 2). The BSCC system simultaneously characterizes both bacteria and cancer cells and is compatible with many standard assays and instruments for downstream experiments and analysis that are difficult to attain with other experimental setups (Fig. 3). For example, time-lapse microscopy can profile the spatiotemporal dynamics of the cells, such as spheroid size, bacterial distributions and gene expression kinetics. At any time point, individual tumor spheroids can easily be extracted for assays such as viability, histology and bacteria colony counting.

Table 1 | Comparison of existing bacteria–cancer coculture systems

Coculture methods	Stability	Throughput	Physiological relevance
Monolayer cancer cells			
Direct inoculation[24–26]	Low stability. Bacteria rapidly overgrow in the medium	High throughput. Most systems are amenable to standard multi-well screening	Low-medium relevance. Monolayers typically do not recapitulate the microenvironment. It may be amenable to the addition of various cell types
Transwell[27–29]	Medium stability. Physical separation does not fully prevent rapid bacteria nutrient consumption	High throughput. Some Transwell systems are amenable to standard multi-well screening	Low relevance. Monolayers typically do not recapitulate the microenvironment. No physical interactions between bacteria and cancer cells
Microfluidics[30–33]	High stability. The fluidics system washes away excessive bacteria and provides new medium	Low throughput. The fluidics system is technically difficult to test multiple bacteria in parallel	Low-medium relevance. Monolayers typically do not recapitulate the microenvironment. It may be amenable to the addition of various cell types
3D tumor culture			
Direct inoculation[34–36]	Low stability. Bacteria rapidly overgrow in the medium	High throughput. Some 3D culture systems are amenable to standard multi-well screening	Medium relevance. The 3D culture recapitulates the key microenvironment but lacks many cell types. Some 3D cultures such as organoids provide various cell types
Microinjection[10]	Medium stability. Direct injection does not fully contain bacteria within the tumor, because bacteria will eventually overgrow in the surrounding culture medium	Low throughput. Slow incremental injections are required for each spheroid/organoid	Medium-high relevance. Some 3D cultures such as organoids provide various cell types, but direct injection does not recapitulate natural tumor infiltration
Tumor-on-a-chip[37,38]	High stability. The fluidics system washes away excessive bacteria and provides new medium	Low throughput. The fluidics system is technically difficult to test multiple bacteria in parallel	Medium-high relevance. The 3D culture recapitulates the key microenvironment but lacks many cell types. Some 3D cultures such as organoids provide various cell types
BSCC[20,21]	High stability. Poorly diffusible antibiotic contains bacteria within tumor spheroids	High throughput. Ultralow-attachment plates are amenable to standard multi-well screening	Medium-high relevance. The 3D culture recapitulates the key microenvironment but lacks many cell types

Overview of the procedure

Our protocol details the culturing of bacteria (Box 1) and the generation of tumor spheroids, which consists of the expansion of tumor cells (Steps 1–35) and seeding of the cells in an ultra-low-attachment round-bottom plate (Steps 36–47). We then detail the following procedures for coculturing bacteria within the tumor spheroids: incubation of bacteria with tumor spheroids (Steps 48–62), washing steps to remove extratumoral bacteria and the addition of an antibiotic (Steps 63–73). We also provide details for the use of our coculture in the analysis of bacterial growth trajectory (Step 74A–D), gene circuit dynamics (Box 2) and therapeutic efficacy (Box 3). Lastly, we provide details for validating the lead therapeutic candidates from BSCC screening in vivo (Box 4).

Comparison with other methods

Many early studies on bacterial interactions with cancer cells were conducted by adding bacteria onto a monolayer of tumor cells[24–26,39]. This approach is simple to set up and allows testing in a multi-well format. However, because of the sheer difference in growth rate between the two cellular populations, bacteria rapidly overtake the tumor cells and exhaust nutrients in the culture medium. This results in cancer cell death and expended medium, limiting the analysis to a few hours, which is typically too short to observe cellular interactions and responses. Several modifications had been developed to address this issue; they included the addition of the Transwell system to physically separate bacteria and cancer cells and the use of inactivated and lysed bacteria[27–29,32,40]. While these approaches limit bacterial growth, they compromise cell–cell interactions and do not allow characterization of live bacteria, respectively.

Fluidic systems have emerged as alternative methods to control cellular growth and nutrient supply. Specifically, microfluidic systems allow sophisticated control over cellular positions and have

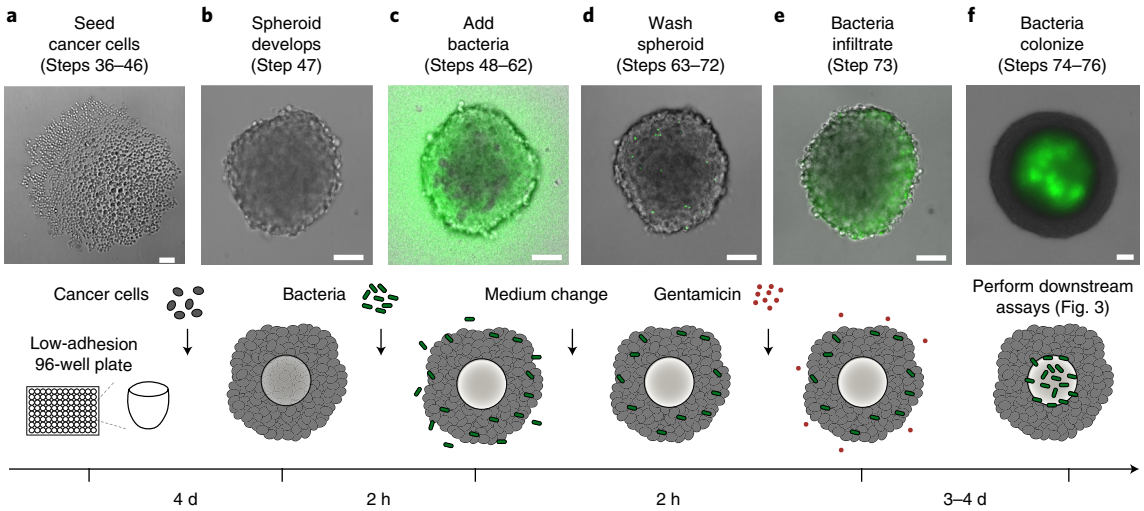

Fig. 2 | Schematic workflow to establish BSCC. The top panel shows representative images of tumor spheroids cocultured with super folder GFP (sfGFP)-expressing *S. typhimurium*. Scale bars, 200 μm. The bottom schematic shows steps and descriptions of the protocol. **a**, Cancer cells are seeded in the low-adhesion, round-bottom plate (Steps 36–46). **b**, Tumor cells aggregate and form tumor spheroids (Step 47). **c**, Bacteria are added to the medium and incubated to allow infiltration (Steps 48–62). **d**, Extratumoral bacteria are removed by repeatedly changing the culture medium, followed by adding the poorly diffusible antibiotic gentamicin (Steps 63–72). **e**, Bacteria selectively infiltrate the growing tumor spheroids (Step 73). **f**, Bacteria stably colonize tumor spheroid cores (Steps 74–76). The coculture is compatible with a number of downstream assays described in Fig. 3. All data were adapted from ref. [20].

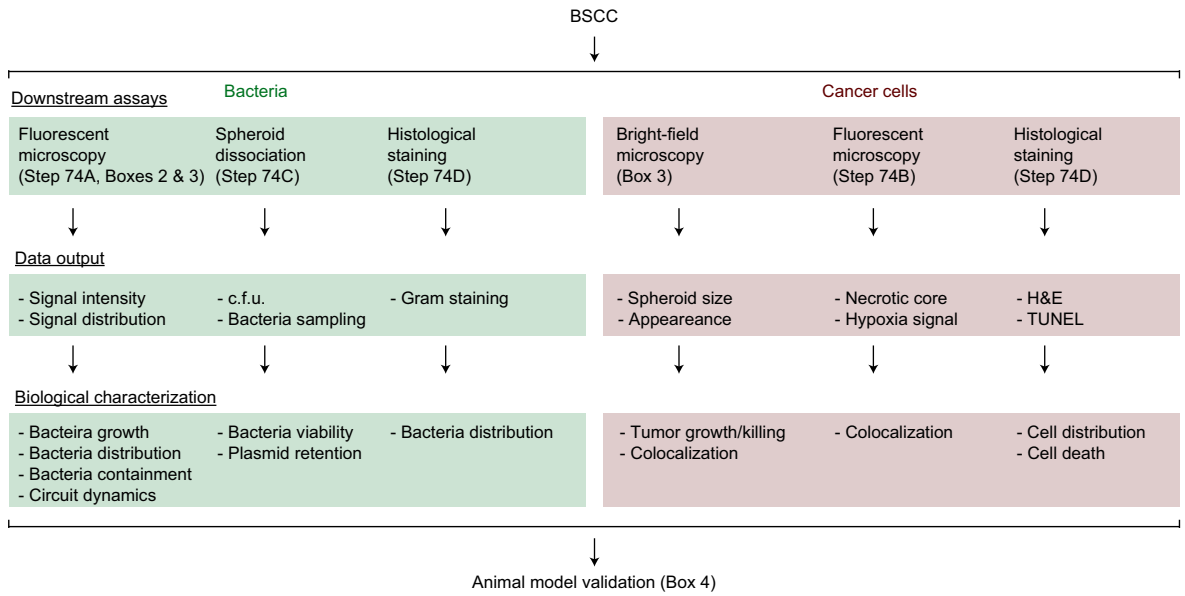

Fig. 3 | Overview of the downstream assays and their utilities. The coculture system is compatible with various standard assays, enabling characterization of both bacteria and cancer cell populations. The stability and high-throughput nature of the setup makes the coculture method suitable for kinetic studies and drug screenings, respectively.

been used widely to study bacteria and cancer cells independently in monoculture settings. Several coculture designs have been developed in which cancer cells are initially seeded in the microfluidics channel followed by bacterial inoculation[30–33]. A single fluidic system can harbor multiple traps to grow cancer cells of various types and densities, allowing comparisons in one experimental setup. For example, tumor cells and nontumorous cells have been placed in separate traps of a device to study bacterial chemotaxis toward cancer cells[30,31]. In another study, a microfluidic device was designed to grow bacteria in a trap, characterizing the effect of bacterial density and antitumor efficacy[32]. Although these microfluidics coculture systems allow longitudinal studies by washing away extra bacteria, these systems inevitably require specialized equipment and are difficult to run multiple

Box 1 | Bacteria culture ● Timing 0.5 h (setup), overnight (bacterial growth)

Procedure

Prepare the bacterial culture after 3 d of spheroid seeding.

▲ **CRITICAL** This procedure gives details for preparing a bacterial culture for *S. typhimurium* ELH1301. See Table 2 for parameters to consider for optimizing the procedure for bacterial strains.

1 Prepare bacteria culture medium as described in Materials. 50 μg/ml kanamycin is added to the medium as a selection marker for constitutive sfGFP plasmid. Transfer 2 ml of culture medium to a 12-ml culture tube.
▲ **CRITICAL STEP** Adjust the type and concentration of the medium and antibiotics depending on the selection marker and bacterial strain of your choice.

2 The night before the day of coculture, obtain glycerol stocks of *S. typhimurium* ELH1301. Place the tube onto a cold block to minimize thawing.
! **CAUTION** Although the ELH1301 strain is attenuated, it should be handled under a biosafety level 2 condition. Institutional guidelines for Environmental Health and Safety should be followed, and materials should be decontaminated and disposed of according to biohazard materials and safety policy.

3 Transfer the glycerol stock into the biosafety cabinet.
▲ **CRITICAL STEP** Using a separate biosafety cabinet from the one used for tumor cell culturing is suggested to avoid cross contamination. If sharing the biosafety cabinet space, handle with extra caution to avoid spills, and frequently sterilize the work area by wiping with 70% (vol/vol) ethanol.

4 Scrape the top surface of the glycerol stock by using a sterile disposable loop or pipette tip.

5 Inoculate the bacteria into the culture tube containing bacteria growth medium. Cap the tube to avoid spills.
▲ **CRITICAL STEP** The bacteria culture tube has a dual-position snap-cap. Cap to the first position to allow aeration for better growth of facultative anaerobic bacteria including *S. typhimurium*.

6 Place the bacterial culture in a 37 °C shaker incubator. Set the shaking speed to 200–250 rpm to allow aeration.

7 On the next morning (after 12–16 h of inoculation), take the culture out of the shaker. The medium should be densely cloudy, indicating the growth of bacteria to a stationary stage of growth.
? TROUBLESHOOTING

Box 2 | Gene circuit dynamics

We describe the procedure for inducing and imaging an AHL-inducible gene circuit, by using *S. typhimurium* strain ELH1301 transformed with AHL-inducible sfGFP plasmid (see Materials). Refer to ref. [20] for further details of the gene circuit constructs used in the original studies.

Procedure

Activate bacterial gene circuits in coculture ● Timing 0.5 h

1 To study gene circuit dynamics, establish coculture by using bacteria carrying desired gene circuits by following Steps 1–73.

2 For AHL-inducible circuits, prepare 10 μM stock of AHL in MilliQ water (see Materials).
▲ **CRITICAL STEP** For biosensors or auto-inducible circuits, skip to Box 2, steps 8–11 for time-lapse microscopy.

3 Prepare coculture medium containing 20 nM AHL.

4 Sterilize the biosafety cabinet.

5 At the desired time point, take out the coculture plate from the 37 °C CO_2 incubator and place it in the biosafety cabinet.

6 Aspirate the medium by slowly pipetting up 100 μl of the medium from the well. The remaining 100 μl of medium should contain the spheroid in each well.

7 Slowly add 100 μl of the coculture medium containing 20 nM AHL, making the final concentration of the medium in a well 10 nM AHL.

Acquire time-lapse images ● Timing 0.5 h (setup), 3–5 d (image acquisition)

8 Set up the Nikon TiE microscope equipped with an Okolab stage-top incubator. Adjust to 20% oxygen, 5% CO_2, 95% humidity and 37 °C.

9 Transfer the coculture plate onto the Okolab stage-top incubator.

10 Using the Nikon Elements software, program the scope to image the spheroids every 60 min.
▲ **CRITICAL STEP** Adjust parameters according to the microscope setup of your choice. For the original experiments, phase-contrast images are taken at 10× magnification at 50–200-ms exposure times. Fluorescent imaging at 10× is performed at 70 ms for sfGFP, with a 30% setting on the Lumencor Spectra-X Light.

11 Continue time-lapse imaging for the desired time. Place the coculture plate back into the 37 °C CO_2 incubator once completed.

experiments in a parallel manner. Importantly, typical microfluidic systems allow for only one bacteria strain to be tested in a single experiment.

A key drawback for most monolayer and microfluidics systems is the lack of certain physiological conditions including cellular geometry, metabolites and oxygen gradients. In vivo, the necrotic and hypoxic cores of a solid tumor provide a unique set of metabolite and nutrient gradients that facilitate bacteria growth and competition with cancer cells[3,4]. These solid tumor microenvironments are known to be important for tumor progression and affect critical parameters for therapies such as gene expression, metabolism and growth[41]. Although some attempts had been made to compensate for some aspects of the tumor environment, such as hypoxic chambers to recapitulate tumor oxygen levels[42,43], they often do not address many other critical tumor signatures.

Box 3 | Therapeutic efficacy screen

▲ **CRITICAL** Using an inducible system is recommended to express the therapeutic gene. This enables therapeutic expression from the tumor core, recapitulating an in vivo therapy scenario. Refer to ref. [20] for details of gene circuit constructs used in the original studies.

Procedure

Induce therapeutic expression ● Timing ~0.5 h

1 Establish coculture by following Steps 1–73.
2 When bacteria colonize the spheroid core (days 3 and 4), induce therapeutic expression by following Box 2, steps 1–7.
3 Image bacteria (sfGFP) and spheroids (brightfield) daily by following Step 74A.

Analyze microscope images and assess therapeutic efficacy ● Timing 1–3 h

4 Transfer imaging data and open in FIJI software (or a software of your choice).
5 To measure spheroid size, set a threshold brightness value for each brightfield image (such as using a minimum method or Yen's method).
6 Set the ROI to distinguish the dark spheroid from the light background.
7 Identify the largest region within the resulting threshold-based image mask as the tumor spheroid and measure the ROI area.
8 To quantify bacterial fluorescence, measure the mean intensity of sfGFP fluorescence within the spheroid region.
9 (Optional) To compute radial distribution, compute fluorescence for multiple thin annuli centered on the centroid of the spheroid mask region. For time-course images, align the images for spheroid rotation and movement. Source code is provided in Supplementary Information.

3D culture systems have been increasingly used to study a wide range of biological questions and are considered more relevant models to perform drug efficacy studies[44,45]. Previous studies using 3D culture systems have attempted to address issues of rapid microbial overgrowth by the addition of microfluidics to create tumor-on-a-chip devices, microinjection of bacteria into the core of tumor organoids to facilitate bacterial tumor localization and the use of strict anaerobic bacterial species or inactivated bacteria to control overgrowth outside of the tumor[10,37,38,46,47]. However, these methods in turn can compromise simplicity, throughput and generalizability. Tumor-on-a-chip systems require high technical complexity that can limit the use to specialized laboratories and do not allow parallel testing of multiple bacteria and cancer types. Researchers require substantial technical training to be competent in microinjection assays, and these are laborious to run on a large scale. In addition, local injection is not sufficient to restrict bacterial growth within tumors; thus, frequent washing or clearance of bacteria is necessary. Lastly, restricting the types of bacteria to anaerobic species severely limits the scope of the study. Coculture models for microorganisms and mammalian cells in the context of pathogens and infections have also been developed, but they are typically not adapted for studying bacterial therapy for cancer[48–50]. In comparison to these systems, BSCC provides a simple and stable 3D coculture system that allows high-throughput and longitudinal characterization of bacteria in a physiological tumor environment.

Advantages and limitations of the protocol

An advantage of the BSCC system is the recapitulation of bacterial colonization in the tumor environment. The use of a 3D model provides many features that are important for bacterial colonization such as metabolites, oxygen gradients and cell–cell interactions. Notably, this protocol recapitulates the selective growth of bacteria in necrotic and hypoxic cores of the tumors as seen in vivo. Previous studies have demonstrated the relevance of the tumor environment provided by the 3D models, including the hypoxic core that changes bacterial transcriptional state and supports the growth of anaerobic bacteria[23,46]. Our results confirm the protocol's suitability for assessing behaviors and responses of both bacteria and cancer cells in conditions similar to their native context.

Importantly, this protocol enables coculturing of bacteria and cancer cells in a simple and high-throughput manner. Unlike other 3D coculture systems that are limited by complex and specialized arrangements, we took advantage of the 96-well and suspension culture setups that require only standard tissue-culture equipment. The use of a broad-spectrum antibiotic with reduced diffusion inside of the spheroids enabled simultaneous testing of a large diversity of bacterial strains, tumor types, gene circuits and therapeutic payloads. For example, we screened a library of therapeutic payloads expressed at various levels via a small molecule-inducible gene circuit by using the BSCC system described in this protocol[20].

The capability to stably coculture bacteria within tumors allows for careful characterization over space and time, which is critical for assessing live microbial therapeutics that undergo chemotaxis and replicate. For example, our BSCC system measured ~3×10^8 c.f.u./mm^3 of *Salmonella typhimurium* in

Box 4 | In vivo therapeutic validation

Procedure

Inject mice subcutaneously with tumor cells ● Timing 2–3 h (preparation), 1–3 weeks (tumor grafting)

1 Obtain BALB/c mice and acclimate them in the facility for 1 week before cancer cell injections.
▲ **CRITICAL STEP** For reproducibility and calculating statistical significance, we suggest at least five mice per experimental condition.
2 Culture tumor cells by following Steps 1–34.
3 Cool down sterile 1× DPBS by placing the bottle on ice.
4 Count tumor cells by following Steps 39 and 40.
5 Centrifuge the tube containing tumor cells at 200*g* for 5 min at room temperature.
6 Aspirate the medium and resuspend the cell pellet in an appropriate volume of ice-cold sterile 1× DPBS. Dilute to 5×10^7 cells per 1 ml of 1× DPBS.
7 1 million cells in 100 µl should be injected per mouse. Divide the cell suspension in 1.5-ml Eppendorf tubes for easily filling the injection syringe in the upcoming step.
▲ **CRITICAL** Always prepare 20% more volume of tumor cell suspension than the calculated volume, because some cells will be lost during the counting and filling in the syringe.
8 Anesthetize animals by following the appropriate IACUC protocol.
9 Load 200–1,000 µl of cell suspension into a 1-ml syringe using a 29-gauge needle.
10 With forceps, gently grab the region of the skin in between the hind leg and tail of the mouse.
11 Insert the needle by 1/8–1/4 inches and inject cells slowly. Each mouse can have bilateral tumors, with 100 µl (5×10^6 cells) per tumor.
▲ **CRITICAL STEP** Take care that the needle does not puncture the skin at the other side of the grabbed region, to avoid injected cells leaking out.
12 Release forceps before pulling out the needle.
13 As you are pulling out the needle, use the forceps to seal the point of injection and slowly and gently remove the syringe to avoid spills.
14 Monitor the mice, and after the mice wake up and start moving inside the cage, place the cage in the appropriate place in the animal facility. Tumors will be visible and palpable over the next 1–3 weeks.
? TROUBLESHOOTING

In vivo therapeutic validation ● Timing 2–3 h (injection), 1–2 weeks (tumor growth), 1–3 weeks (treatment)

15 Measure and record the tumor volume of BALB/c mice harboring subcutaneous CT26 tumors by using calipers twice a week. Tumor volumes are calculated by measuring the length, width and height of each tumor ($V = L \times W \times H$). Tumors are ready for treatment when they grow to ~150 mm^3.
16 Randomize the mice so that the control and treatment groups have approximately comparable tumor volumes.
17 On the night before the start of treatment, grow bacterial strains overnight in LB medium containing appropriate antibiotics. Bacteria expressing sfGFP instead of therapeutics can be used as a control.
! CAUTION Use a biosafety cabinet for all bacteria handlings.
18 On the next morning, subculture the bacteria by making a 1:100 dilution into a new 50-ml medium with an appropriate antibiotic selection marker. Grow the culture in the 37 °C incubator shaker until the OD_{600} reaches ~0.1 (early log growth phase).
19 Obtain a bucket of ice and place the bacterial culture in it for 10 min to halt growth.
20 Centrifuge the bacteria culture at 3,000*g* for 10 min at 4 °C and discard the supernatant.
21 Resuspend the pellet with 50 ml of sterile 1× DPBS by gently pipetting up and down.
22 Wash bacteria by repeating Box 4, steps 20 and 21 three times.
23 Adjust the OD_{600} to 0.5 ($1–2 \times 10^8$ bacteria per ml).
24 Load the bacteria into a 1-ml syringe with a 27-gauge needle.
25 Insert the needle in the tumor, aiming for the central area of the tumor, pull out slightly to make a small empty space inside the tumor and then very slowly inject a total volume of 20–40 µl per tumor.
▲ **CRITICAL STEP** Insertion, injection and extraction should be carried out slowly to avoid bacteria suspension squirting out of the dense tumor.
? TROUBLESHOOTING
26 Induce bacterial therapeutic expression 1 d after bacterial injection. Prepare 10 µM AHL (see Materials) and inject 0.5 ml subcutaneously behind the neck of each mouse.
27 Measure tumor volume every 2–3 d as described in Box 4, step 15.
28 (Optional) For multiple dosing, follow Box 4, steps 17–27 every 2–4 d until the study end point.
! CAUTION Check mice frequently to ensure the health status of the animals. Follow the appropriate IACUC-approved protocol.

a single spheroid after 1-week coculture. This is roughly similar to the numbers of bacteria found in mouse models[51,52]. In addition to bacteria growth, many synthetic gene circuits are dynamic, requiring monitoring over time for proper characterization in native contexts. The BSCC protocol successfully tested various kinetic parameters including bacterial growth and lysis and gene activation and biosensing in tumor environments[20,21]. Finally, the BSCC platform also allows assessment of tumor growth over time to characterize the response and resistance to novel therapies.

Using the high-throughput format, we have screened multiple aspects of engineered microbial therapy including bacterial species and strains, therapeutic payloads and payload-delivery mechanisms[20]. This screen rapidly identified efficacious strains against colon cancer in an animal model. In a separate study, we have characterized the performance of the engineered biosensors[21]. The natural solid tumor microenvironment provided by the BSCC system enabled monitoring of the oxygen, lactate and pH sensor performance. We envision that the use of this technique will accelerate the

development of bacteria cancer therapies and address questions of multicellular interactions between microorganisms and cancer cells.

Although the BSCC system introduces new opportunities in synthetic biology, there are some limitations of the technique, such as the lack of cell type heterogeneity found in the solid tumor microenvironment and the incompatibility with several bacteria species. Previous studies show the importance of various cell types present in the tumor environment such as immune cells, stroma cells and vasculature[41]. Our current protocol uses unicellular tumor spheroids and does not include other cell types such as immune cells that have been targeted in some studies using immunotherapy approaches, limiting the scope of the system. However, future iterations could potentially include additional cell types or account for spatial heterogeneity. This protocol also depends on the use of gentamicin to limit bacterial overgrowth. Although gentamicin possesses broad-spectrum bactericidal effects, some bacteria have been reported to be resistant to this antibiotic[53]. It should also be noted that antibiotics may influence cancer cell biology and may affect the experimental results[54]. In addition, different bacteria have different sensitivity to gentamicin, infiltration efficiency and growth rate, which may require additional optimization. In this protocol, we provide guidance to customizing the coculture protocol for applications with various cell types.

Experimental design

The use of tumor spheroids enables a simple and parallel culturing of the 3D tumors while providing several physiological conditions that are critical for bacterial therapy including hypoxia, tumor metabolites and central necrosis[5,19]. In this paper, we define tumor spheroids as 3D aggregations of tumor cells derived from a cell line. Specifically, this protocol is primarily focused on the use of the mouse colorectal cancer cell line CT26 to generate tumor spheroids. Other types of 3D culture systems such as ones derived from primary cells and containing heterogeneous cell types may be used but would probably require optimization.

A key consideration for the BSCC coculture is the optimization of the conditions to maintain stable growth of bacteria while limiting bacterial overgrowth in the surrounding media. This is influenced by many factors including the bacteria and cell types used, media composition, washing steps and gentamicin concentration. Optimizing the coculture procedures before setting up the experiments is recommended. If possible, using optically labeled bacteria (such as the bacteria expressing sfGFP as described in this paper) is helpful in determining the distribution and growth of bacteria in tumor spheroids. To maintain robust experimental results, the protocol should also include proper controls. For example, a bacterial strain with non-therapeutic payload (such as sfGFP) can be used as a negative control to assess the contribution of the therapeutics to anticancer efficacy. A control with no bacteria can also be included to study the contribution of live bacteria on tumor spheroid growth. Biological replicates should always be included to account for experimental variations. This is particularly important for live bacterial therapy because stochastic variations in bacterial behavior and mutations may influence the experimental outcomes.

The BSCC protocol can be adapted to diverse bacterial strains, species and cancer cell types. Although this protocol mainly focused on the use of an attenuated *S. typhimurium* (ELH1301) that is widely used for cancer therapy applications[19,32,51], we have previously demonstrated successful colonization of tumor spheroids by using various *S. typhimurium* strains (ELH430, SL7207 and VNP20009) and bacterial species (*Listeria monocytogenes*, *Proteus mirabilis* and *Escherichia coli*) (Fig. 4,a and b). In addition, tumor spheroids of various tissue types (colon and breast) from different hosts (human and mouse) (Fig. 4C) were also tested. Because of differences in cellular physiochemical properties, several parameters may need to be tuned for optimal coculture. For example, the optimal gentamicin condition for ELH1301 in CT26 colon tumor spheroids was determined by screening various concentrations (Fig. 4D). Table 2 summarizes important parameters and their optimization strategies.

Lead therapeutic candidates from BSCC screening can be validated in an animal model composed of the same cancer cell type. For example, this paper describes the use of the CT26 cell line to generate a murine tumor model. Female 4–6-week-old BALB/c mice were used as a syngeneic host with an intact immune system that plays a critical role in bacterial containment and efficacy[55,56]. CT26 cells were injected subcutaneously to establish solid tumors, and the tumors were treated with intratumoral bacterial injections. This model enables characterization of the engineered bacteria by using the same cancer cells as tumor spheroids. Subcutaneous tumors provide access to track tumor growth via calipering and downstream procedures such as tumor biopsy for further analysis. We expect that a

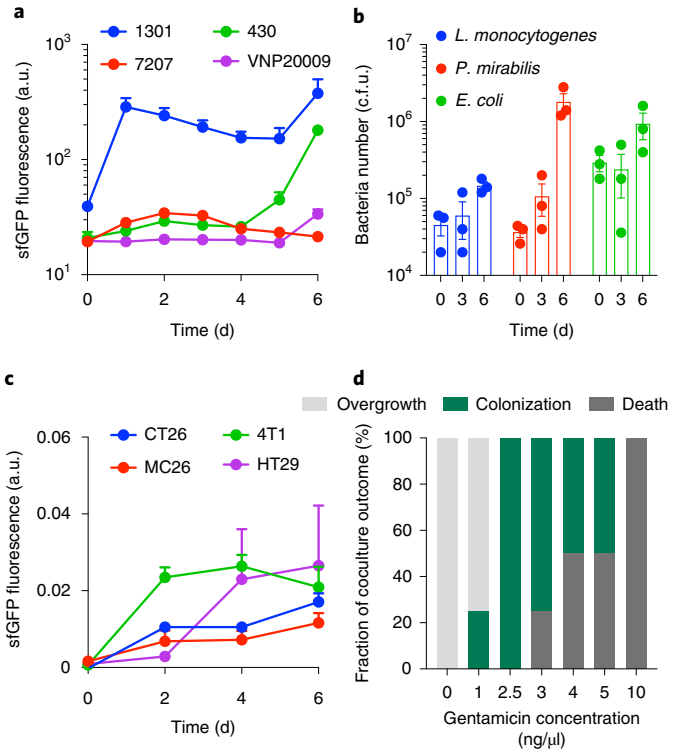

Fig. 4 | Coculture with various bacteria and tumor cell types. a, Growth of *S. typhimurium* strains (ELH430, SL7207, ELH1301 and VNP20009) within spheroids measured by sfGFP fluorescence. **b**, Growth of *L. monocytogenes, P. mirabilis* and *E. coli* within spheroids measured by CFU. **c**, Growth of *S. typhimurium* ELH1301 within tumor spheroids of various tissue origins (CT26 and MC26 from mouse colon, 4T1 from mouse breast and HT29 from human colon). **d**, Fraction of coculture outcome with various gentamicin concentrations. Low gentamicin allows overgrowth of bacteria outside of tumor spheroids. High gentamicin rapidly kills bacteria before establishing colonization. The fraction of coculture outcome is calculated out of 10 replicates. Error bars indicate ±s.e.m. sfGFP, super-folder GFP. All data were adapted from ref. [20].

similar strategy can be used for various mouse cell lines in syngeneic models, as well as human cell lines by using immunocompromised mouse models as previously described[57].

Materials

Biological materials

Cell lines

- CT26, 4T1 and HT29 cell lines purchased from American Type Culture Collection, cat. nos. CRL-2638 (RRID: CVCL_7256), CRL-2539 (RRID: CVCL_0125) and HTB-38 (RRID: CVCL_0320), respectively
- MC26 cell line obtained from K. Tanabe and B. Fuchs (Massachusetts General Hospital, RRID: CVCL_0240)
- Infrared RFP (iRFP)-transfected CT26 cell line obtained from the Danino laboratory stock

! CAUTION Authenticity and sterility of the cell lines should be maintained by routine check-up. Morphology should be checked by microscope, and animal cell lines should be tested for mycoplasma contamination.

Bacterial strains

- Attenuated strains of *S. typhimurium* ELH1301 and ELH430 obtained from from Dr. Elizabeth Hohmann; SL7207 obtained from Siegfried Weiss
- *L. monocytogenes* obtained from Eric Pamer
- *S. typhimurium* strain VNP20009, *P. mirabilis* and *E. coli* purchased from American Type Culture Collection (cat. nos. 202165, 29906 and 23506, respectively) **! CAUTION** Biosafety level 2 hoods are required for handling *S. typhimurium*. Institutional guidelines for Environmental Health and Safety should be followed, and materials should be decontaminated and disposed of according to biohazard materials and safety policy. **▲ CRITICAL** Bacteria were transformed with a plasmid to express sfGFP

Table 2 | Important parameters for coculture customization

Parameters	Optimization	Examples	Steps
Cancer cells			
Culture medium	Some cells require special culture medium. Adjust according to the recommended medium for each cell type	RPMI (CT26, MC26 and 4T1) and DMEM (HT29)	Steps 1–20: tumor cell culture and growth
Seeding density	Spheroid density may differ depending on cell types. Screen various densities to achieve ~500 μm in diameter after growth, which facilitates the generation of a hypoxic and necrotic center	2,500 cells/well (CT26 and MC26), 5,250 cells/well (4T1) and 5,000 cells/well (HT29)	Steps 36–47: seeding tumor cells for spheroid growth
Spheroid growth time	Cell growth rate may differ depending on cell types. Test various spheroid growth times to facilitate the generation of a hypoxic and necrotic center	4 d (CT26, 4T1 and HT29)	Step 48: formation of a necrotic core inside the spheroids
Bacteria			
Growth condition	Some bacteria require a special culture medium. Adjust according to the recommended medium for each bacteria species	LB (*S. typhimurium*, *P. mirabilis and E. coli*) and BHI (*L. monocytogenes*)	Box 1: growing bacteria
Inoculation density	Screen various densities to achieve maximum spheroid colonization for the particular bacteria species. Generally, it helps to maximize density, but adding too many bacteria will lead to rapid growth of bacteria in the culture mediu, leading to toxicity to cancer cells	10^6 c.f.u./spheroid (*S. typhimurium*), 3×10^7 c.f.u./spheroid (*L. monocytogenes*), 5×10^5 c.f.u./spheroid (*P. mirabilis*) and 3×10^6 c.f.u./spheroid (*E. coli*)	Steps 48–62: add bacteria to tumor spheroids
Initial incubation time	Screen various times to achieve maximum spheroid colonization for the particular bacteria species. Generally, it helps to maximize time, but incubating bacteria for too long before the washing will lead to overgrowth of bacteria in the culture medium, leading to toxicity to cancer cells	2 h (*S. typhimurium*), 1 h (*L. monocytogenes*), 4 h (*P. mirabilis*) and 6 h (*E. coli*)	Steps 48–62: add bacteria to tumor spheroids
Gentamicin concentration	Gentamicin sensitivity may differ depending on bacteria species. Screen various gentamicin concentrations to ensure killing of extratumoral bacteria. An excessive concentration may kill bacteria while infiltrating. See example data in Supplementary Fig. 2	2.5 μg/ml (*S. typhimurium*), 10 μg/ml (*L. monocytogenes*), 4 μg/ml (*P. mirabilis*) and 1.5 μg/ml (*E. coli*)	Steps 63–73: remove extra-tumoral bacteria

for visualization. For gene circuits and therapeutics applications, bacteria were genetically engineered to express necessary genes on plasmids or genomic DNA. Full details of these bacteria can be found in refs. [20,21]. *S. typhimurium* strain ELH1301 was engineered with gene circuits in this protocol.

Mice

- Female BALB/c mice (4–6 weeks old) purchased from Taconic Biosciences **! CAUTION** All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC; Columbia University, protocols ACAAAN8002 and AC-AAAZ4470). **! CAUTION** All procedures described here should be approved by the IACUC. All personnel should be trained (including but not limited to health clearance, humane handling of laboratory animals and barrier facilities) and follow the approved protocols at all times. **! CAUTION** The use of biosafety level 2 bacteria (including the attenuated *S. typhimurium* strain used in this protocol) should be approved by the IACUC, be in accordance with institutional guidelines for Environmental Health and Safety and be performed in a proper dedicated space. **▲ CRITICAL** It is important to note the difference in mouse sex when performing animal experiments. This may influence the results and should accordingly be disclosed.

Reagents

- RPMI 1640 (Gibco Thermo-Fisher, cat. no.11835030)
- DMEM F12 GlutaMAX supplement (Gibco; Thermo-Fisher, cat. no. 11320033)
- FBS (Gibco; Thermo-Fisher, cat. no.10-437-028)
- Penicillin-streptomycin (CellGro; Thermo-Fisher, cat. no. 15-070-063)
- LB broth (Lennox; Thermo-Fisher, cat. no. BP9722-500)
- Kanamycin (Sigma-Aldrich, cat. no. 10106801001)

- Gentamicin (Thermo-Fisher, cat. no. 15-750-060)
- 0.05% trypsin EDTA (Gibco; Thermo-Fisher, cat. no. 25-300-054)
- Trypan blue (Gibco; Thermo-Fisher, cat. no. 15250061)
- Hypoxia dye Image-IT hypoxia reagent (Thermo-Fisher, cat. no. H10498)
- Dimethyl sulfoxide (Thermo-Fisher, cat. no. 50-147-371) **! CAUTION** Avoid inhalation of vapor or mist. Keep away from sources of ignition. Take measures to prevent the buildup of electrostatic charge.
- LB agar (Lennox; Thermo-Fisher, cat. no. 22700025)
- 1× Dulbecco's PBS (Thermo-Fisher, cat. no. 14190144)
- 190 proof ethanol (Thermo-Fisher, cat. no. 22-032-600)
- Bleach (Clorox) **! CAUTION** Avoid contact with skin, eyes and clothing. Reclose the cap tightly after each use.
- *N*-(β-Ketocaproyl)-L-homoserine lactone (Sigma, cat. no. K3007)
- Paraformaldehyde (PFA), 4% (vol/vol) in PBS (Fisher Scientific, cat. no. AAJ61899AP) **! CAUTION** Avoid contact with skin and eyes. Avoid formation of dust and aerosols. Provide appropriate exhaust ventilation at places where dust is formed. Keep away from sources of ignition. Take measures to prevent the buildup of electrostatic charge.

Equipment

Tumor cell culture

- T75 tissue-culture flask (Falcon, cat. no. 13-680-65)
- Serological stripettes (Falcon, cat. no. 13-675-49)
- Serological pipette gun (Rainin, cat. no. 17011733)
- Multi-channel pipette (Rainin, cat. no. 17013805)
- Pipette (Rainin, cat. no. 30456871)
- Pipette tips (Rainin, cat. no. 30389225)
- Cell counter slides (Thermo-Fisher, cat. no. C10228)
- 15-ml conical tubes (Falcon; ThermoFisher, cat. no. 14-959-70C)
- 50-ml liquid reservoir (ThermoFisher, cat. no. 07-200-128)
- 96-well, clear, round-bottom, ultra-low attachment plates with lids (Corning Costar; Sigma, cat. no. CLS7007-24EA)
- Class II A2 biosafety cabinet (Fisher Scientific, cat. no. 30-261-1010)
- 37 °C CO_2 incubator (Thermo Scientific Forma Steri-Cycle Model 370)
- 37 °C water bath (Thermo Fisher Scientific, cat. no. TSGP02)
- Centrifuge (Eppendorf 5804R, cat. no. 022628146)
- Cell counter (Invitrogen; Thermo-Fisher, cat. no. AMQAX2000)
- Vacuum line

Bacterial culture

- 12-ml culture tubes (Falcon; ThermoFisher, cat. no. 14-959-11B)
- Class II A2 biosafety cabinet (Fisher Scientific, cat. no. 30-261-1010)
- 37 °C incubator shaker (ThermoFisher, cat. no. SHK6000)
- NanoDrop One^C (ThermoFisher, cat. no. ND-ONEC-W)
- Plastic cuvettes (Fisher/BrandTech, cat. no. 14-377-017)
- 37 °C CO_2 incubator (Thermo Scientific Forma Steri-Cycle Model 370)
- Petri dishes (VWR, cat. no. 25384-094)

Plasmids/strains

- Constitutive sfGFP (pTH01 from ref. [20])
- *N*-(β-Ketocaproyl)-L-homoserine lactone (AHL)-inducible sfGFP (pTD103 sfGFP from ref. [20])
- AHL-inducible therapeutics (pTH05 from ref. [20])
- Hypoxia-inducible *asd* (pTH06-1 from ref. [21])
- Lactate-inducible *glms* (pTC66 from ref. [21])

Imaging and software

- EVOS FL Auto 2 cell imaging systems
- Celleste imaging analysis software
- Nikon TiE microscope (equipped with Okolab stage top incubator, Andor Zyla complementary metal–oxide–semiconductor (sCMOS) camera and Lumencor Spectra-X Light)

- Nikon Elements software
- Image-J or FIJI
- Graphpad Prism v.8.0

In vivo experiments
- Syringe, 1 ml (Fisher Scientific, cat. no. 14-841-32)
- Isoflurane isothesia solution (Henry Schein, cat. no. 029405)
- Compressed oxygen (Airgas)
- Anesthesia machine (Avante, cat. no. 10150)
- Caliper (Fisher Scientific, cat. no. 14-648-17)
- Mouse diet (PicoLab Rodent Diet 20 and 5053)
- Animal cages—five mice per cage according to the IACUC protocol

Reagent setup

Bacteria culture medium
Prepare LB broth and 50 μg/ml kanamycin. This can be stored at 4 °C for 6 months.

Tumor cell culture medium
Prepare RPMI 1640, 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin. This can be stored at 4 °C for 2 weeks.

Tumor spheroid culture medium
Prepare RPMI 1640 and 10% (vol/vol) FBS. This can be stored at 4 °C for 1 month.

Coculture medium
Prepare RPMI 1640, 10% (vol/vol) FBS and 2.5 μg/ml gentamicin. This can be stored at 4 °C for 2 weeks. ▲ **CRITICAL** The gentamicin concentration is important for successful establishment of the coculture. The appropriate concentration should be determined before the experiment and should be used consistently throughout the course of the experiment.

AHL stock solution
Prepare 10 μM AHL in MiliQ water. This can be stored at 4 °C for 2 weeks.

Procedure

Tumor spheroid preparation

▲ **CRITICAL** This procedure gives details for generating tumour spheroids by using the CT26 cell line. See Table 2 for parameters to consider for optimizing the procedure for other cell lines.

Thaw tumor cells ● Timing 1 h (tumor cell thawing), 2–3 d (tumor cell growth)

1 Prepare tumor cell culture medium as described in Materials.
2 Warm up tumor cell culture medium to 37 °C in a water bath.
3 Sterilize the biosafety cabinet by using UV light and wipe down all working surfaces with 70% (vol/vol) ethanol.
▲ **CRITICAL STEP** This step needs to be sterile of bacterial contamination. Separating the biosafety cabinet for tumor cell monoculture and bacteria coculture is recommended to avoid cross contamination.
4 Thoroughly spray 70% (vol/vol) ethanol onto the outer surface of the bottles containing growth medium and bring them inside the biosafety cabinet.
5 Transfer 9 ml of tumor cell culture medium into a 15-ml conical tube and place it into the 37 °C CO_2 incubator.
6 Thaw a cryovial of cancer cells (CT26) by placing it into a 37 °C water bath (<1 min).
7 Take out the cryovial from the water bath when half the content is thawed, spray 70% (vol/vol) ethanol and quickly place it into the biosafety cabinet.
8 Add 1 ml of tumor cell culture medium to the cryovial and gently resuspend the tumor cells.
9 Move the 15-ml conical tube containing warmed medium into the biosafety cabinet and add the cryovial content slowly.
10 Centrifuge at 125*g* for 5 min.

Table 3 | Suggested cell culture conditions for tumor cells

Tissue culture container	Reagent volume (ml)			Cell density (cells/container)	
	1× DPBS	0.05% trypsin EDTA	Tumor cell culture medium	Seeding	Confluency
CT26					
T25 flask	2	1	5	0.3×10^6	1.2×10^6
T75 flask	3	2	10	0.9×10^6	3.6×10^6
T150 flask	7	4	20	1.8×10^6	7.2×10^6
4T1					
T25 flask	2	1	5	1.8×10^5	1.1×10^6
T75 flask	3	2	10	5.8×10^5	3.5×10^6
T150 flask	7	4	20	1.2×10^6	7×10^6
HT29					
T25 flask	2	1	5	0.6×10^6	2.4×10^6
T75 flask	3	2	10	1.8×10^6	7.2×10^6
T150 flask	7	4	20	3.6×10^6	1.4×10^7
MC26					
T25 flask	2	1	5	0.3×10^6	1.2×10^6
T75 flask	3	2	10	0.9×10^6	3.6×10^6
T150 flask	7	4	20	1.8×10^6	7.2×10^6

11 After centrifugation, check to see aggregates of tumor cells at the bottom of the conical tube. The supernatant should be clear.
? TROUBLESHOOTING

12 Spray 70% (vol/vol) ethanol on to the outer surface of the conical tube and bring it inside the biosafety cabinet.

13 Aspirate the growth medium by using vacuum suction inside the biosafety cabinet.

14 Gently resuspend the tumor cells in 1 ml of warm tumor cell culture medium.

15 Take out 10 µl of tumor cells and mix with 10 µl of trypan blue stain (1:1 ratio). Transfer the solution to cell-counting chamber slides to calculate the concentration of cells.

16 Add 0.3×10^6 tumor cells to 5 ml of tumor cell culture medium in a T25 flask. Refer to Table 3 for the respective number for various other flask sizes.

17 Transfer the medium containing tumor cells into a culture flask. Gently shake to evenly distribute the tumor cells in the flask.

18 Take out the culture flask from the biosafety cabinet and observe it under a brightfield microscope. Tumor cells should be round and floating in the medium.

19 Place the culture flask into the 37 °C CO_2 incubator.
▲CRITICAL STEP Check tumor cells under a microscope the next day. Healthy cells should be adherent to the bottom of the culture flask.

20 After 2–3 d of culture, observe the tumor cells under the brightfield microscope. The cells are ready to be expanded when they cover ~80% of the bottom surface.

Expand tumor cells ● Timing 0.5 h (tumor cell expansion), 2–3 d (tumor cell growth)

21 Warm up tumor cell growth medium to 37 °C in a water bath. Warm up the dissociation reagent, 0.05% (vol/vol) trypsin EDTA, to room temperature (20–24 °C).

22 Sterilize the biosafety cabinet. Details are provided in Step 3.

23 Using 70% (vol/vol) ethanol, wipe the outer surface of the flask containing healthy and adherent tumor cell lines that grew in the 37 °C CO_2 incubator (from Step 20) and bring them inside the biosafety cabinet.

24 Thoroughly spray 70% (vol/vol) ethanol on to the outer surface of the bottles containing tumor cell growth medium and bring them inside the biosafety cabinet.

25 Aspirate the growth medium by using vacuum suction inside the biosafety cabinet. Tilt the flask to avoid touching tumor cells directly.

26 Add an appropriate volume of 1× DPBS at room temperature onto the cells depending on the surface area of the flask or plate. Make sure that the DPBS covers the entire surface of adherent cells by slightly tilting the plate. Refer to Table 3 for suggested volumes of different types of flasks.
27 Aspirate the 1× DPBS by using vacuum suction.
28 Add 0.05% (vol/vol) trypsin EDTA to the flask. Make sure that 0.05% (vol/vol) trypsin EDTA covers the entire surface of adherent cells by slightly tilting the plate. Refer to Table 3 for suggested volumes of different types of flasks.
29 Place the flask back in the 37 °C CO_2 incubator and wait for 3–5 min.
30 Take the flask out of the CO_2 incubator. Gently tap the side of the flask with the palm of one hand while holding the flask securely with the other hand. This will allow most cells to dissociate from the surface. Observe for cell dissociation from the surface under a microscope.
▲ **CRITICAL STEP** The necessary incubation time may vary. If most of the cells are still attached, repeat Step 29 for an additional 2–3 min. Be careful not to over-trypsinize the cells (cells start to form visible clumps).
31 Take the flask back inside the biosafety cabinet. Add an equal volume of tumor cell culture medium. Tilt the flask and flush the bottom surface where the cells attached with culture medium. Repeat the flushing step four to five times to cover different parts of the surface.
▲ **CRITICAL STEP** Minimize creating air bubbles while pipetting.
32 Transfer the cell-containing solution to a 15-ml conical tube.
33 Centrifuge the tube at 200*g* for 5 min at room temperature.
34 Repeat Steps 11–15 to count cell numbers and determine if there are enough cells for generating the number of spheroids of your choice. Roughly 0.25×10^6 cells are required to generate spheroids in a 96-well plate. Refer to Table 3 to estimate the number of tumor cells in the flask.
35 If cells need to be expanded, repeat Steps 16–34. Otherwise, skip to Step 36.

Seed tumor cell spheroids ● Timing 1 h (tumor spheroid seeding), 4 d (tumor spheroid growth)
36 Prepare tumor spheroid culture medium as described in Materials.
▲ **CRITICAL STEP** Tumor spheroids are generated by using tumor spheroid culture medium that does not contain antibiotics. Handle with extra caution to ensure sterile culture.
37 Warm up tumor spheroid growth medium to 37 °C in a water bath. Warm up 0.05% (vol/vol) trypsin EDTA to room temperature.
38 Prepare tumor cells by trypsinizing and resuspending the cells. Repeat Steps 22–34, then Steps 11–13.
39 Gently resuspend the tumor cells in 1 ml of warm tumor spheroid culture medium.
40 Take out 10 µl of tumor cells and mix with 10 µl of trypan blue stain (1:1 ratio). Transfer the solution to cell-counting chamber slides to calculate the concentration of cells.
41 To seed tumor spheroids in a 96-well plate, add 0.25×10^6 cells in 10 ml of tumor spheroid culture medium.
42 Transfer the tumor cells to a 50-ml liquid reservoir.
43 Using a multi-channel pipette, transfer 100 µl of cell suspension in each well of the 96-well, clear, round-bottom, ultra-low attachment plate.
44 Centrifuge the 96-well round-bottom plate at 100*g* for 5 min at room temperature.
45 After centrifugation, check to see aggregates of tumor cells at the bottom of the 96-well plate.
▲ **CRITICAL STEP** Handle gently to avoid disturbing tumor cell aggregates.
46 Transfer the plate to a 37 °C CO_2 incubator.
47 Check tumor spheroids daily under a brightfield microscope. They will aggregate and grow in size over the days. Incubate spheroids for 4 d.
? TROUBLESHOOTING

Bacteria coculture with tumor spheroids

Add bacteria to tumor spheroids ● Timing 0.5 h (setup), 2 h (incubation)
! CAUTION Although ELH1301 strain is attenuated, it should be handled under the biosafety level 2 condition.
▲ **CRITICAL** Bacterial cultures should be set up 3 d after spheroid seeding (Box 1). *S. typhimurium* strain ELH1301 carrying a constitutive sfGFP plasmid is used in this protocol (see Materials), unless otherwise noted. See Table 2 for parameters to consider for optimizing the procedure for bacterial strains.

48 After 4 d of spheroid seeding, check the appearance of tumor spheroids. The cores should appear slightly darker than the periphery under a brightfield microscope, indicating the start of necrotic center formation.
? TROUBLESHOOTING

49 Sterilize the biosafety cabinet. Details are provided in Step 3.

50 Prepare a beaker containing bleach for bacteria disposal. Place it in the biosafety cabinet.

51 Prepare coculture medium as described in Materials.

52 Warm up tumor spheroid growth medium and coculture medium to 37 °C in a water bath.

53 Centrifuge the bacterial culture tube at 3,000*g* for 5 min.

54 Sterilize the outer surface of the bacterial culture tube and gently place it onto the biosafety hood.

55 Carefully decant the supernatant into bleach while saving the bacterial pellet at the bottom of the culture tube.

56 Add 2 ml of 1× DPBS to the tube and resuspend the bacterial pellet by pipetting.

57 Transfer 1 ml of the bacteria into a plastic cuvette.

58 Measure the optical density of the bacterial culture. Place the plastic cuvette into a NanoDrop spectrophotometer and measure the OD_{600} value. The OD_{600} value should be ~3 for a stationary phase *S. typhimurium* overnight culture.
? TROUBLESHOOTING

59 Adjust the OD_{600} of the bacterial solution to 0.5 by dilution with 1× DPBS. 1 ml of the solution should now contain ~$0.1–0.2 \times 10^9$ c.f.u. of *S. typhimurium* (Supplementary Fig. 1).

60 Take the spheroid plates (from Step 47) out of the 37 °C CO_2 incubator and place them in the biosafety cabinet.
▲ CRITICAL STEP Changing gloves and sterilizing hands before reaching into the CO_2 incubator is recommended to avoid bacterial contamination.

61 Add 2 µl of the bacteria solution to each spheroid well on the 96-well plate. An OD_{600} value of 0.5 should equate to $10^5–10^6$ c.f.u. of *S. typhimurium* per spheroid well.

62 Transfer the plates to a 37 °C CO_2 incubator and incubate for 2 h.
▲ CRITICAL STEP Using a separate 37 °C CO_2 incubator from the one used for tumor cell culturing is suggested to avoid cross contamination.

Remove extratumoral bacteria ● Timing 1–2 h (setup), 3–4 d (coculture)

63 After a 2-h incubation, take out the coculture plate and observe it under the brightfield microscope. Small speckles of bacteria should be seen at the bottom of the wells surrounding tumor spheroids.
▲ CRITICAL STEP Observe the plate under a fluorescent microscope to more clearly see bacteria via sfGFP.

64 Bring the coculture plate back to the biosafety cabinet.

65 Wash out the extratumoral bacteria. Aspirate the medium by using a p200 pipette to slowly pipette up 60 µl of growth medium from each well of the 96-well plate. The remaining 40 µl of medium should contain the spheroid in each well. A multi-channel pipette can be used to simultaneously change medium.
▲ CRITICAL STEP Set up a clean, white background at the rear end of the workspace. Spheroids can be identified quickly as small, dark spheres under adequate light when observed against the white background. To avoid aspirating the spheroid, angle the pipette tips at 45 °, touching the top part of the medium in each well during aspiration. Before discarding the tips, observe carefully to make sure that no spheroids entered the pipette tips. This also applies to Steps 67 and 68.

66 Slowly add 160 µl of 1× DPBS toward the side of each well, angling the pipette tips at 45 °, touching the top part of the well.
▲ CRITICAL STEP Spheroids may float up when adding the medium, which helps wash bacteria at the bottom of the plate. However, avoid pipetting strongly to prevent breaking the spheroids.

67 To aspirate the medium, slowly pipette up 160 µl of the medium from each well of the 96-well plate. The remaining 40 µl of medium should contain the spheroid in each well. A multi-channel pipette can be used to simultaneously change medium.

68 Repeat Steps 66 and 67 a further two times to ensure that all extra bacteria are removed.

69 Use a p20 pipette to aspirate the 1× DPBS as much as possible without aspirating the spheroids.

70 Slowly add 200 µl of the coculture medium containing gentamicin. Add the medium toward the side of each well, angling the pipette tips at 45 °, touching the top part of the well.

71 Check under a brightfield microscope for spheroid integrity. A tumor spheroid typically contains ~1 × 10^4 c.f.u. of bacteria after the washing steps. Extratumoral bacteria should be washed away at this point.
? TROUBLESHOOTING

72 Transfer the coculture plate to a 37 °C CO_2 incubator.

73 For long-term experiments, medium should be replaced every 3–4 d. To change the medium, follow Step 67 and then Step 66. Use coculture medium instead of 1× DPBS.

Bacterial infiltration and colonization

74 Perform further analyses to characterize tumour spheroids by following option A (imaging bacteria within spheroids), option B (imaging regions of hypoxia within the spheroids), option C (tracking bacterial growth) or option D (histology):

(A) **Image bacteria within tumor spheroids ● Timing 0.5 h**

(i) Take out the coculture plate from the 37 °C CO_2 incubator and check the appearance of the tumor spheroids under a brightfield microscope daily. The tumor spheroids should be intact and round. Over time, they will continue to grow in size and appear darker in the center (necrotic core).
▲ CRITICAL STEP You should not see bacteria outside of the tumor spheroids at this point, given sufficient washing and addition of gentamicin (Supplementary Fig. 2A and 2C).
? TROUBLESHOOTING

(ii) To image bacterial fluorescence, transfer the coculture plate onto the fluorescent microscope (EVOS FL Auto 2 cell imaging systems).

(iii) Set the focal plane to the tumor spheroids and acquire brightfield and fluorescence (sfGFP) images. Daily imaging of the spheroids is recommended.
▲ CRITICAL STEP You should see bacterial signal infiltrating toward the center of the growing spheroids during the first 2–3 d. Once bacteria reach the spheroid core, they will colonize the necrotic region, which can be observed as increasing sfGFP signal.

(iv) Quickly put the coculture plate back into the 37 °C CO_2 incubator.

(B) **Image hypoxia and tumor spheroids ● Timing 1 h (setup), 2 h (incubation)**

(i) To image hypoxic regions within tumor spheroids, prepare a 1 mM stock solution of Image-IT hypoxia reagent in 1× dimethyl sulfoxide.

(ii) Sterilize the biosafety cabinet. Details are provided in Step 3.

(iii) Take out the coculture plate from the 37 °C CO_2 incubator and place it in the biosafety cabinet.

(iv) Add the hypoxia reagent directly to the medium containing the tumor spheroids at a final concentration of 10 μM.

(v) Transfer the coculture plate back into the 37 °C CO_2 incubator for 1 h.

(vi) Take out the coculture plate from the 37 °C CO_2 incubator and place it in the biosafety cabinet.

(vii) Exchange the medium to wash out the hypoxia reagent. Repeat Step 67 and then Step 66, twice.

(viii) Transfer the coculture plate back into the 37 °C CO_2 incubator and incubate overnight.

(ix) Image the tumor spheroids on the fluorescence microscope. Hypoxia reagent can be imaged at 490/610 nm (e.g. by using a Texas Red filter). Refer to Step 74A(i–iv) for imaging details.
? TROUBLESHOOTING

(x) (Optional) Tumor cell distribution can be imaged by using CT26 cells transfected to express iRFP (or fluorescence of your choice). Over time, the core will appear dim, indicating the necrotic center of the spheroid.

(C) **Track bacterial growth with c.f.u. ● Timing 1 h (setup), overnight (incubation)**

(i) Melt previously prepared and autoclaved LB agar in a microwave for 30–60 s.
! CAUTION The agar can get very hot. Handle with care.

(ii) Sterilize the biosafety cabinet. Details are provided in Step 3.

(iii) Prepare agar plates by transferring the LB agar and Petri dishes into the biosafety cabinet. Avoid making any air bubbles.

(iv) Add 50 μg/ml kanamycin to the LB agar.
▲ CRITICAL STEP Wait until the agar cools down so you can hold it. This prevents antibiotic degradation due to excessive heat. Avoid waiting longer than necessary to prevent clumps of solid agar forming on the plates.
(v) Before the agar solidifies, transfer 15 ml of LB agar into a Petri dish.
(vi) Air-dry the LB agar to solidify it by keeping the lid open.
▲ CRITICAL STEP Handle with sterile technique to avoid contamination. Completely solidify the LB agar before moving.
(vii) Take out the coculture plate from the 37 °C CO_2 incubator and place it in the biosafety cabinet.
(viii) Wash the tumor spheroids. Repeat Step 67 and then Step 66, twice.
▲ CRITICAL STEP Gentamicin needs to be removed from the medium to avoid killing the bacteria.
(ix) Slowly add 100 μl of 1× DPBS to the well.
(x) Use sterile tips to mechanically dissociate tumor spheroids. A sterile scalpel can be used to cut the tumor spheroid into four or five small pieces. Pipette tips can be used to pierce the spheroids to promote dissociation. After initial disruption of the spheroids, repeated pipetting of the spheroids can further break the cell aggregation. Repeat this step to make sure that the bacteria from the central core are exposed.
(xi) Using a p200 pipette, repeatedly pipette up and down to further dissociate the tumor spheroid.
(xii) Serially dilute 10× the solution containing dissociated spheroid with 1× DPBS. We recommend eight dilutions to ensure that individual colonies can be counted.
(xiii) Place 5-μl droplets of each dilution onto the LB agar plate.
▲ CRITICAL STEP Leave ~0.5 inches of space between droplets to avoid merging of colonies.
(xiv) Leave the plate upright with the lid open to air-dry the droplets.
(xv) Place the lid on the agar plate and transfer it to a 37 °C incubator. Incubate overnight to allow colony formation.
▲ CRITICAL STEP Place the agar plate upside down to avoid condensation.
(xvi) On the next morning, take out the agar plate and count the number of colonies on each droplet. Spot dilutions that allow visualization of individual colonies.
? TROUBLESHOOTING
(xvii) Enumerate the c.f.u. per spheroid by multiplying with the appropriate dilution factor.
(xviii) (Optional) Measure bacterial plasmid retention by plating bacteria to agars with no plasmid selection antibiotic marker. The ratio of c.f.u. in selected to non-selected plates can be calculated.

(D) **Prepare histological samples** ● Timing **1 h (setup), 1 d (processing)**
(i) Place 4% (vol/vol) PFA on ice.
! CAUTION Formaldehyde is a Group 1 carcinogen. Handle it in a fume hood and dispose of it with precaution.
(ii) Sterilize the biosafety cabinet.
(iii) At the desired time point, take out the coculture plate from the 37 °C CO_2 incubator and bring it to the biosafety cabinet.
(iv) Remove culture medium from each well by following Step 67 and then Step 69.
(v) Add 100 μl of 4% PFA to each well.
(vi) Incubate on ice (or 4 °C environment) for 20 min.
(vii) Wash out 4% PFA by following Steps 67–69 (wash spheroids three times total).
(viii) Preserve the spheroid in 70% (vol/vol) ethanol solution at 4 °C.
(ix) Transfer fixated spheroid samples to a holder for downstream embedding, cutting and staining. In our original study, H&E, Gram and TUNEL staining were performed to study spheroid architecture, bacterial distribution and tumor cell death, respectively[20].

75 (Optional) Perform further analyses of tumour spheroids. Spheroids can be used for the following:
- Analysis of gene circuits (Box 2).
- Therapeutic efficacy screening (Box 3).

76 (Optional) Validate therapeutics with an in vivo mouse model (Box 4).

Troubleshooting

Troubleshooting advice can be found in Table 4.

Table 4 | Troubleshooting table

Step	Problem	Possible reason	Solution
11	The supernatant is not clear after centrifugation	Centrifugation did not work	Ensure that the centrifuge is working at the appropriate speed, temperature and time
		The medium is contaminated	Ensure sterile techniques
47	Spheroids do not form	Cancer cells are dead	Ensure that viability is high before forming spheroids
			Change the medium regularly. Ensure that the spheroids are not damaged when pipetting
		Spheroids are still in the process of forming	The initial phase will be aggregation of tumor cells (~1 d). Spheroids will start to grow in size after aggregation is complete
		Contamination (e.g. *Mycoplasma*)	Check for contamination regularly
48	Tumor spheroid cores do not appear darker than the periphery under a brightfield microscope after 4 d of spheroid seeding	Spheroids did not form necrotic cores	Ensure that spheroids are seeded at the appropriate cell numbers and conditions
		The brightfield microscope is not properly set up	Follow the manufacturer's manual to set up the brightfield microscope. Check that the focal plane is set at the right level
58	The OD_{600} value is not ~3 for a stationary phase *S. typhimurium* overnight culture	*S. typhimurium* did not grow enough to reach the stationary phase	Ensure that the culture is grown in the appropriate condition overnight
		S. typhimurium overgrew and died	Ensure that the culture does not grow beyond overnight and that appropriate antibiotics are used
71	Spheroids are dissociated	Spheroids enter the pipette tips during aspiration	Optimize the pipetting process. Find a comfortable angle and slowly aspirate the medium
		Pipetting is too rigorous	Gently pipette
	Bacteria are still seen in the medium	Not enough washing	Repeat washing steps until extratumoral bacteria are washed away
74A(i)	Bacteria leaking out from spheroid (Supplementary Fig. 2A and C)	The spheroid is dissociated	Ensure that the spheroid was not damaged during the washing process
		Not enough washing	Ensure that all extratumoral bacteria are washed out
		The gentamicin concentration is low	Test various gentamicin concentrations for optimization
74B(ix)	No signal is observed from the hypoxia imaging assay	No hypoxia in the core	Check for a darker core within the spheroids under the brightfield microscope, which suggests hypoxia and necrosis. Hypoxia may take time to develop depending on the cell type and spheroid size
		Not enough dye	Optimize the hypoxia dye assay with concentration and incubation time
74C(xvi)	No CFU observed (Supplementary Fig. 2B)	No bacteria in spheroid	Bacteria did not survive in tumor spheroids. Check that the coculture procedures have been followed properly
		The tumor spheroid was not completely dissociated	Ensure that spheroids are completely dissociated by checking under the brightfield microscope
Box 1, 7	Bacteria do not grow	Wrong medium, temperature or antibiotic selection marker	Check the growth condition
		Need more time	Depending on the inoculation volume, the culture needs more time. Some bacteria grow more slowly
Box 4, 14	Tumors not grafting	Tumor cells were not viable	Ensure tumor cell viability by using trypan blue and Cell Countess as described in step 14. Viability should be >90%
		The mouse strain is not compatible with tumor cells	Find an appropriately graft-compatible mouse strain for the specific tumor cells
	Tumor sizes vary across the mice	Variability in number of tumor cells grafted	Ensure that the same number of tumor cells is being injected in each mouse. Gently pipette the tumor cell solution up and down five to seven times before filling up the injection syringe to use a homogeneous solution. Randomize the mice across cages on the basis of their tumor sizes before treatment starts

Table continued

Table 4 (continued)

Step	Problem	Possible reason	Solution
Box 4, 25	Bacteria leak out after injection in the subcutaneous tumor	The injection was in the outer region of the tumor	Insert the needle in the tumor, aiming for the central area of the tumor, pull out slightly to make a small empty space inside the tumor and then very slowly inject a total volume of 20–40 µl per tumor
		The tumor is too small to contain the bacteria	Change the location of the injection and split the injection in multiple small volumes to reach a final volume of 20–40 µl per tumor

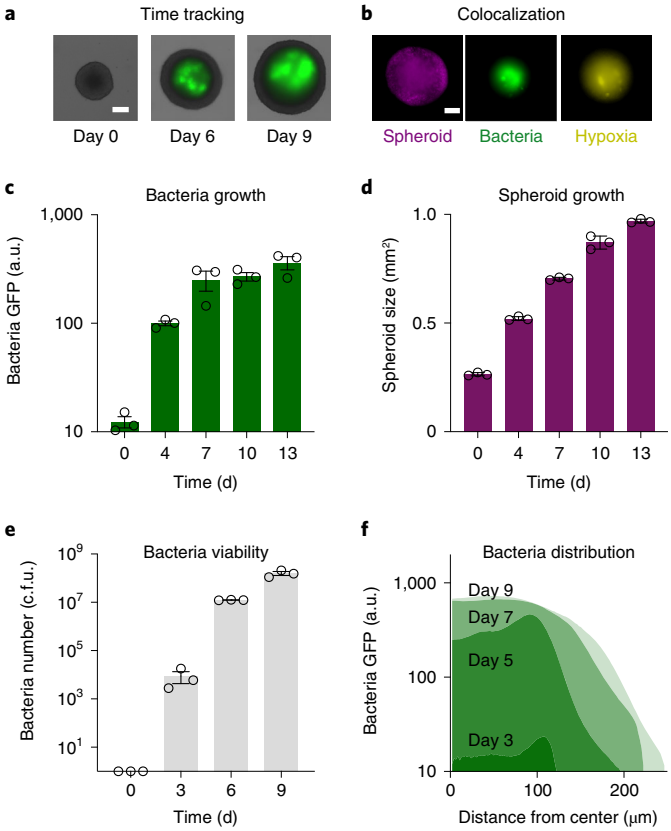

Fig. 5 | Characterization of bacteria and tumor spheroids in coculture. a, Representative images of sfGFP expressing *S. typhimurium* in CT26 tumor spheroids over time. Scale bar, 200 µm. **b**, Distribution of the cancer cells (iRFP, magenta), bacteria (sfGFP, green) and hypoxia (hypoxic probe dye, yellow) in coculture after 14 d of coculture. Scale bar, 200 µm. **C**, Bacteria growth within tumor spheroids measured by sfGFP fluorescence. **d**, Growth of tumor spheroids colonized by bacteria. **e**, Bacteria viability measured by c.f.u. after spheroid dissociation. **f**, Radial distribution of bacteria measured by sfGFP levels within tumor spheroids over time. Error bars indicate ±s.e.m. of three replicates in panels **c–e**. All data were adapted from ref. [20].

Timing

Steps 1–20, thaw tumor cells: 1 h (thawing), 2–3 d (growth)
Steps 21–35, expand tumor cells (optional): 0.5 h (expansion), 2–3 d (growth)
Steps 36–47, seed tumor spheroids: 1 h (seeding), 4 d (growth)
Steps 48–62, add bacteria to tumor spheroids: 0.5 h (setup), 2 h (incubation)
Steps 63–73, remove extratumoral bacteria: 1–2 h (setup), 3–4 d (coculture)
Step 74A, image bacteria within tumor spheroids: 0.5 h
Step 74B, image hypoxic regions and tumor spheroids: 1 h (setup), 2 h (incubation)
Step 74C, track bacterial growth with c.f.u.: 1 h (setup), overnight (incubation)
Step 74D, prepare histological samples: 1 h (setup), 1 d (processing)

Box 1, grow bacteria: 0.5 h (setup), overnight (growth)
Box 2, steps 1–7, activate bacterial gene circuits: 0.5 h
Box 2, steps 8–11, acquire time-lapse images: 0.5 h (setup), 3–5 d (image acquisition)
Box 3, steps 1–3, induce therapeutic expression: ~0.5 h
Box 3, steps 4–9, analyze microscope images: 1–3 h
Box 4, steps 1–14, inject mice subcutaneously with tumor cells: 2–3 h (preparation), 1–3 weeks (tumor grafting)
Box 4, steps 15–28, in vivo therapeutic validation: 2–3 h (injection), 1–2 weeks (tumor growth), 1–3 weeks (treatment)

Anticipated results

We envision that the BSCC protocol described here will facilitate a broad range of experimental studies for topics including bacteria–cancer interactions, genetic circuit dynamics and

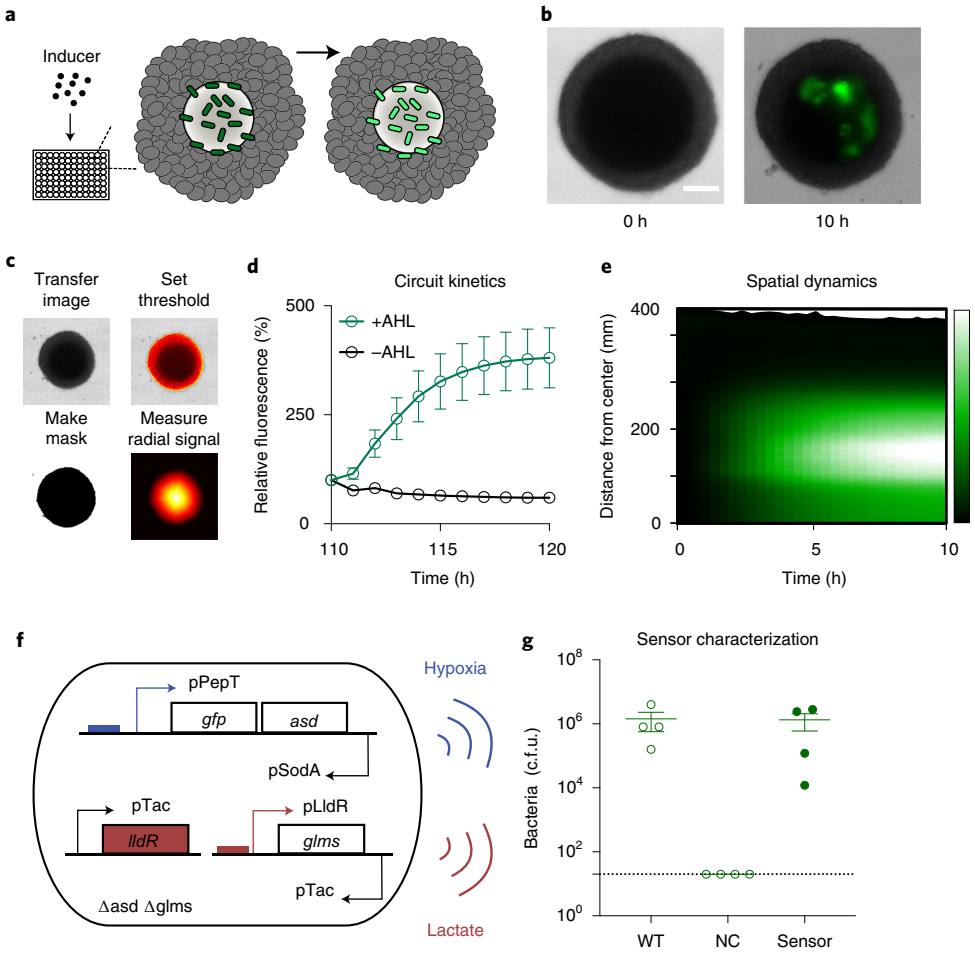

Fig. 6 | Gene circuit performance in tumor spheroids. a, An inducible system from bacteria is activated by adding an inducer molecule, AHL, to the coculture. **b**, Representative images of bacterial sfGFP expression from the inducible circuit. Scale bar, 200 μm. **c**, An example workflow of image analysis. Images were imported to a standard analysis tool (e.g. ImageJ). The spheroid outline is set with thresholding. Masks are made to assign spheroid area. sfGFP signal is measured within tumor spheroids in total or with radial average. **d**, Quantification of the bacterial sfGFP signal within tumor spheroids over time. Error bars indicate ±s.e.m. of three replicates. **e**, Space-time diagram showing radial average sfGFP levels within tumor spheroids over time. **f**, Gene circuit diagram of the engineered bacteria programmed to grow only when they encounter hypoxia and high lactate conditions. The gene circuit consists of hyoxia promoter pPepT expressing an essential gene (*asd*) and lactate promoter pLldR expressing an orthogonal essential gene (*glms*). **g**, Engineered bacteria harboring a biosensor circuit in **f** were cocultured in tumor spheroids and monitored for growth. Similar levels of bacterial c.f.u. were recovered after 6-d coculture from the engineered strain (Sensor) compared to the wildtype strain (WT). Negative control (NC) without sensors (*ΔasdΔglms*) failed to grow in tumor spheroids. The dotted line indicates the limit of detection at 20 c.f.u. Error bars indicate ±s.e.m. All data were adapted from ref. [20] (**b–e**) and ref. [21] (**g**).

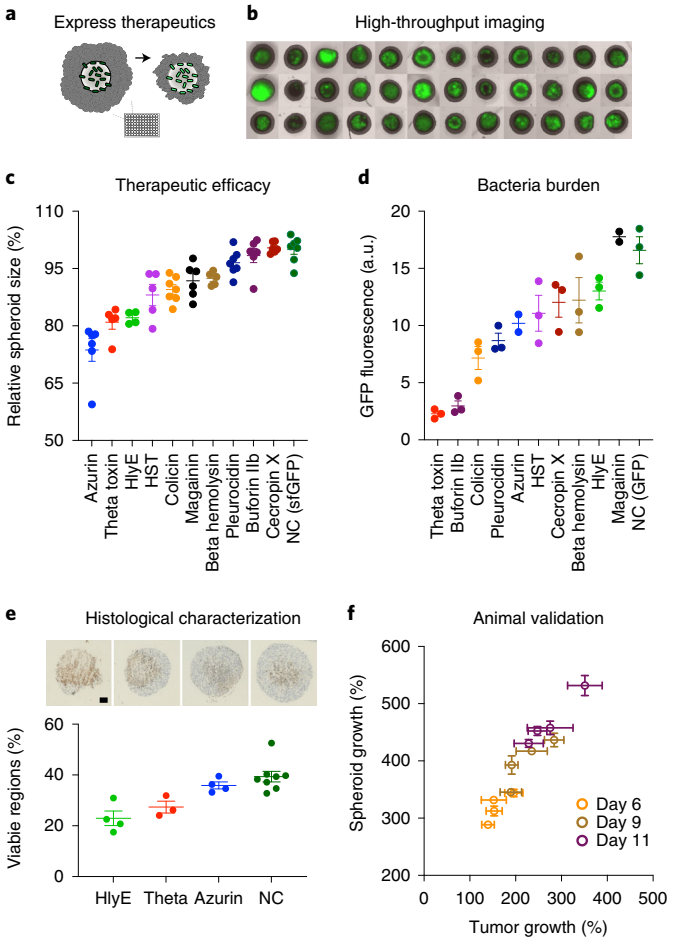

Fig. 7 | Therapeutic screening and validation. a, Bacteria express therapeutic payloads from within the tumor spheroid core. sfGFP is constitutively expressed by bacteria. Values are normalized by control (bacteria expressing sfGFP). 10 nM AHL was added to the culture medium to induce therapeutic expression from bacteria. **b**, Images of tumor spheroids colonized by bacteria carrying various payloads. **c**, Therapeutic efficacy of various therapeutic payloads measured by spheroid size after 10-d colonization. **d**, Bacteria burden measured by sfGFP expressed from bacteria within spheroids after 10-d colonization. **e**, Representative images of tumor spheroids with TUNEL staining. The graph below shows quantification of viable regions within tumor spheroids treated with bacteria expressing therapeutics. Scale bar, 100 μm. **f**, Comparison of tumor growth in tumor spheroids and in an animal model. Linear regression shows correlation between the two models as $R^2 = 0.92$. For animal experiments, subcutaneous CT26 tumors were treated by intratumorally injecting bacteria expressing beta hemolysin, theta toxin, azurin and hemolysin E. Error bars indicate ±s.e.m. of at least four replicates. HlyE, hemolysin E; HST, heat-stable enterotoxin. All data were adapted from ref. [20].

high-throughput therapeutic testing. Initial establishment of bacterial tumor colonization in tumor spheroids is a critical step and the basis of many of the downstream studies. To characterize bacterial invasion and growth, we tracked sfGFP fluorescence expressed primarily in *S. typhimurium* ETH1301 over time. Upon incubating with tumor spheroids and replacing the growth medium, we initially observed that the bacteria attached to the spheroid surface. After adding gentamicin to the medium to contain bacterial growth, the bacteria infiltrated, localized and proliferated in the interior of the growing tumor spheroid (Fig. 5a). *S. typhymurium* was found to localize in the necrotic and hypoxic regions of the spheroid, recapitulating the bacterial colonization condition in vivo (Fig. 5b). Both bacterial sfGFP and c.f.u. levels increased for up to 14 d (Fig. 5, c–f). Using this protocol, we also tested different combinations of bacterial strains, species and tumor cell types, which resulted in a diverse array of bacterial colonization dynamics and tumor responses (Fig. 4, a–c).

The ability to maintain stable coculture over time makes this protocol suitable for quantitative monitoring of cellular dynamics. We characterized dynamics of gene circuits including inducible, biosensing and logic-gate constructs within the tumor spheroid. Small-molecule AHL-inducible circuits were tested by adding the inducer in the culture medium and monitoring dynamics via

time-lapse microscopy (Fig. 6a–c). This revealed an increase in sfGFP signal upon induction, reaching maximum expression at ~4 h near the core of the tumor (Fig. 6d,e). Taking advantage of the natural tumor environment provided by the 3D tumor model, we also tested the ability of bacteria to sense tumoral hypoxia, lactate and acidity. Coupling two biosensors to bacterial growth (hypoxia and lactate-inducible systems; see Materials), we recently constructed an AND logic-gate system in which bacteria are allowed to proliferate only upon encountering both signals (Fig. 6f). The bacteria's performance was tested by coculturing with tumor spheroids, demonstrating that the engineered bacteria are capable of sensing the environment in tumor spheroids and growing in response (Fig. 6g).

This coculture protocol is compatible with the standard 96-well format, allowing high-throughput drug testing (Fig. 7,a and b). Because bacteria cancer therapy studies usually test only a limited number of therapeutic payloads and are generally not compared side by side, we tested the efficacy of bacteria expressing a library of cytotoxic antitumor payloads. We induced therapeutic expression when bacteria colonized the CT26 spheroid core and subsequently observed that many therapeutics demonstrated reduction in tumor spheroid growth (Fig. 7c). The growth burden on bacteria from payload expression was quantified by measuring bacterially expressed sfGFP (Fig. 7d). Histopathological analysis (TUNEL staining) of treated spheroids revealed tumor cell death within tumor spheroids after treatment (Fig. 7e). To facilitate mechanistic understanding of the therapeutic efficacy, various factors such as bacterial growth, payload effectiveness and expression level can be tested in combination by assessing bacterial c.f.u., lysate efficacy and fluorescence expression level, respectively. Lastly, we validated in vivo efficacy by using a syngeneic CT26 mouse model. Several strains identified to be effective from the in vitro screen elicited response in tumor growth when compared to the control strain. Linear regression analysis on efficacy revealed a high correlation (Fig. 7f), demonstrating predictive power of the in vitro screening platform.

Data availability

The main data discussed in this protocol were generated as part of the studies published in the supporting primary research papers[20,21]. Source data are provided for Figs. 4–7. Source data are provided with this paper.

Code availability

The computational code used for image analysis is provided in Supplementary Information.

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Author contributions

T.H. and T.D. conceived and designed the study. T.H., D.D. and T.D. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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