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(54) **Title:** THREE-DIMENSIONAL CO-CULTURE SYSTEM FOR HIGH-THROUGHPUT TESTING OF THERAPEUTICS AND
 DIAGNOSTICS

Fig. 1

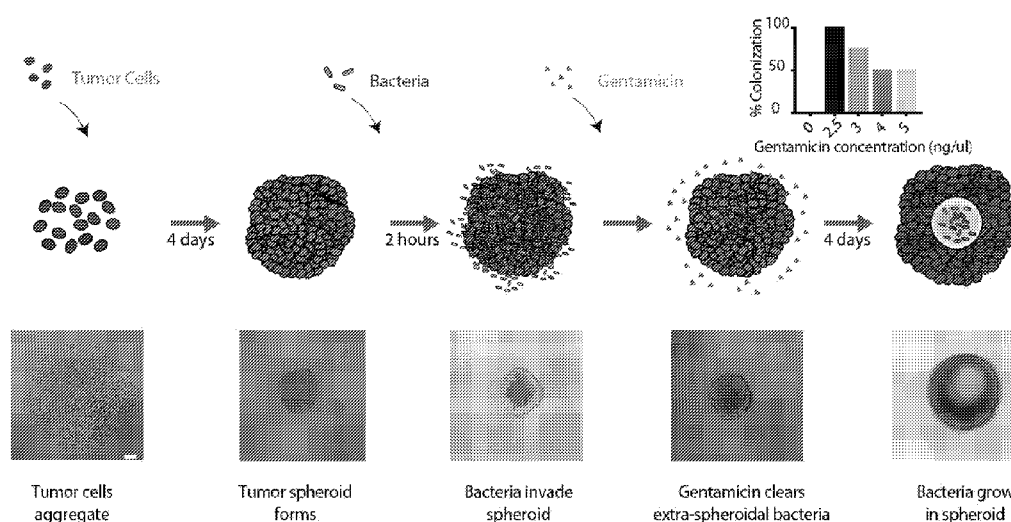


Fig. 1A

(57) **Abstract:** The disclosure herein relates to a three-dimensional co-culture system or platform for testing, screening or evaluating potential therapeutics and diagnostics for a variety of diseases, including cancer. The three-dimensional co-culture system includes a tumor spheroid or organoid, and a bacteria, virus, or eukaryotic cell. The disclosure also includes methods of generating and using the three-dimensional co-culture system or platform.



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THREE-DIMENSIONAL CO-CULTURE SYSTEM FOR HIGH-THROUGHPUT TESTING OF THERAPEUTICS AND DIAGNOSTICS

CROSS-REFERENCE TO OTHER APPLICATIONS

The present application claims priority to U.S. Patent Application Serial No. 62/739,501 filed October 1, 2018 which is hereby incorporated by reference in its entirety.

STATEMENT OF GOVERNMENT SUPPORT

This invention was made with government support under W81XWH-17-1-0356 and W81XWH-17-1-0395 awarded by the Army Medical Research and Material Command and CA197649 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

The disclosure herein relates to a three-dimensional co-culture system for high throughput testing, screening or evaluating potential therapeutics and diagnostics for a variety of diseases, including cancer. The three-dimensional co-culture system includes a tumor spheroid or organoid, and a bacteria, virus, or eukaryotic cell.

The disclosure also includes methods of generating and using the three-dimensional co-culture system.

BACKGROUND OF THE INVENTION

An emerging area of synthetic biology is the engineering of bacteria to sense and respond to various diseases states in the body such as gut inflammation, infections, and cancer (Rigler *et al.* (2017); Daeffler *et al.* (2017); Hwang *et al.* (2017); Mao *et al.* (2018); Din *et al.* 2016); Danino *et al.* (2015); Anderson *et al.* (2006)). Despite these efforts, the lack of physiologically-relevant *in vitro* testing environments to evaluate engineered bacteria limits rapid development for clinical use (Goers *et al.* (2014); Riglar and Silver (2018)).

In particular, bacteria-mediated tumor therapy, in which specially selected or genetically engineered bacteria are used as anti-cancer therapeutics, is a promising approach for cancer treatment. However, screening methods for engineered bacterial therapeutics remain limited as

conventional two-dimensional (2-D) cell monolayer models do not adequately recapitulate the complex *in vivo* microenvironment of cancer tumors.

Due to the rapid proliferation rate of bacteria, co-culture with mammalian cells has been a challenge, limiting assays to short time periods or necessitating the use of heat-killed bacteria (Bhave *et al.* (2015); Goers *et al.* (2014)). Previous attempts to control the imbalance in growth rates have fluidically controlled excess bacteria, directly injected bacteria into multicellular aggregates, or utilized obligate anaerobes to prevent overgrowth (Kim *et al.* (2016); Bartfeld *et al.* (2015); Osswald *et al.* (2015)). As a result, these systems expectedly increase technical complexity and restrict species types, reducing throughput and broad-applicability, respectively. Furthermore, long-term analysis of bacteria circuit dynamics has yet to be employed in multicellular co-cultures or applied towards therapeutic development (Goers *et al.* (2014)). In order to accelerate development for *in vivo* applications, simple and high-throughput three-dimensional (3-D) co-culture platforms are needed to rapidly identify effective microbial therapy candidates.

SUMMARY OF THE INVENTION

Disclosed herein a high-throughput system or platform to characterize bacteria as well as viruses and eukaryotic cells in a three-dimensional (3-D) co-culture system or platform comprising tumor spheroids or organoids. The disclosure herein presents a novel *in vitro* platform to test, screen or evaluate potential therapeutics or diagnostics, delivery systems, and genetic circuit dynamics in a high-throughput manner.

One embodiment is a three-dimensional co-culture system comprising: a tumor spheroid or organoid; and bacteria, virus or eukaryotic cell.

In some embodiments, the spheroid or organoid is genetically engineered to in order to have a means of detection or visualization.

In some embodiments, the bacteria, virus or eukaryotic cell is naturally occurring. In some embodiments, the bacteria, virus or eukaryotic cell is genetically engineered to comprise a therapeutic or diagnostic agent. In some embodiments, the bacteria, virus or cell is genetically engineered to release a therapeutic or diagnostic agent. In some embodiments, the bacteria, virus or cell is genetically engineered to contain a genetic circuit. In some embodiments, the genetic

circuit is a means to deliver a therapeutic or diagnostic agent. In some embodiments, the genetic circuit is a biosensor.

In some embodiments, the bacteria, virus or cell is being tested, screened or evaluated as a therapeutic or diagnostic. In other embodiments, the bacteria, virus or cell comprise an agent to be tested, screened or evaluated as a therapeutic or diagnostic. In other embodiments, the bacteria, virus or cell comprise a genetic circuit to be tested, screened or evaluated. In some embodiments, the genetic circuit is a means to deliver a therapeutic or diagnostic agent. In some embodiments, the genetic circuit is a biosensor.

In some embodiments, the bacteria, virus or cell is genetically engineered in order to have a means of detection or visualization.

A further embodiment is a method of generating or establishing the three-dimensional co-culture system comprising: a tumor spheroid or organoid; and bacteria, virus or eukaryotic cell.

In some embodiments, the spheroid or organoid is genetically engineered to in order to have a means of detection or visualization.

In some embodiments, the bacteria, virus or eukaryotic cell is naturally occurring. In some embodiments, the bacteria, virus or eukaryotic cell is genetically engineered to comprise a therapeutic or diagnostic agent. In some embodiments, the bacteria, virus or cell is genetically engineered to release a therapeutic or diagnostic agent. In some embodiments, the bacteria, virus or cell is genetically engineered to contain a genetic circuit. In some embodiments, the genetic circuit is a means to deliver a therapeutic or diagnostic agent. In some embodiments, the genetic circuit is a biosensor.

In some embodiments, the bacteria, virus or cell is genetically engineered in order to have a means of detection or visualization.

Yet a further embodiment is a method using the three-dimensional co-culture system comprising a tumor spheroid or organoid and bacteria, virus or eukaryotic cell to test, screen or evaluate a therapeutic or diagnostic agent, a delivery system, and/or a genetic circuit.

In some embodiments, the spheroid or organoid is genetically engineered to in order to have a means of detection or visualization.

In some embodiments, the bacteria, virus or cell is tested, screened or evaluated as a therapeutic or diagnostic. In some embodiments, the bacteria, virus or cell comprise an agent to be tested, screened or evaluated as a therapeutic or diagnostic. In some embodiments, the

bacteria, virus or cell comprise a genetic circuit to be tested, screened or evaluated. In some embodiments of the method of using the three-dimensional co-culture system, an agent is added to the system to induce the bacteria, virus or cell to release the potential therapeutic or diagnostic agent and/or induce a genetic circuit.

The present disclosure also provides for kits for generating the three-dimensional co-culture system and using the three-dimensional co-culture system.

BRIEF DESCRIPTION OF THE DRAWINGS

For the purpose of illustrating the invention, there are depicted in drawings certain embodiments of the invention. However, the invention is not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

Fig. 1 shows the design and characterization of high-throughput bacteria spheroid (HTBS) co-culture platform. Fig. 1A is a schematic of the exemplified protocol for establishing the spheroid / bacteria co-culture system. Fig. 1B is a schematic of workflow for testing microbial therapeutics in tumor spheroids. Bacteria selectively colonize tumor spheroids in 96-well low adhesion plates. Host strains, synthetic gene circuits, and anticancer agents were characterized *in vitro* and validated in mouse tumor models. Fig. 1C is images and a graph of total sfGFP of bacteria (green triangles) and colony forming units (CFU, blue squares) over time. Day 0 values are after inoculation and washing of bacteria. Error bars indicate $\pm$ s.e. averaged over three measurements. a.u., arbitrary unit. Representative images from this data. Scale bar, 200 μ m. Fig. 1D shows images and graphs of the spatial distribution of *S. Typhimurium* in tumor spheroid 14 days post colonization. CT26 tumor spheroids (iRFP, magenta) developed hypoxic regions in spheroid core as shown by hypoxic probe dye (blue). *S. Typhimurium* (sfGFP, green) grew inside of the necrotic core. Scale bar, 200 μ m. The graph shows the distribution of fluorescence intensity of tumor cells, bacteria, and hypoxic regions from the center of spheroid. Fig. 1E are graphs of *S. typhimurium* growth in tumor spheroids derived from multiple cell types. The graph on the left shows bacteria growth over time in tumor spheroids as measured by sfGFP expression. The graph on the right shows tumor spheroid growth over time after colonization by bacteria. Bars indicate from left to right, days 0, 2, 4, 6 post inoculation of bacteria. Error bars indicate $\pm$ s.e. averaged over three measurements. Fig. 1F shows a space-time diagram showing radially averaged fluorescence intensity of sfGFP expressing bacteria. The gray top boundary

indicates the edge of the spheroid. Fig. 1G is a graph of total sfGFP of bacteria (green) and colony forming units (CFU, blue) over time. Day 0 values are after inoculation and washing of bacteria. Error bars indicate $\pm$ s.e. averaged over three measurements. Figure 1H are images of bacteria (purple) detected in the necrotic region in tumor spheroid via gram staining. Scale bar, 100 μ m (left hand image) and 10 μ m (right hand image).

Fig. 2 shows the comparison of *S. typhimurium* strains in tumor spheroids. Fig. 2A is a graph showing the growth of *S. typhimurium* strains (ELH1301, SL7207, ELH430, and VNP20009) in batch culture. Fig. 2B is a graph showing the constitutively expressed sfGFP signal of *S. typhimurium* strains (ELH1301, SL7207, ELH430, and VNP20009) in batch culture. Fig. 2C is a graph of the tumor spheroid growth of *S. typhimurium* strains (ELH1301, SL7207, and ELH430) recovered from dissociated spheroid. Fig. 2D is a graph of the CFU of *S. typhimurium* strains (ELH1301, SL7207, and ELH430) recovered from dissociated spheroid. Fig. 2E is a graph of sfGFP fluorescence of *S. typhimurium* strains in tumor spheroids. Fig. 2F is a graph of the percent plasmid retention recovered from dissociated tumor spheroid, 10 days post bacterial inoculation. Plasmid retention was calculated by colony counts on either plasmid-selective (kanamycin) or non- plasmid-selective plates. Fig. 2G is a graph of CFU of *S. typhimurium* strains (ELH1301 and VNP20009) recovered from tumor spheroids. Fig 2H is a graph of the sfGFP fluorescence from *S. typhimurium* strains in tumor spheroids, 7 days post bacterial inoculation. Figs. 2A-2H- Error bars indicate $\pm$ s.e. averaged over three measurements. NC, negative control. Fig. 2I is a graph of sfGFP fluorescence of *S. typhimurium* strains within spheroids over time (ELH1201- back squares; ELH430- gold upside down triangles; SL7207- red triangles; VNP20009- purple diamonds).

Fig. 3 is a graph showing the percent viability of CT26 cell monolayer (y axis) when bacteria lysate expressing anticancer agents (x axis) were applied. MTT assay was used to obtain a measure of viability of the cells 48 hours post lysate treatment. The values are normalized by negative control (NC, bacteria expressing sfGFP). Error bars indicate $\pm$ s.e. averaged over > three measurements. HlyE, hemolysin E. NC, negative control.

Fig. 4 shows use of the inducible circuit consisting of the *luxI* promoter driving sfGFP that is activated via externally supplied AHL. Fig. 4A is a schematic of the inducible circuit. Fig 4B shows the representative images of bacteria in tumor spheroids for the inducible circuit and a graph showing quantification of sfGFP signal 5 days post bacteria inoculation. *LuxR* is

constitutively expressed in all systems. Bold trajectories correspond to the images above. Scale bar, 200 μ m. Fig. 4C is a graph showing percentage tumor spheroid growth containing engineered *S. typhimurium* expressing various anticancer agents with an inducible circuit after adding 10nM AHL post bacteria colonization (****P<0.0001, ***P=0.0002, **P=0.0086, *P=0.0301 compared to control, one-way ANOVA with Bonferroni post-test). Values are normalized by control (bacteria expressing sfGFP) 10 days post bacterial administration. Error bars indicate $\pm$ s.e. averaged over four or more samples. HST, heat stable enterotoxin. HlyE, hemolysin E. NC, negative control. Fig. 4D is a graph showing the percentage cell death of histological tumor sections treated with bacteria expressing inducible therapeutics with 10 nM AHL 10 days post administration. TUNEL staining for tissue sections indicating apoptotic regions of tumors were used to obtain a measure of live and dead regions (*P=0.0112, ***P=0.0003, one-way ANOVA with Bonferroni post-test, error bars indicate $\pm$ s.e. averaged over > three measurements).

Fig. 5 shows use of the quorum-sensing (QS) circuit containing the *luxI* promoter regulated production of autoinducer AHL, leading to self-activation via positive feedback and the synchronized lysis circuit (SLC) carrying an additional lysis gene under the control of *luxI* promoter to achieve coordinated periodic lysis. Fig. 5A is schematic of the quorum-sensing circuit. Fig. 5B is a representative image of bacteria in tumor spheroids for the quorum-sensing circuit and a graph showing quantification of sfGFP signal 5 days post bacteria inoculation. *LuxR* is constitutively expressed in all systems. Bold trajectories correspond to the images above. Scale bar, 200 μ m. Fig. 5C is a graph showing percentage tumor spheroid growth containing engineered *S. typhimurium* expressing various anticancer agents with quorum-sensing (QS) circuit (****P<0.0001, **P=0.0022, *P=0.0344 compared to control, one-way ANOVA with Bonferroni post-test). Values are normalized by control (bacteria expressing sfGFP) 10 days post bacterial administration. Error bars indicate $\pm$ s.e. averaged over four or more samples. HST, heat stable enterotoxin. HlyE, hemolysin E. NC, negative control. Fig. 5D shows a schematic of the synchronized lysis circuit (SLC). Fig. 5E is a representative image of bacteria in tumor spheroids for the SLC gene circuit and a graph of the quantification of sfGFP signal 5 days post bacteria inoculation. *LuxR* is constitutively expressed in all systems. Bold trajectories correspond to the images above. Scale bar, 200 μ m. Fig. 5F is a graph showing the percentage tumor spheroid growth containing engineered *S. typhimurium* expressing various anticancer agents with

synchronized lysis circuit (SLC) (**** $P < 0.0001$, *** $P = 0.0005$, ** $P = 0.0075$, colicin * $P = 0.0394$, * $P = 0.0015$ cecropin X compared to control, one-way ANOVA with Bonferroni post-test). Values are normalized by control (bacteria expressing sfGFP) 10 days post bacterial administration. Error bars indicate $\pm$ s.e. averaged over four or more samples. HST, heat stable enterotoxin. HlyE, hemolysin E. NC, negative control. Fig. 5G shows the biocontainment of bacteria within tumor spheroids both typical images of sfGFP-expressing bacteria contained in tumor spheroids, or growing outside after removal of gentamicin from the media, respectively (Scale bar, 200 μ m) and a graph of the time for bacteria to escape containment and grow outside of tumor spheroid (* $P = 0.0401$, student's t-test, error bars show s.e.).

Fig. 6 shows the use of the three-dimensional co-culture system to correctly predict the efficacy of cancer therapeutic agents. Fig. 6A is a graph of the percent tumor growth over time for subcutaneous tumor bearing mice injected with *S. Typhimurium* carrying inducible gene circuit expressing beta hemolysin, theta-toxin, azurin, hemolysin E, and sfGFP (control) (**** $P < 0.0001$, *** $P = 0.0003$, two-way ANOVA with Bonferroni post-test, $n = 8, 7, 10, 7, 5$ tumors respectively, error bars show s.e.). Black arrows indicate bacteria injection. Fig. 6B is a graph of the analysis of correlation between tumor growth over time in spheroid and *in vivo* model (Linear regression, $R^2 = 0.92$). Fig. 6C is a graph of percentage cell death of histological tumor sections treated with bacteria expressing inducible therapeutics 14 days post administration. TUNEL staining for tissue sections indicating apoptotic regions of tumors were used to obtain a measure of live and dead regions (*** $P = 0.0009$ theta toxin, *** $P = 0.0003$ HlyE, one-way ANOVA with Bonferroni post-test, error bars indicate $\pm$ s.e. averaged over three measurements). Fig. 6D is a graph showing the comparison of efficacy between monolayer cell growth *in vitro* and *in vivo* tumor model. The plot shows analysis of correlation (Linear regression, $R^2 = 0.38$) treated with *S. typhimurium* carrying an inducible gene circuit expressing beta hemolysin, theta-toxin, azurin, hemolysin E, and sfGFP (control).

Fig. 7 further shows the use of the three-dimensional co-culture system to correctly predict the efficacy of cancer therapeutic agents. Fig. 7A shows the efficacy of combinatorial therapy in co-culture platform. The numbers in each box indicate average percent spheroid growth over two or more measurements 8 days post bacteria administration. Colors indicate additional efficacy of combinatorial therapy. Additional efficacy represents the increased efficacy observed compared to expected from additive effect alone. Fig. 7B is a graph of the

percent tumor growth over time for subcutaneous tumor bearing mice injected with *S. Typhimurium* carrying inducible circuit expressing theta-toxin, azurin, combination (theta-toxin and azurin) and sfGFP (control) (****P<0.0001, *P=0.0117, two-way ANOVA with Bonferroni post-test, n=7,9,9,5 tumors respectively, error bars show s.e.). Fig. 7C is a graph of the percent tumor growth over time for subcutaneous tumor bearing mice injected with bacteria carrying SLC expressing beta hemolysin, theta-toxin, azurin, hemolysin E, and sfGFP (control) (*P=0.0363, ****P<0.0001, two-way ANOVA with Bonferroni post-test, n=8,9,6,8,8 tumors respectively, error bars show s.e.). Fig. 7D is a graph of the percent tumor growth over time for subcutaneous tumor bearing mice injected with bacteria carrying SLC or QS circuit expressing theta-toxin, azurin, and sfGFP (control) (****P<0.0001, **P=0.0018, two-way ANOVA with Bonferroni post-test, n=8,5,10,10,9 tumors respectively, error bars show s.e.). Fig. 7B-D- Black arrows on the X axis indicate bacteria injections. Fig. 7E shows the percent change in body weight over time for the mice with subcutaneous tumor injected with *S. Typhimurium* carrying inducible gene circuit and synchronized lysis circuit expressing sfGFP. Black arrows indicate bacteria injections (***P=0.0008, two-way ANOVA with Bonferroni post-test, n=5 mice, error bars show s.e.).

Fig. 8 is a map of the main plasmids used in the study.

Fig. 9 shows the co-culture of diverse species in three-dimensional culture platform. Fig. 9A shows the colonization dynamics of *L. monocytogenes*, within spheroids. Fig. 9B shows the colonization dynamics of *P. mirabilis*, within spheroids. Fig. 9C shows the colonization dynamics of *E. coli*, within spheroids. Space-time diagram showing fluorescence intensity of sfGFP expressing bacteria radially averaged over time. Typical images of sfGFP expressing bacteria in spheroids 6 days post bacteria administration. Scale bar, 100 μ m. (bottom) Total sfGFP of bacteria (green) colony forming units (CFU, blue) over time. Day 0 values are after inoculation and washing of bacteria. Scale bar, 100 μ m. a.u., arbitrary unit. Error bars indicate $\pm$ s.e. Fig. 9D shows the growth of *P. aeruginosa* within spheroids at day 9.

DETAILED DESCRIPTION OF THE INVENTION

Disclosed herein is a three-dimensional (3D) co-culture platform or system that enables long-term growth of engineered bacteria in spheroid necrotic cores entitled “High-Throughput Bacteria-Spheroid (HTBS).” The three-dimensional co-culture platform recapitulates properties

often seen in bacterial tumor colonization *in vivo*, and allows for rapid testing of bacteria strains, synthetic gene circuits, and therapeutic payloads or agents in various cancer types to define novel lead candidates for the treatment of disease. The co-culture platform was used to evaluate *S. typhimurium* strains with inducible, quorum-sensing, and lysis-mediated delivery of therapeutic payloads or agents and identified potent engineered bacterial therapeutics from a small library of anticancer agents. A combination screen identified novel pairs of therapeutics resulting in significant tumor reduction compared to individual therapies. Using a syngeneic mouse model, a high similarity between *in vitro* and *in vivo* results was demonstrated. Other bacteria including *L. monocytogenes*, *P. mirabilis*, *E. coli* and *Pseudomonas aeruginosa* were also used successfully in the co-culture platform. It is anticipated that the 3-D co-culture platform will provide a quantitative platform to engineer effective microbial therapeutics in cancer and other disease areas in a high-throughput manner.

While previous studies have tested up to five bacteria-produced therapeutics in monolayers using bacterial lysate and culture supernatants, the three-dimensional co-culture platform enabled screening of approximately 40 bacterial therapy candidates for efficacy while simultaneously monitoring bacteria dynamics and disease progression over a time scale of weeks. This long-term monitoring aspect is critical to the success of bacterial therapies, as it is necessary to characterize the effects of dynamic therapeutic expression from genetic circuits on bacteria and cellular growth in physiological conditions.

Shown herein is a platform to simultaneously profile a vast number of engineered bacteria. By utilizing a scalable methodology, the three-dimensional co-culture platform enables rapid ‘build-test’ cycles of bacterial dynamics, therapeutics, and species for *in vivo* applications of synthetic biology. The key features of the system are: 1) quantification of bacterial population and circuit dynamics in a 3-D disease model; 2) accurate prediction of long-term disease progression *in vivo* from *in vitro* screening; and 3) a simple and broadly applicable system for high-throughput development of novel bacteria therapies.

This platform has high potential for commercial opportunities with respect to the development of therapeutics and diagnostics for the microbiome, cancer, and personalized medicine fields. Despite the emerging effort to develop living therapeutics with synthetic biology tools, the lack of physiologically-relevant *in vitro* testing environments to evaluate engineered cells limits rapid development to clinical use.

Notably, recent findings indicate the role of bacteria in modulating tumor immune environment, with increasing reports on developing effective cancer immunotherapy utilizing bacteria (Zheng *et al.* (2017); Gopalakrishnan *et al.* (2018)). By incorporating various cell types such as immune cell co-culture or organoids, the three-dimensional co-culture platform may also be amenable to investigation of such aspects, potentially capable of screening various other bacteria-based therapeutics including immunotherapeutics. As the field of engineered cell therapy evolves with the use of more complex synthetic gene circuits (Weinberg *et al.* (2017); Nielsen *et al.* (2016)), incorporation of multi-cellular ecosystems (Scott *et al.* (2017)) and organoid models (Clevers (2016)), the three-dimensional co-culture platform will be a starting point for accelerating clinical development of therapies to treat a broad range of diseases.

As shown herein, the platform can be used to screen both combinations of agents and genetic circuits as well as larger libraries of agents. Since multiple bacteria strains expressing different therapeutics will compete for resources within a tumor, reducing the effective dosage of each therapeutic, the three-dimensional co-culture platform can assess how combination therapy will perform when increasing the number of bacterial therapies. In addition, the broad applicability of the three-dimensional co-culture system can be employed to assess combinations of bacterial therapy with chemotherapy and radiotherapy to improve upon existing treatments. The three-dimensional co-culture platform is precisely suited to quantitatively explore large libraries and combinatorial strategies in a high-throughput fashion.

While cancer therapy was the focus, the three-dimensional co-culture platform may be expanded to characterize bacteria-based therapeutics for various diseases and explore fundamental biological questions about bacteria in host tissue types. For example, the three-dimensional co-culture platform can provide a platform to generally address safety concerns such as escape of bacteria from host tissue. Here three-dimensional co-culture platform can enable development and comparison of various biocontainment strategies such as antibiotic treatments and genetic kill switch circuits. Additionally, by incorporating immune cells into the co-culture platform, the platform can also be adapted to investigate bacteria-based immunotherapeutics that have been reported to augment immunotherapies (Zheng *et al.* (2017); Gopalakrishnan *et al.* (2018)). Lastly, this multicellular spheroid model sets the stage for development of an organoid-based system that incorporates cells with different lineages and may enable further investigation of host-microbe interactions. As the field of engineered cell and microbial therapies evolves with

the use of more complex synthetic gene circuits and incorporation of multi-cellular ecosystems (Weinberg *et al.* (2017); Nielsen *et al.* (2016); Scott *et al.* (2017)), the three-dimensional co-culture platform will accelerate therapeutic development for wide range of diseases towards clinical translation.

Thus, the current disclosure provides for a three-dimensional co-culture system; a method for establishing and generating the three-dimensional co-culture system; and a method for using the three-dimensional co-culture system for testing, screening and evaluating therapeutics and diagnostics.

The three-dimensional co-culture platform includes two main components: a three-dimensional structure made of cells, *e.g.*, a tumor spheroid or an organoid; and a microbe, *e.g.*, bacteria or virus, or cell.

Exemplified herein is a tumor spheroid derived from mouse colorectal and breast cancers and human colorectal and breast cancers. The tumor spheroids were generated by seeding cells in round bottom ultra-low attachment plates. Each well was seeded with about 2000-15,000 cells in about 100 μ l of appropriate media. The numbers were adjusted for each cell line in order to obtain a particular desired diameter of spheroid. It is within the skill of the art to adjust numbers of cells to seed in order to obtain a particular diameter. The cells were aggregated and then place in an incubator prior to co-culture with bacteria. Other way of generating spheroids include hanging-drop, use of coated wells (such as alginate), and stirring. See generally Lv, *et al.* (2017).

Spheroids can be derived from any other tumor type using methods known in the art such as the one exemplified herein as well as others. Additionally, tumor spheroids are available commercially. Tumor spheroids can also be derived from a patient's tumor and used in the three-dimensional co-culture system for use in a personalized medicine protocol. Tumor spheroids that can be used in the system include but are not limited to brain, prostate, pancreatic, stomach, colorectal, breast, lung, ovarian, liver, kidney, skin, and bone.

Organoids can also be used in the three-dimensional co-culture system. Organoids can be derived from many cell/organs and are also available commercially. Organoids can also be derived from a patient's organ and used in the three-dimensional co-culture system for use in a personalized medicine protocol. Organoids can also be derived from stem cells and induced pluripotent stem cells using methods known in the art (see, *e.g.*, McCauley and Wells (2017)).

Organoids that can be used in the system include but are not limited to bone, muscle, cerebral, gut, intestinal, stomach, thyroid, hepatic, pancreatic, epithelial, lung, kidney and cardiac.

Spheroids and organoids later developed can also be used in the system of the present method.

Spheroids and organoids for use in the three-dimensional co-culture system can also be comprised of more than one cell type. In particular, immune cells can be added to the spheroids or organoids. Immune cell populations that can be added include wide variety of cell types including innate immune population such as macrophages and neutrophils and adaptive cell population such as CD4+ helper T cells, CD8+ cytotoxic T cells, and regulatory T cells.

In addition, tumors are often comprised of non-cancerous cells that can be incorporated into the spheroid co-culture. For example, fibroblasts can be a major component of surrounding stroma and plays important role in modulating tumor microenvironment and extracellular matrix (Erdogan and Webb (2017)). Endothelial cells and other cell types that make vasculature structures may also be added to model tumor-vasculature interaction as well as drug transport system in the tumor (Laschke and Menger (2017))

Additionally, the spheroids or organoids can be engineered in order to have a means of detection or visualization as a further way to determine the growth of the spheroid or organoid in the system. One example is to transfect the cell line used for the spheroid or organoid with a fluorescent reporter gene.

The second component of the three-dimensional is a bacteria, virus or eukaryotic cell. In some embodiments, the bacteria, virus or eukaryotic cell itself is being tested, screened or evaluated as a therapeutic or diagnostic. In some embodiments, the bacteria, virus or cell comprise an agent to be tested, screened or evaluated as a therapeutic or diagnostic. In other embodiments, the bacteria, virus or cell comprise a genetic circuit to be tested, screened or evaluated.

The bacteria, virus or eukaryotic cell can be naturally occurring or genetically engineered. Exemplified herein was the use of *S. Typhimurium* engineered with various genetic systems for delivery of the therapeutic agents to be tested. Therapeutics or diagnostics that can be tested in the system include but are not limited to natural probiotic cocktails, engineered bacteria for cancer and microbiome applications, viral therapeutic delivery (*e.g.* oncolytic viruses), and engineered cells (*e.g.* CAR-T, NK).

Examples of bacteria that can be used in the three-dimensional co-culture platform include but are not limited to *Salmonella typhimurium* (*S. typhimurium*), *Listeria monocytogenes* (*L. monocytogenes*), *Proteus mirabilis* (*P. mirabilis*), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*). It is contemplated that any bacteria that can be used as a therapeutic or diagnostic, comprises a therapeutic or diagnostic agent, comprises a genetic circuit, and/or can be used for delivering therapeutic or diagnostic agents could be used in the three-dimensional co-culture platform in order to test its efficacy. Thus additional bacteria that can be used in the three-dimensional co-culture platform include but are not limited to *Bacillus*, *Bacteroides*, *Bifidobacterium*, *Brevibacteria*, *Caulobacter*, *Clostridium*, *Enterococcus*, *Escherichia coli*, *Lactobacillus*, *Lactococcus*, *Listeria*, *Mycobacterium*, *Saccharomyces*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Vibrio*, *Bacillus coagulans*, *Bacillus subtilis*, *Bacteroides fragilis*, *Bacteroides subtilis*, *Bacteroides thetaiotaomicron*, *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium breve* UCC2003, *Bifidobacterium infantis*, *Bifidobacterium lactis*, *Bifidobacterium longum*, *Clostridium acetobutylicum*, *Clostridium butyricum*, *Clostridium butyricum* M-55, *Clostridium butyricum* miyairi, *Clostridium cochlearum*, *Clostridium felsineum*, *Clostridium histolyticum*, *Clostridium multifementans*, *Clostridium novyi-NT*, *Clostridium paraputrificum*, *Clostridium pasteurianum*, *Clostridium pectinovorum*, *Clostridium perfringens*, *Clostridium roseum*, *Clostridium sporogenes*, *Clostridium tertium*, *Clostridium tetani*, *Clostridium tyrobutyricum*, *Corynebacterium parvum*, *Escherichia coli* MG1655, *Escherichia coli* Nissle 1917, *Mycobacterium bovis*, *Salmonella choleraesuis*, *Salmonella typhimurium*, and *Vibrio cholera*.

In some embodiments, the natural bacteria, virus or cell itself are being tested as a therapeutic or diagnostic. Bacteria are advantageous in that they can generate an antitumor immune response, e.g., a local or innate immune response that develops into a systemic or adaptive immune response. For example, bacteria can stimulate the antigen-presenting ability of immune cells that infiltrate the tumor, the antigen-presenting ability of immune cells that infiltrate the tumor microenvironment (e.g., B cells, plasmacytoid and myeloid dendritic cells (DCs), CD4+ T cells, CD8+ T cells, T regs, natural killer cells (NK cells), and tumor-associated macrophages (TAMs)). Many immune cells found in the tumor microenvironment express pattern recognition receptors (PRRs), which receptors play a key role in the innate immune response through the activation of pro-inflammatory signaling pathways, stimulation of

phagocytic responses (macrophages, neutrophils and dendritic cells) or binding to micro-organisms as secreted proteins.

In some embodiments, the bacteria, virus or cells are engineered to comprise a therapeutic or diagnostic agent for testing. In some embodiments, the therapeutic agent is produced by a plasmid which is introduced into the bacteria, virus, or cell. In some embodiments, the plasmid comprises a nucleic acid encoding a therapeutic agent. In some embodiments, the nucleic acid is operably linked to a promoter. In some embodiments, the promoter is constitutive. In some embodiments, the promoter is inducible.

Examples of plasmid include but are not limited to pColE1, p15A, pAH162, and pSC101.

In some embodiments, the therapeutic agent is for treating cancer and includes but is not limited to checkpoint inhibitors, cell surface markers, immunostimulatory molecules, peptides, and toxins.

In some embodiments, the therapeutic agent is for treating disease other than cancer, including but not limited to infectious diseases and inflammation.

In some embodiments, the bacteria, virus or cells are engineered to deliver more than one therapeutic or diagnostic agent. In some embodiments, the therapeutic or diagnostic agents are contained on one plasmid. In some embodiments, the therapeutic or diagnostic agents are contained on more than one plasmid.

In some embodiments, the bacteria, virus or cells are engineered to deliver a therapeutic or diagnostic agent. In some embodiments, the therapeutic agent is for treating cancer. In some embodiments, the therapeutic agent is for treating disease other than cancer, including but not limited to infectious diseases and inflammation.

In some embodiments, the bacteria, virus or cells are engineered to contain genetic circuits or delivery systems for therapeutic or diagnostic agents. Such genetic delivery systems include but are not limited to inducible, quorum-sensing, and lysis-mediated delivery systems. A lysis-mediated system (SLC) can comprise at least the following components: a nucleic acid encoding a quorum-sensing gene; a nucleic acid encoding a lysis gene; a promoter; and a terminator. In some embodiments, the SLC comprises more than one of each component. Additional components of the SLC may include antibiotic resistance genes. In some embodiments, the genetic circuits are contained on a plasmid that is introduced into the bacteria.

In some embodiments, the bacteria, virus or cells are engineered to contain genetic circuits that serve as biosensors. In some embodiments, the genetic circuits are contained on a plasmid that is introduced into the bacteria

In some embodiments, the bacteria, virus or cells are genetically engineered to contain a means of visualization of the bacteria, virus or cell to determine if it has grown within the spheroid or organoid. Examples of such means include but are not limited to fluorescence and luminescence. Plasmids containing luminescent or fluorescent proteins can be introduced into the bacteria. Luminescent proteins include but are not limited to *Oplophorus* luciferase. Fluorescent proteins are known in the art and include green fluorescent protein (*e.g.*, sfGFP, AcGFP1, moxGFP, mEmerald, EGFP, MGFP), yellow fluorescent protein (*e.g.*, EYFP, mTopaz, mVenus, SYFP), cyan (*e.g.*, ECFP, mCerulean, SCFP3A), and red (*e.g.*, mRuby2, mScarlet-H, mApple).

The current disclosure also provides a method of producing or generating the three-dimensional co-culture system comprising the following steps:

obtaining or generating a spheroid or organoid;

co-culturing the spheroid or organoid with bacteria, virus or eukaryotic cells under conditions that allow the bacteria, virus or eukaryotic cells to invade the spheroid or organoid and grow within the core of the spheroid or organoid.

Optional steps include: introducing an antibiotic to clear extra-spheroidal or organoidal bacteria; and monitoring for the growth of the bacteria, virus or eukaryotic cells in the spheroid or organoid.

The first step of obtaining or generating a spheroid or organoid can be done by methods known in the art. Tumor cells can be obtained from many sources including a specific patient. In the exemplified method, tumor spheroids were generated by seeding cells in round-bottom ultra-low attachment 96 well plate. Cells were seeded in each well to adjust for each cell line to maintain a similar diameter of spheroid and is varied accordingly. The plate was centrifuged to aggregate cells at the bottom of the plate and placed inside a tissue culture incubator for 4 days before co-cultured with bacteria.

Also as discussed above, any method known in the art can be used to obtain the spheroids and organoids. Also spheroids and organoids are commercially available.

The next step of the method is to co-culture the spheroid or organoid with bacteria, virus or cells.

In the exemplified method, bacteria, virus or cells are cultured in order to reach stationary phase before use. The bacteria, virus or cells are inoculated into the wells at an amount ranging from 10^5 to 10^8 cells containing the spheroids or organoids at approximately 4 days. After approximately 2-6 hours of co-culture, the media is removed and the tumor spheroids are washed repeatedly while leaving spheroids at the bottom of plate. After washing, media is added to the wells and tumor spheroids were monitored for growth. For long-term experiments, media was replaced every 3-4 days. Schematic of the overall protocol is provided (Fig. 1A). The co-culture can be maintained for weeks, ranging from one week to over 8-12 weeks.

Alternatively, the bacteria can be introduced into the aggregating spheroids or organoids, rather than when the aggregated spheroids or organoids have already formed.

In some embodiments of the method, an antimicrobial is added to the spheroids after washing. This is to allow the growth of the bacteria inside the spheroid or organoid by reducing permeability through the eukaryotic membrane. Antibiotics that can be used include but are not limited to gentamycin in an amount ranging from about 1 $\mu\text{g/mL}$ to 1 mg/mL , or an amount ranging from about 0.5 $\mu\text{g/mL}$ to 500 $\mu\text{g/mL}$, or in amount ranging from about 0.5 $\mu\text{g/mL}$ to 50 $\mu\text{g/mL}$. Other antimicrobials and concentrations that can be used in the method include but are not limited to those listed in Table 1.

Table 1- Antimicrobials for use in the method

Categories	Antimicrobials	Concentrations
Antibiotics	Erythromycin	25-500 $\mu\text{g/mL}$
	Tetracycline	1-100 $\mu\text{g/mL}$
Antimicrobial peptides	Pyrrhocorcin	0.1-60 μM
	Apidaecin Ib	0.1-60 μM

	Drosocin	0.1-60 μ M
Metals	Silver nanoparticle (100 nm diameter)	1-50 μ g/mL

In some embodiments, the growth and/or localization of the bacteria, virus or eukaryotic cells in the spheroid or organoid is monitored. This can be done by methods known in the art for visualizing the bacteria, virus or eukaryotic cell such as monitoring fluorescence and microscopy.

As shown herein when the spheroids or organoids are monitored for growth, the three-dimensional co-culture system, it was shown that the *S. typhimurium* attached to the spheroid surface and subsequently localized and proliferated in the interior of the growing tumor spheroid. An increasing level of GFP (sfGFP) fluorescence from *S. typhimurium* within tumor spheroids was detected over 9 days, confirming growth of bacteria (Fig. 1C). Notably, the bacteria were localized to the necrotic and hypoxic regions of the spheroids (Fig. 1D). Additionally other bacteria also colonized in the spheroid (Fig. 9). These results show that the three-dimensional co-culture system can be adapted for long-term high-throughput characterization of engineered microbial therapeutics in physiologically relevant conditions.

The current disclosure also provides for a method of using the three-dimensional co-culture system for testing therapeutic and diagnostic agents and genetic circuits.

In some embodiments, when the bacteria, virus or cell is being tested as a therapeutic or diagnostic, the three-dimensional co-culture system is cultured in appropriate medium and the spheroid or organoid are monitored for growth and proliferation, or lack of growth and death of the spheroid or organoid. If lack of growth or cell death is the desired outcome, such as with a cancer spheroid, if the bacteria, virus or cell inhibits the growth or kills the spheroid or organoid, the bacteria, virus or cell would be considered a therapeutic. If proliferation or stimulation of growth is the desired outcome, if the bacteria, virus or cell stimulates the growth of the spheroid or organoid, the bacteria, virus or cell would be considered a therapeutic. If the bacteria, virus or cell is being tested as a diagnostic, its localization and visualization within the spheroid or organoid is monitored.

In some embodiments, where the bacteria, virus or cell comprises a therapeutic or diagnostic agent for testing, the method can comprise the steps of culturing the three-dimensional co-culture system in appropriate medium and of introducing a second agent which induces the bacteria, virus or cell to deliver a therapeutic or diagnostic agent after the bacteria, virus or cell has localized in the spheroid or organoid. In some embodiments, the second agent is added to the cell culture medium. Then, the spheroid or organoid are monitored for growth and proliferation, or lack of growth and death of the spheroid or organoid. If lack of growth or cell death is the desired outcome, such as with a cancer spheroid, if the agent inhibits the growth or kills the spheroid or organoid, the agent would be considered a therapeutic. If proliferation or stimulation of growth is the desired outcome, if the agent stimulates the growth of the spheroid or organoid, the agent would be considered a therapeutic. If the agent is being tested as a diagnostic, its localization and visualization within the spheroid or organoid is monitored.

In some embodiments, the bacteria, virus or cell comprises a genetic circuit. In some embodiments, the genetic circuit is being used to deliver a therapeutic or diagnostic agent for testing. The method can comprise the steps of culturing the three-dimensional co-culture system in appropriate medium and of introducing a second agent which induces the genetic circuit to deliver a therapeutic or diagnostic agent after the bacteria, virus or cell has localized in the spheroid or organoid. In some embodiments, the genetic circuit lyses the bacteria, virus or cell and the therapeutic or diagnostic agent is released into the three-dimensional co-culture platform. In some embodiments, the second agent is added to the cell culture medium. Then, the spheroid or organoid are monitored for growth and proliferation, or lack of growth and death of the spheroid or organoid. If lack of growth or cell death is the desired outcome, such as with a cancer spheroid, if the agent inhibits the growth or kills the spheroid or organoid, the agent would be considered a therapeutic. If proliferation or stimulation of growth is the desired outcome, if the agent stimulates the growth of the spheroid or organoid, the agent would be considered a therapeutic. If the agent is being tested as a diagnostic, its localization and visualization within the spheroid or organoid is monitored.

In some embodiments, the bacteria, virus or cell comprises a genetic circuit. In some embodiments, the genetic circuit is being tested for its effectiveness in delivering a therapeutic or diagnostic agent. The method can comprise the steps of culturing the three-dimensional co-culture system in appropriate medium and of introducing a second agent which induces the

genetic circuit to deliver a therapeutic or diagnostic agent after the bacteria, virus or cell has localized in the spheroid or organoid. In some embodiments, the genetic circuit lyses the bacteria, virus or cell and the therapeutic or diagnostic agent is released into the three-dimensional co-culture platform. In some embodiments, the second agent which induces the genetic circuit to deliver a therapeutic or diagnostic agent is added to the cell culture medium. Then, the spheroid or organoid are monitored for growth and proliferation, or lack of growth and death of the spheroid or organoid. If lack of growth or cell death is the desired outcome, such as with a cancer spheroid, if the agent inhibits the growth or kills the spheroid or organoid, the genetic circuit would be considered effective. If proliferation or stimulation of growth is the desired outcome, if the agent stimulates the growth of the spheroid or organoid, the genetic circuit would be considered effective. If the agent is being tested as a diagnostic, its localization and visualization within the spheroid or organoid is monitored.

In some embodiments, the bacteria, virus or cell comprises a genetic circuit. In some embodiments, the genetic circuit is being tested for its effectiveness as a biosensor. The method can comprise the steps of culturing the three-dimensional co-culture system in appropriate medium. Then, the bacteria, virus or cell are monitored for their ability to colonize in the spheroid or organoid. If lack of colonization is the desired outcome, if the bacteria, virus or cell does not colonize then the genetic circuit is an effective biosensor. If colonization is the desired outcome, if the bacteria, virus or cell colonize then the genetic circuit is an effective biosensor.

The spheroids and organoids can be monitored for growth, either increased or decreased, by methods known in the art including imaging and microscopy. In some embodiments, the spheroids or organoids have fluorescent reporters that can be used to monitor growth.

In some embodiments, combinations of different types of bacteria, viruses or cells can be tested in one spheroid or organoid. In some embodiments, the bacteria, viruses or cells are of a different type or species. In some embodiments, the bacteria, virus or cell comprise different agents. In some embodiments, the bacteria, virus or cell comprise different delivery systems. In some embodiments, the bacteria, virus or cell comprise different genetic circuits.

As shown herein, using a three-dimensional co-culture system comprising tumor spheroids and genetically modified *S. typhimurium* which delivered potential anti-cancer therapeutics to tumor spheroids, potential anti-cancer therapeutics were identified and confirmed in an *in vivo* mouse model. In contrast, bacteria in a tumor monolayer did not correctly predict

the effectiveness of the anti-cancer therapeutics. Additionally, using three different genetic circuits in the *S. typhimurium* identified the most effective for delivering the therapeutic agents.

The current method can be used for high-throughput screening of therapeutics and diagnostics. As shown herein, 96-well plated can be produced containing the same spheroid or organoid, or different spheroids or organoids. The bacteria, virus or cell is co-cultured with the spheroid or organoid. The same bacteria, virus or cell can be used or different bacteria, viruses or cell can be used. The bacteria, virus or cell can comprise the same or different therapeutic or diagnostic agents and/or genetic circuits. In this way, the same potential therapeutic or diagnostic agent or genetic circuit can be tested on different spheroids or organoids. Alternatively, different potential therapeutic or diagnostic agents or genetic circuits can be tested on the same or different spheroids or organoids. The co-culture platform allows the testing of a multitude of combinations in a high-throughput fashion.

The present invention also provides for kits for establishing and generating and using the three-dimensional co-culture system. In some embodiments, the kit comprises a tumor spheroid or an organoid. In some embodiments, the tumor spheroid or organoid is contained in a well of a plate. In some embodiments, the kit comprises more than one plate. In some embodiments, the wells contain the same spheroid or organoid. In some embodiments, the wells contain different spheroids or organoids. In some embodiments, the kit further comprises bacteria, virus or cells for co-culturing. In some embodiments, the bacteria, virus or cell are naturally occurring and can be genetically engineered to comprise a plasmid comprising an agent, delivery system and/or a genetic circuit to be tested. The kits can further comprise instructions for co-culturing the bacteria, virus or cell of interest with the tumor spheroid or organoid and for using the three-dimensional co-culture system to test, screen or evaluate therapeutic or diagnostic agents, delivery systems and/or genetic circuits, as well as reagents for the co-culturing including but not limited to appropriate media and antibiotics and inducing agents.

EXAMPLES

The present invention may be better understood by reference to the following non-limiting examples, which are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed to limit the broad scope of the invention.

Example 1 - Materials and Methods for Examples 2-8

Host strains and culturing

ELH1301 and ELH430 were kindly provided by Dr. Elizabeth Hohmann. SL7207 was provided by Dr. Siegfried Weiss. VNP20009 was obtained from ATCC (202165).

L. monocytogenes was kindly provided by Dr. Eric Pamer. VNP20009, *P. mirabilis* and *E. coli* were obtained from ATCC (202165, 29906, 23506).

S. typhimurium, *P. mirabilis* and *E. coli* were cultured in LB media (Sigma-Aldrich). *L. monocytogenes* was cultured in Brain Heart Infusion media (Fisher Scientific). All bacteria were grown with appropriate antibiotics selection (100 µg ml⁻¹ ampicillin, 50 µg ml⁻¹ kanamycin, 25 µg ml⁻¹ chloramphenicol) at 37°C. Synchronized lysis circuit strains were cultured with 0.2% glucose for less than 16 hours. The glucose was added in order to decrease expression from the *luxI* promoters.

For full strain information, see Table 2.

Mammalian cells and spheroid generation

The MC-26 cell line was provided by K. Tanabe and B. Fuchs (Massachusetts General Hospital). Mammalian cells were cultured in DMEM/F-12 media with GlutaMAX supplement (Gibco; for HT-29 and MCF-7) or RPMI 1640 media (Gibco; for CT-26, CT-26-iRFP, MC-26, 4T1) and supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (CellGro), placed inside a tissue culture incubator at 37 °C maintained at 5% CO₂.

For full cell line information, see the Table 3.

CT26 cells were transfected with genomic integration of *iRFP* gene with nuclear localization signal sequence to construct CT26-iRFP cell line. Specifically, pNLS-iRFP670 plasmid was obtained from Addgene (Plasmid #45466). The plasmid was prepared by miniprep of an overnight culture and subsequently transfected to CT26 cells using lipofectamine (Invitrogen). Monoclonal cells were generated by suspending single cells in 96-well tissue culture plates (Falcon) and subsequently expanding from single wells. Selected iRFP-expressing monoclonal cells were expanded and used to seed spheroids in all corresponding assays.

Tumor spheroids were generated by seeding cells in round-bottom ultra-low attachment 96 well plate (Corning). Each well contains 2,500 CT-26 cells in 100 µl of appropriate media without antibiotics. Number of cells seeded was adjusted for each cell line to maintain a similar

diameter of spheroids generated across cell lines (MC-26 2,500 cells, 4T1 5,250 cells, HT-29 5,000 cells, MCF-7 12,500 cells). The plate was centrifuged at 3,000 rcf for 5 minutes to aggregate cells at the bottom of the plate and placed inside a tissue culture incubator for 4 days before co-cultured with bacteria.

Plasmids and therapeutic library

Plasmids were constructed using Gibson Assembly or using standard restriction digest and ligation cloning and transformed into Mach1 competent cells (Invitrogen). Previously constructed pTD103 sfGFP plasmid containing kanamycin resistance cassette and ColE1 origin of replication was used to characterize the inducible gene circuit (Danino *et al.* (2010)). The QS circuit (pTH02) was constructed by switching antibiotic resistance cassette and origin of replication of previously used pTD103 LuxI sfGFP plasmid (Prindle *et al.* (2011)) to ampicillin and p15A using the modular pZ plasmids (Lutz and Bujard (1997)). The synchronized lysis circuit (SLC) plasmid (pTH03) was constructed by first amplifying a region containing the constitutively expressed *luxR* gene and *luxI* gene under the control of *luxI* promoter from pTD103 LuxI sfGFP plasmid. Next, the bacteriophage ϕ X174E was synthesized from IDT and cloned next to the *luxI* gene with intergenic RBS sequence between genes to allow for operon expression. Finally, the antibiotic resistance cassette and origin of replication was replaced with ampicillin and sc101* from a modular pZ plasmid. Maps of main plasmids used in this study (Fig. 8) are provided.

The therapeutics library was constructed by synthesizing therapeutic genes from IDT, except for the hemolysin E gene obtained via PCR from a plasmid in previous work (Din *et al.* (2016)). Therapeutics were cloned under the control of the *luxI* promoter by replacing *sfGFP* gene in a previously used ColE1 pTD103 sfGFP plasmid to construct therapeutics with inducible control (pTH05) (Danino *et al.* (2010)) To combine QS circuit and SLC with therapeutic expression, plasmids containing these circuits were co-transformed into bacteria and plated on full antibiotics. To make therapeutic characterization comparable between circuits, the QS circuit was swapped to a sc101* origin of replication (pTH06). A detailed table of therapeutics in the library (Tables 2 and 3) and main plasmids used in this study (Fig. 8) are provided.

Bacteria co-culture with monolayer cancer cells

Tumor cell monolayer viability experiment was performed in 96-well tissue culture plates (Falcon) to confirm therapeutic expression. CT26 cells were allowed to adhere to the wells for 24

hours before the addition of bacterial lysate. Overnight bacteria carrying therapeutic payloads were subcultured to a starting OD₆₀₀ = 0.1 with 10 nM AHL to induce therapeutic production for 6 hours. Subsequently, the bacterial cultures were resuspended in PBS and bacterial cells were lysed by repeated freeze-thaw cycles. Viability was assessed using an MTT assay by measuring the colorimetric output in a TECAN Infinite M200 Pro plate reader.

Bacteria co-culture with tumor spheroids

Bacteria were cultured in a 37°C shaker overnight to reach stationary phase before use. 10⁶ CFU bacteria were inoculated into wells containing 4-day old tumor spheroids and placed back into the tissue culture incubator. After 2 hours of co-culture, media was removed and tumor spheroids were washed with 200 µl of PBS repeatedly while leaving spheroids at the bottom of plate. After washing, 200 µl of media containing 2.5 µg/ml Gentamicin (Gibco) were added and tumor spheroids were monitored for growth. For long-term experiments, media was replaced every 3-4 days. Bacterial therapeutics expression was induced by replacing media containing 10 nM N-(β-Ketocaproyl)-L-homoserine lactone (AHL) (Sigma Aldrich) every other day. Schematic of the overall protocol is provided (Fig. 1A).

For other bacteria, identical procedures were followed with modifications in bacterial inoculation density, incubation time, and gentamicin concentration. For full protocol, please refer to Table 3. ELH1301 (SL1344 *phoPQ-laroA-*) was used for all co-culture experiments unless otherwise noted.

Bacterial colonization quantification via colony counts

Spheroids containing bacteria were washed with 200 µl of PBS repeatedly while leaving spheroids at the bottom of plate. After washing, spheroids were re-suspended in 100 µl of PBS and homogenized using mechanical dissociation with sterile tips and repeated pipetting. Destruction of spheroids were confirmed by microscopy. Serial 10-fold dilutions of the samples were inoculated on appropriate agar plates.

Microscopy

Acquisition of spheroid still images was performed with EVOS FL Auto 2 Cell Imaging Systems. The scope and accessories were programmed using the Celleste Imaging Analysis software. For analysis of synthetic gene circuit dynamics, Nikon TiE microscope equipped with Okolab stage top incubator was used to maintain the culture at 37°C with 5% CO₂ for time-lapse movies. The scope and accessories were programmed using the Nikon Elements software and

images were taken every 60 minutes. For the acquisition of images, an Andor Zyla sCMOS camera was used. The microscope and acquisition were controlled by the Nikon Elements software. Phase-contrast images were taken at 10x magnification at 50-200ms exposure times. Fluorescent imaging at 10x was performed at 50ms for GFP, 30% setting on the Lumencor Spectra-X Light Engine. Further information on the analysis of these images is presented in the sections below.

Image alignment and localized fluorescence measurement for spheroids

Time-course imaging of tumor spheroids required image registration to properly align the resulting images, since the spheroid would both rotate and translate significantly within the field of view. For image registration, the popular registration method ORB (Rublee *et al.* (2011)) in the Python implementation of OpenCV was leveraged to find matching key points in images at adjacent time points. An implementation of RANSAC (Fischler and Bolles (1981)) in scikit-image (Van der Walt *et al.* (2012)) determined the corresponding Euclidean transform between adjacent images. For each time point, edge-filtered transmitted light (TL) and edge-filtered sfGFP images was averaged to form the input for our registration pipeline, which allowed both TL and sfGFP to inform alignment. Once this method was used to align a time-course experiment, Fiji (Schindelin *et al.* 2012) was used for data analysis. sfGFP fluorescence trajectories for a spheroid are based on averaged signal of this aligned image set within several circular regions of interest (ROI's) of the same size. These circular ROI's are fixed in position and chosen to highlight representative dynamics of the spheroid. Images showing ROI's used in this study are provided in Figs. 4B, 5B and 5E.

sfGFP average fluorescence and radial histograms for spheroids

To measure the spatiotemporal dynamics of bacteria invading tumor spheroids, first a threshold brightness value for each TL image was found to distinguish the dark spheroid from the light background. Scikit-image implementations of two popular thresholding methods were used: the minimum method (Prewitt and Mendelsohn (1966)) for images taken daily; and Yen's method (Yen *et al.* (1995)) for other images. Then the largest region within the resulting threshold-based image mask was identified as the tumor spheroid, and mean intensity of sfGFP fluorescence within this region determined. To compute radial histograms, mean sfGFP fluorescence for many thin annuli with variable mean radius was computed and centered on the

centroid of the spheroid mask region. To compute radial fluorescence in Fig. 1C, fluorescence was calculated based on a line across the radius of the spheroid.

Animal models

All animal experiments were approved by the Institutional Animal Care and Use Committee (Columbia University, protocol AC-AAAN8002). The protocol requires animals to be euthanized when tumor burden reaches 2 cm in diameter, or under veterinary staff recommendation. Mice were blindly randomized into various groups.

Animal experiments were performed on 4-6 weeks-old female BALB/c mice (Taconic Biosciences) with bilateral subcutaneous hind flank tumors from CT26 colorectal cells. The concentration for implantation of the tumor cells was 5×10^7 cells per ml in RPMI (no phenol red). Cells were injected at a volume of 100 μ l per flank, with each implant consisting of 5×10^6 cells. Tumors were grown to an average of approximately 150 mm³ before experiments. Tumor volume was quantified using calipers to measure the length, width, and height of each tumor ($V = L \times W \times H$). Volumes were normalized to pre-injection values to calculate relative or % tumor growth on a per mouse basis. Animals with tumors less than 75 mm³ or greater than 400 mm³, and tumors presenting extremely blistering (assessed by an independent researcher who was blinded) were excluded.

Bacterial administration for *in vivo* experiments

Bacterial strains were grown overnight in LB media containing appropriate antibiotics and 0.2% glucose. A 1:100 dilution into media with antibiotics was started the day of injection and grown until an OD of approximately 0.1. Bacteria were spun down and washed 3 times with sterile PBS before injection into mice. Intratumoral injections of bacteria were performed at a concentration of 5×10^8 cells per ml in PBS with a total volume of 20–40 μ l injected per tumor. For bacteria carrying the inducible circuit, 0.5 mL of 10 μ M AHL was injected subcutaneously the day after bacterial treatment to induce therapeutic expression. For combination therapy, bacteria cultures were combined after washing with PBS at 1:1 ratio to reach total concentration of 10^9 cells per ml and injected the total volume of 20-40 μ l per tumor.

Tissue histological analysis

Tumors were extracted from mice at the termination of trials according to protocol. Immediately after extraction, tumor tissues were rinsed with PBS and fixed in 4% paraformaldehyde for 24 hours in 4°C. Tumor spheroids were fixed for 20 minutes to prevent

over fixation. After fixation, the tissues were rinsed with PBS and preserved in 70% ethanol in 4 °C. For histological analysis, tissues were paraffin embedded and sectioned into 5µl onto slides. Gram staining was performed to confirm presence of bacteria inside the tumors. TUNEL staining was performed to obtain measurement of apoptosis. Tumor cell death was quantified using ImageJ software to measure the area of the viable and dead area by setting a pixel threshold to make binary images.

Statistical analysis

Statistical tests were calculated either in GraphPad Prism 7.0 (Student's *t*-test and ANOVA). The details of the statistical tests carried out are indicated in the respective figure legends. Where data were approximately normally distributed, values were compared using either a Student's *t*-test or one-way ANOVA for single variable, or a two-way ANOVA for two variables with Bonferroni correction for multiple comparisons. Mice were randomized in different groups before experiments.

Calculation of additional efficacy

Additional efficacy was calculated, which represents the increased efficacy observed compared to expected from additive effect alone, based on past calculations of synergy and combination therapies for other drugs (Foucquier and Guedj (2015)). Unique to bacterial therapy combinations, when two different bacteria share the tumor space, each can only grow to half of the possible maximum bacterial volume of a single therapy. Efficacy of a single therapy (for example, therapy A) was calculated by measuring the percent reduction of spheroid growth as a result of treatment with that therapy alone. For example, 80% relative tumor growth after treatment with A was counted as A = 20% efficacy. The expected additive efficacy between two therapies was calculated as $(A+B)/2$. Thus, to calculate additional efficacy, we subtracted the expected additive efficacy from the measured efficacy from combinatorial treatment with A and B ("AB" term in the equation), with a formula of additional efficacy = $AB - (A+B)/2$, so that any result greater than 1 indicated the combinatorial therapeutic strategy had greater efficacy than additive effect of the component therapies.

Table 2 – Therapeutics tested

#	Label	Therapeutic	Host Species	Category
1	Colicin	Colicin E1	<i>E. coli</i>	Pore forming toxin
2	Beta	Beta Hemolysin	<i>S. agalactiae</i>	Pore forming toxin
3	Theta	Theta Toxin	<i>C. perfringens</i>	Pore forming toxin
4	Azurin	Azurin	<i>P. aeruginosa</i>	Apoptosis inducing
5	HST	Heat Stable Enterotoxin 1	<i>E. coli</i>	Apoptosis inducing
6	Magainin	Magainin	<i>Xenopus laevis</i>	Anti-cancer peptide
7	Pleurocidin	Pleurocidin	<i>Pseudopleuronectes americanus</i>	Anti-cancer peptide
8	Buforin IIb	Buforin IIb	<i>Bufo garagrios</i>	Anti-cancer peptide
9	Cecropin X	Cecropin X	<i>Hyalophora cecropia</i>	Anti-cancer peptide
10	HlyE	Hemolysin E	<i>E. coli</i>	Pore forming toxin

Table 3 – Bacteria and mammalian cells used with respective plasmids/genomic changes

Label	Bacteria/Cell line	Plasmid(s)/Genomic
TH01	<i>S. typhimurium</i> (ELH1301)	pTH01 (ptac sfGFP)
TH02	<i>S. typhimurium</i> (ELH430)	pTH01
TH03	<i>S. typhimurium</i> (SL7207)	pTH01
TH04	<i>S. typhimurium</i> (VNP20009)	pTH01
TH05	<i>S. typhimurium</i> (ELH1301)	pTD103 sfGFP
TH06	<i>S. typhimurium</i> (ELH1301)	pTH02 (QS)
TH07	<i>S. typhimurium</i> (ELH1301)	pTH03 (SLC) + pTH01
TH08	<i>S. typhimurium</i> (ELH1301)	pTH05 (luxI Therapeutics) + pTH04
TH09	<i>S. typhimurium</i> (ELH1301)	pTH05+ pTH06 (pTD103 LuxI)
TH10	<i>S. typhimurium</i> (ELH1301)	pTH05 + pTH03
TH11	<i>L. monocytogenes</i>	GFP (genomic)
TH12	<i>P. mirabilis</i>	pTH01
TH13	<i>E. coli</i>	pTH01
CT26	Mouse colorectal carcinoma	N/A
CT26- iRFP	Mouse colorectal carcinoma	iRFP NLS (genomic)
MC26	Mouse metastatic colorectal	N/A
4T1	Mouse metastatic mammary gland	N/A
HT29	Human colorectal adenocarcinoma	N/A
MCF7	Human metastatic breast	N/A

Table 4 – Co-Culture protocols

Bacteria Species	Inoculation CFU	Incubation Times (hours)	Gentamicin Concentration (μg/mL)
<i>S. Typhimurium</i>	10 ⁶	2	2.5
<i>L. monocytogenes</i>	3X10 ⁷	1	10
<i>P. mirabilis</i>	5X10 ⁵	4	4
<i>E. coli</i>	3X10 ⁶	6	1.5

Example 2- Choice of Bacterium for Three-Dimensional Co-culture System

To more accurately recapitulate bacterial tumor colonization *in vitro*, selective bacterial growth in the hypoxic core of 3-dimensional tumor spheroids was sought (Figs. 1A and 1B). Tumor spheroids from a mouse colorectal tumor cell line, CT26, were generated by aggregation in low adhesion 96-well plates to allow for high-throughput testing (Friedrich *et al.* (2009)).

Next, *S. typhimurium* was chosen as a model bacterium, as it has been extensively studied for its anticancer applications and shown safety in human clinical trials (Toso *et al.* (2002); Forbes (2010)). Due to the rapid proliferation rate of *S. Typhimurium*, long-term co-culturing has been a challenge, limiting use to native or engineered obligate anaerobes (Osswald *et al.* (2015)). It was hypothesized that addition of a poorly diffusible antimicrobial agent to the external media would restrict growth of bacteria to inside of spheroids, analogous to *in vivo* bacterial containment by the host immune system (Westphal *et al.* (2008)). Gentamicin, a broad-spectrum aminoglycoside with reduced permeability through the eukaryotic membrane, was chosen (Edwards and Massey (2001)). Scanning of gentamicin concentrations in the co-culture identified a range that confined bacterial growth to within tumor spheroids. Here *S. Typhimurium* attached to the spheroid surface and subsequently localized and proliferated in the interior of the growing tumor spheroid. An increasing level of GFP (sfGFP) fluorescence from *S. Typhimurium* within tumor spheroids was detected over 9 days, confirming growth of bacteria (Fig. 1C). Notably, the bacteria were localized to the necrotic and hypoxic regions of the spheroids,

resembling typical bacterial colonization conditions *in vivo* (Chien (2017)) (Fig. 1D). Additionally, spheroids derived from human colorectal (HT-29) and breast (MCF-7) cancers, as well as murine colorectal (MC-26) cancers, and breast (4T1) cancers were generated. An increasing sfGFP fluorescence from bacteria was observed over time (Fig. 1E), indicating growth of *S. Typhimurium* in a range of cancer types. These results suggested that the co-culturing platform could be adapted for long-term and high-throughput characterization of engineered microbial therapeutics in physiologically relevant conditions.

To investigate the spatiotemporal dynamics of bacteria in detail, automated image analysis was used to quantify the distribution of fluorescence intensity within the spheroid over time. Bacteria fluorescence was observed to increase near the spheroid boundary (40 hours), reach a steady state level of fluorescence (110 hours), and eventually grow but remain contained within the spheroid (140 hours) (Fig. 1F). Bacterial proliferation over time was confirmed by plating dissociated spheroids on selective agar (Fig. 1G). Bacteria achieved stable colonization for up to 2 weeks and localized to necrotic and hypoxic regions of spheroids (Figs. 1D, and 1H), analogous to bacteria colonization conditions *in vivo* (Chien (2017)). These results suggested the platform enables long-term, quantitative characterization of bacterial dynamics within physiologically-relevant 3-D models in a highly parallel manner.

Example 3- Choice of Bacterial Strain

The development of bacterial therapies involves optimizing the host strain, therapeutic payload and synthetic gene circuit (Forbes (2010)). To this end, multiple clinically relevant *S. typhimurium* strains were first tested in tumor spheroids to identify an appropriate host strain for further engineering. The colonization capacity and protein expression level of commonly used tumor-targeting strains derived from the same parent including SL7207 (SL1344 *phoPQ*-), ELH430 (SL1344 *aroA*-), and ELH1301 (SL1344 *phoPQ-laroA*-) were tested (Hoiseth and Stocker (1981); Hohmann *et al.* (1996)), as well as VNP20009 (14028S *msbB*-), which has been tested in clinical trials (Toso *et al.* (2002)). All strains successfully colonized tumor spheroids and had minimal effect on spheroid growth, allowing for stable co-culturing (Figs. 2A-H). ELH1301 demonstrated the highest colonization levels and sfGFP expression within spheroids (Figs. 2A-H). Analysis of dynamics revealed increased initial invasion by ELH1301 compared to

others (Fig. 2I), possibly contributing to its high colonization capacity at day six and was chosen as the host strain in which to engineer synthetic gene circuits expressing a therapeutic cargo.

Example 4- Use of Engineered Bacteria in the Three-Dimensional Co-culture System – Inducible System

Bacteria cancer therapy studies thus far have only tested a limited number of therapeutic payloads which have generally not been compared to one another (Forbes (2010)). Here various therapeutic payloads were incorporated to identify novel engineered bacterial therapies. Given the emerging interest in mining natural products from the natural environment and microbiota (Smanski *et al.* (2016)), a library of ten therapeutics was created consisting of compounds from various organisms with cytotoxic potential, including bacterial toxins and anti-cancer peptides to produce in bacteria (Table 2). When bacterial lysates containing therapeutics were incubated with a monolayer CT26 cells, all therapeutics caused tumor cell death to varying degrees (Fig. 3).

Next engineered bacteria in the three-dimensional co-culture system were evaluated to assess efficacy in physiologically relevant stoichiometry, geometry, and environmental conditions that mimic tumors *in vivo*. An inducible system was engineered, which have been previously utilized to achieve remote control over production of therapeutics at disease sites (Forbes (2010)). Specifically, an acyl-homoserine lactone (AHL) inducible *luxI* promoter was cloned and characterized the circuit dynamics by monitoring the production of downstream sfGFP from the bacteria within the spheroid. A 400% induction in sfGFP signal from bacteria colonized in tumor spheroid was demonstrated within approximately 4 hours post introduction of 10 μ M AHL, reaching a steady-state expression within 10 hours (Figs. 4A and 4B), demonstrating the ability to rapidly trigger therapeutic production within a tumor environment.

Next the efficacy of therapeutic payloads was evaluated using the inducible system. A library of therapeutics was created including previously uncharacterized bacterial toxins and anti-cancer peptides (Table 2). Next, the bacterial therapies were tested by selectively expressing therapeutics from bacteria within spheroids using the inducible system. After adding AHL to spheroids that contain bacteria, many therapeutics demonstrated reduction in tumor spheroid growth (Fig. 4C), corroborating with previous reports on successful delivery of cargo to tumor sites *in vivo* despite additional delivery systems such as secretion (Chien (2017)). The three

candidates that exhibited highest reduction in spheroid growth were azurin (Yamada *et al.* (2002)), theta-toxin (Verherstraeten *et al.* (2015)) and hemolysin E (Ryan *et al.* (2009)). The former is a pro-apoptotic protein, and the latter two are pore-forming toxins, of which theta-toxin had yet to be studied as an engineered bacterial therapeutic. Histopathological analysis (TUNEL staining) of treated spheroids revealed higher levels of tumor cell death following treatment with pore-forming toxins as compared to the control (Fig. 4D).

Example 5- Use of Engineered Bacteria in the Three-Dimensional Co-culture System – Quorum-Sensing Circuit and Synchronized Lysis Circuit

Recent studies have employed increasingly complex gene circuits to achieve optimal control over drug production and release at disease sites (Chien (2017)). Directly comparison of the effect of additional gene circuits on bacterial therapeutic efficacy was examined by building upon the above-mentioned AHL inducible system. First a positive feedback of *luxI* was engineered and hence AHL to build a quorum-sensing (QS) circuit. This circuit enables self-triggered gene expression when bacteria reach a critical density within the tumor core (Swofford *et al.* (2015)), and subsequently sustain production of therapeutics without the need for repeated infusion of chemical inducer. sfGFP was produced from bacteria within tumor spheroids without the addition of external AHL. Although sustained production of sfGFP was demonstrated over 16 hours, induction only reached 40% above baseline, likely due to the use of a low copy number plasmid for AHL production to minimize leaky expression (Figs. 5A and 5B). Incorporation of this circuit to bacteria carrying therapeutic payloads correspondingly resulted in limited efficacy in tumor spheroids (Fig. 5C).

To improve upon this efficacy, a genetic circuit was explored to enhance drug release from bacteria. A recently created synchronized lysis circuit (SLC) was modified, which produces periodic cycles of self-lysis of the host bacteria under quorum-sensing control (Din *et al.* (2016)), by engineering the circuit on a low copy number plasmid in a single operon to minimize leaky expression while allowing for more efficient release of therapeutics into the surrounding environment (Fig. 5D). Monitoring sfGFP expressed from SLC bacteria, pulsatile fluorescence was detected over 72 hours when the bacteria colonized tumor spheroids, indicating periodic lysis (Fig. 5E). Major lysis events were observed every 24-48 hours, roughly corresponding to previously reported dynamics *in vivo* (Din *et al.* (2016)). Additionally, spatiotemporal analysis

indicated localization of bacteria closer to the center of the spheroid, while total sfGFP signal did not increase up to 170 hours of bacterial colonization, suggesting a smaller and spatially-restricted bacterial population due to SLC as well as the finding that SLC maintained containment inside spheroids for 2-fold longer than a non-lysing inducible circuit in the absence of gentamicin (Fig. 5G), suggesting an improved biocontainment property of SLC.

Having characterized dynamics of SLC in tumor spheroids, the efficacy of therapeutic payloads from SLC bacteria was assessed. It was found that SLC increased efficacy compared to the QS circuit on the same copy number for many therapeutics tested, confirming the use of lysis as an effective therapeutic delivery strategy (Fig. 5F). Notably, it was found that theta-toxin expressed from SLC exhibited significantly higher efficacy compared to other therapeutics, achieving approximately 40% reduction in tumor spheroid growth. Given the toxicity of theta-toxin to host bacteria, AHL was varied in the inducible circuit from 0 nM to 10 nM and found that increasing theta-toxin expression did not decrease efficacy (results not shown). Thus, it was reasoned that theta-toxin expression from SLC exhibited high therapeutic efficacy due to efficient release from bacteria. Together, these results demonstrated that microbial therapeutics controlled by dynamic synthetic gene circuits can be studied using the 3D co-culture system to evaluate circuit performance and efficacy.

Example 6 – Three-dimensional Co-culture System was Predictive of the Efficacy of Therapeutics

To determine how predictive the three-dimensional co-culture platform was of an *in vivo* tumor model, the effect of therapeutic strains was assessed in a syngeneic, hind-flank CT26 tumor model in mice. First inducible therapeutics with predicted high efficacy (azurin, theta-toxin and hemolysin E), moderate efficacy (beta-hemolysin), and a control (sfGFP) were investigated. Upon intratumoral injections, bacterial therapeutics identified to be effective from the *in vitro* screen elicited strong response on tumor growth when compared to the control bacteria, with up to approximately 3-fold reduction in tumor growth (Fig. 6A). Tracking therapeutic response over time, good agreement was found in the order of efficacy between the *in vitro* and *in vivo* results (Fig. 6B). Additionally, histopathological analysis was performed on treated tumors at the termination of the trial. Consistent with the spheroid results, tumors treated with theta-toxin and hemolysin E showed increased percentage of cell death relative to tumors

treated with control bacteria (Fig. 6C). In contrast, results from bacteria co-cultured with a monolayer of the same CT-26 cells failed to predict trends of efficacy *in vivo* (Fig. 6D).

Example 7 - Three-dimensional Co-culture System was Predictive of the Efficacy of Combined Therapeutics

Leveraging the high-throughput nature of the three-dimensional co-culture platform, combination therapy was investigated and compared with mouse tumor models. By applying pairwise combinations of strains at equal proportions in the three-dimensional co-culture platform, it was found that theta-toxin and azurin to be the most effective combination, achieving up to 25% spheroid growth reduction within 4 days of therapeutic induction (75% relative growth, Fig. 7A). In addition, this combination and others exhibited higher therapeutic efficacy than the additive effect of single therapies combined, suggesting possible synergy between the therapeutics. When the effect of combination therapy *in vivo* was examined, combinatorial therapy exerted significantly stronger anti-tumor effect than either therapeutic alone, reducing growth of tumor grafts by approximately 4-fold when compared to the control theta toxin and azurin (Fig. 7B). These findings highlight the potential of the three-dimensional co-culture system for the study of multiple therapeutics and their interaction as well as predictively identify potent genetic circuits and therapeutic combinations in a high-throughput manner.

Next, bacteria with SLC was examined to study whether the difference in efficacy of therapeutics observed in the three-dimensional co-culture platform are recapitulated *in vivo*. The general trend in the order of therapeutic efficacy within SLC strains corresponded with the observations from the spheroid screen as well (Fig. 7C). Importantly, when efficacy of SLC and QS circuits was compared, theta-toxin from SLC exerted stronger response than that from the QS circuit, correlating with efficacy observed in spheroids (Fig. 7D). In addition, SLC-mediated bacteria population control improved health on the host animal, indicated by minimal weight drop compared to the bacteria carrying inducible circuits (Fig. 7E). These results support the use of the disclosed technology to rapidly identify effective combinations of microbial therapeutics that would be challenging to optimize in animal models alone.

Example 8 – Use of Other Bacterial Species in the Three-Dimensional Co-culture System

The colonization of *L. monocytogenes*, *P. mirabilis*, and *E. coli* (bacterial species previously tested for cancer therapy) was tested in the three-dimensional co-culture platform (Chien (2017); Zhang *et al.* (2017)). Optimizing bacterial inoculation density, incubation time, and gentamicin concentrations (Table 4), long-term growth of all bacterial species was established, indicated by increase in sfGFP fluorescence and CFU from spheroids over multiple days (Figs. 9A-9C). Spatiotemporal analysis allowed for comparison of fluorescence distribution within spheroids and respective dynamics. *L. monocytogenes* displayed a notably wide area of tumor colonization (Fig. 9A), possibly because of its cell-to-cell spreading ability within tumor mass (Tangeney and Gahan 2010). *P. mirabilis* reached the spheroid core at earlier time compared to other bacteria (Fig. 9B), which might be attributable to its swarming ability. *E. coli* displayed a sharp increase in number at day 6 within the spheroid core (Fig. 9C), demonstrating similar dynamics to *S. typhimurium*.

Additionally, *P. aeruginosa* was tested in the three-dimensional co-culture platform. As shown in Fig. 9D, this bacterial species also colonized in the spheroid.

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CLAIMS

1. A three-dimensional co-culture system comprising:
 - a. a spheroid or organoid; and
 - b. bacteria, virus, or eukaryotic cell, wherein the bacteria, virus or eukaryotic cell is being tested, screened or evaluated as a therapeutic or diagnostic, or the bacteria, virus or cell comprise an agent to be tested, screened or evaluated as a therapeutic or diagnostic.
2. The three-dimensional co-culture system of claim 1, wherein the spheroid is derived from a tumor chosen from the group consisting of brain, prostate, pancreatic, stomach, colorectal, breast, lung, ovarian, liver, kidney, skin, and bone.
3. The three-dimensional co-culture system of claim 1, wherein the organoid is derived from an organ chosen from the group consisting of bone, muscle, cerebral, gut, intestinal, stomach, thyroid, hepatic, pancreatic, epithelial, lung, kidney and cardiac.
4. The three-dimensional co-culture system of claim 1, wherein the spheroid or organoid is generated from a tumor or organ from a patient in which the therapeutic or diagnostic agent is to be administered or delivered after being screened, tested or evaluated.
5. The three-dimensional co-culture system of claim 1, wherein the bacteria, virus or cells are naturally occurring.
6. The three-dimensional co-culture system of claim 1, wherein the bacteria, virus or cells are genetically engineered.
7. The three-dimensional co-culture system of claim 6, wherein the bacteria, virus or cells are genetically engineered to comprise a means for detection or visualization.
8. The three-dimensional co-culture system of claim 7, wherein the means for detection or visualization is fluorescence or luminescence.
9. The three-dimensional co-culture system of claim 6, wherein the bacteria, virus or cells are genetically engineered to comprise a means for delivering the agent to be tested, screened or evaluated as a therapeutic or diagnostic.
10. The three-dimensional co-culture system of claim 6, wherein the bacteria, virus or cells are genetically engineered to comprise the agent to be tested, screened or evaluated as a therapeutic or diagnostic.

11. A three-dimensional co-culture system comprising:
 - a. a spheroid or organoid; and
 - b. bacteria, virus, or eukaryotic cell, wherein the bacteria, virus or eukaryotic cell is genetically engineered to comprise a genetic circuit.
12. The three-dimensional co-culture system of claim 11, wherein the spheroid is derived from a tumor chosen from the group consisting of bone, muscle, cerebral, gut, intestinal, stomach, thyroid, hepatic, pancreatic, epithelial, lung, kidney and cardiac.
13. The three-dimensional co-culture system of claim 11, wherein the organoid is derived from an organ chosen from the group consisting of bone, muscle, cerebral, gut, intestinal, stomach, thyroid, hepatic, pancreatic, epithelial, lung, kidney and cardiac.
14. The three-dimensional co-culture system of claim 11, wherein the spheroid or organoid is generated from a tumor or organ from a specific patient.
15. A method of generating the three-dimensional co-culture system of claim 1 or 11 comprising the steps of:
 - a. obtaining or generating a spheroid or organoid; and
 - b. co-culturing the spheroid or organoid with bacteria, virus or eukaryotic cells under conditions that allow the bacteria, virus or eukaryotic cells to invade the spheroid or organoid and grow within the core of the spheroid or organoid.
16. The method of claim 15, further comprising introducing an antibiotic into the system.
17. The method of claim 16, wherein the antibiotic is gentamycin.
18. The method of claim 15, further comprising the step of monitoring the growth of the bacteria, virus or eukaryotic cells within the core of the spheroid or organoid.
19. A method of using the three-dimensional co-culture system of claim 1 to identify, test, screen or evaluate a therapeutic or diagnostic, comprising the steps of:
 - a. introducing the therapeutic or diagnostic agent into the three-dimensional co-culture system; and
 - b. monitoring the growth or death of the spheroid or organoid in the system.
20. The method of claim 19, wherein the therapeutic or diagnostic agent is the bacteria, virus or cell.
21. The method of claim 19, wherein the bacteria, virus or cell comprises the therapeutic or diagnostic agent.

22. The method of claim 19, further comprising introducing a second agent to the three-dimensional co-culture system which induces delivery of the therapeutic or diagnostic from the bacteria, virus or cell.
23. A method of using the three-dimensional co-culture system of claim 11 to identify, test, screen or evaluate a genetic circuit, comprising the steps of:
- a. introducing an agent which induces the genetic circuit into the three-dimensional co-culture system; and
 - b. monitoring the growth or death of the spheroid or organoid in the system.

Fig. 1

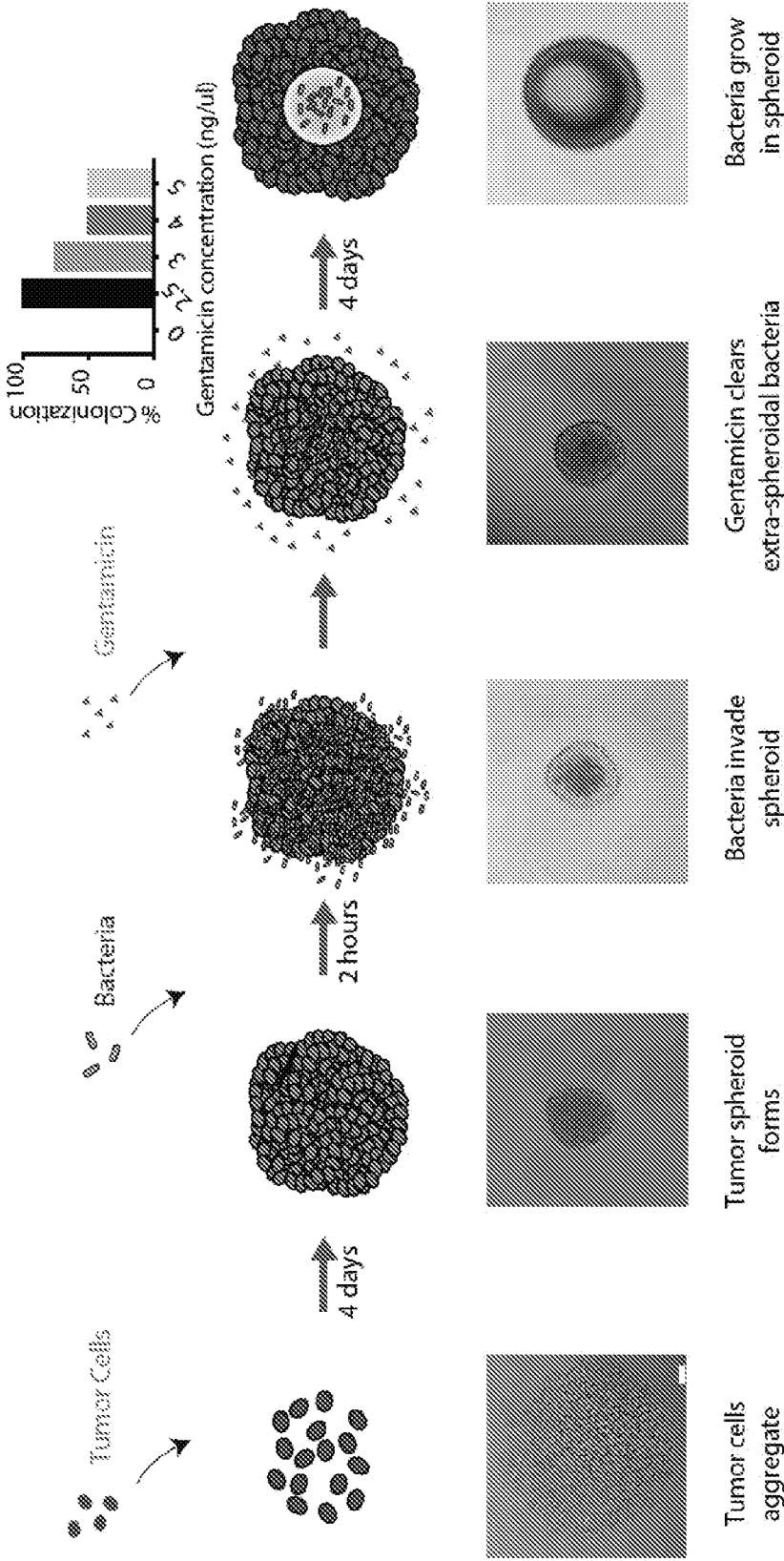


Fig. 1A

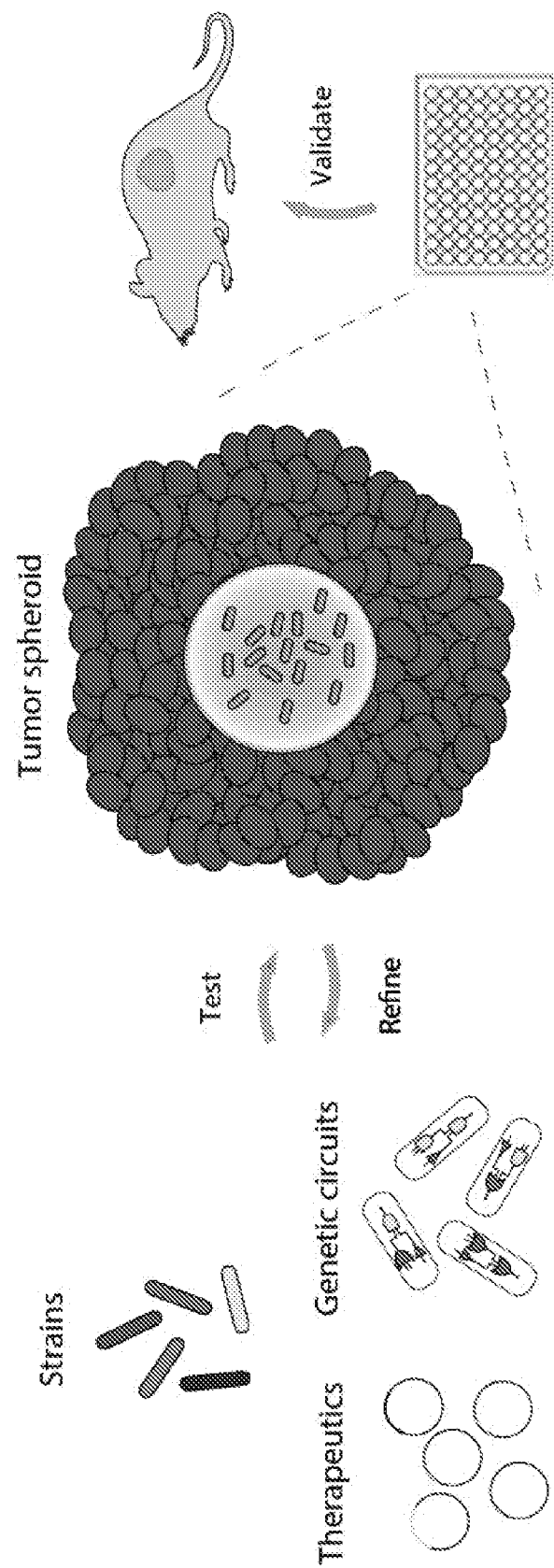


Fig. 1B

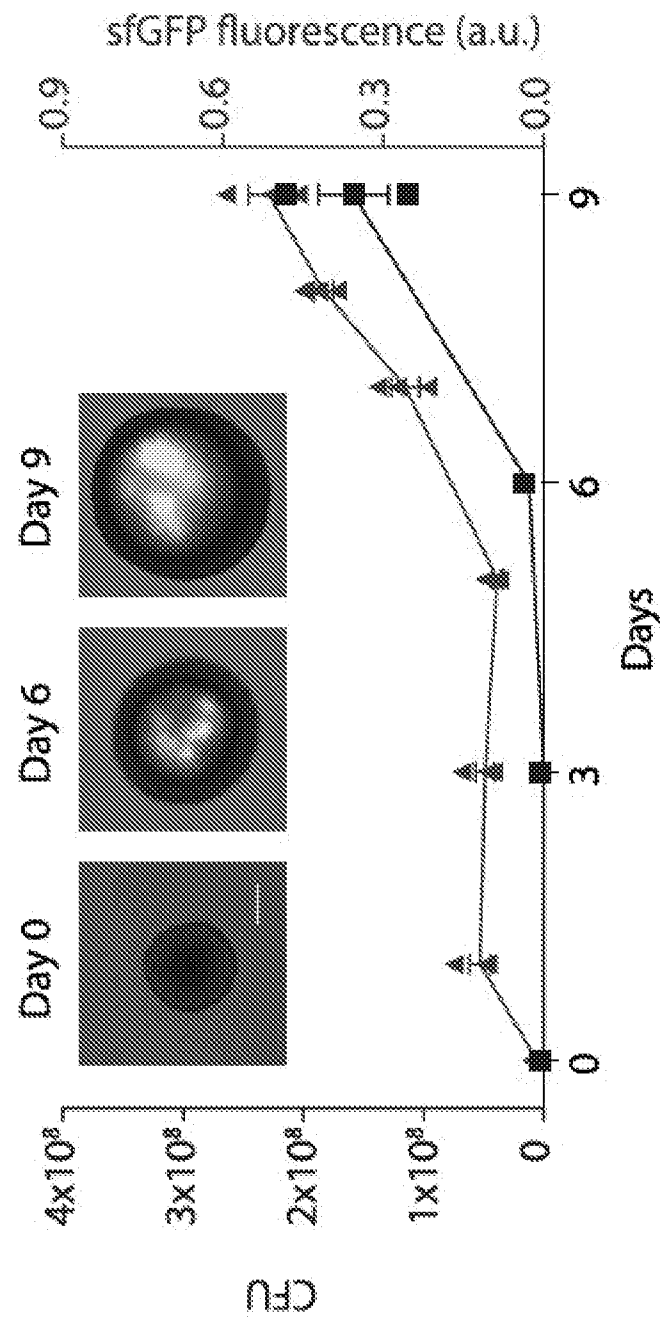


Fig. 1C

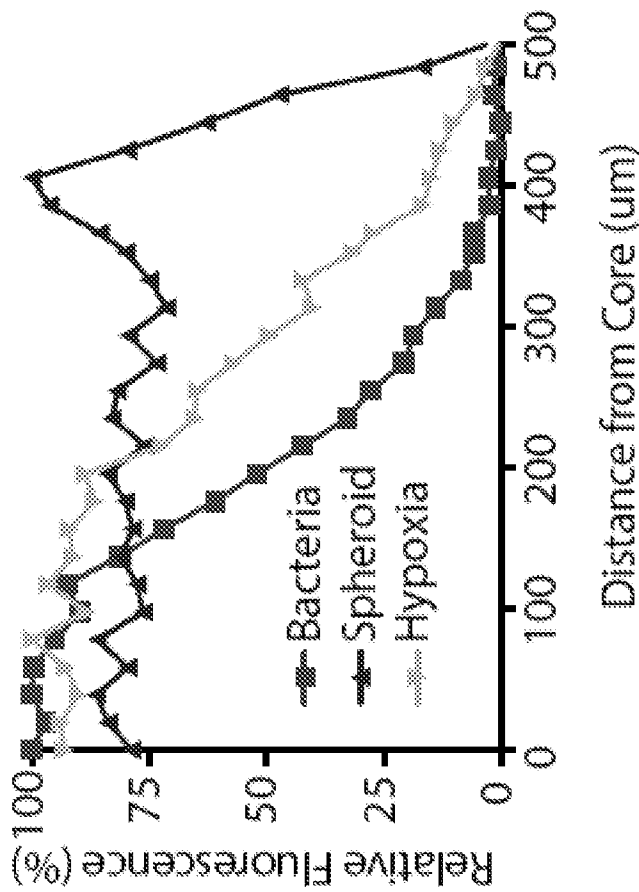
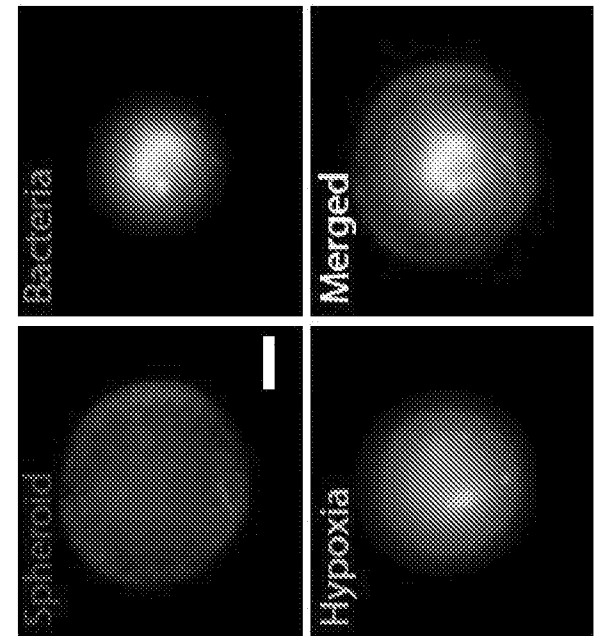


Fig. 1D



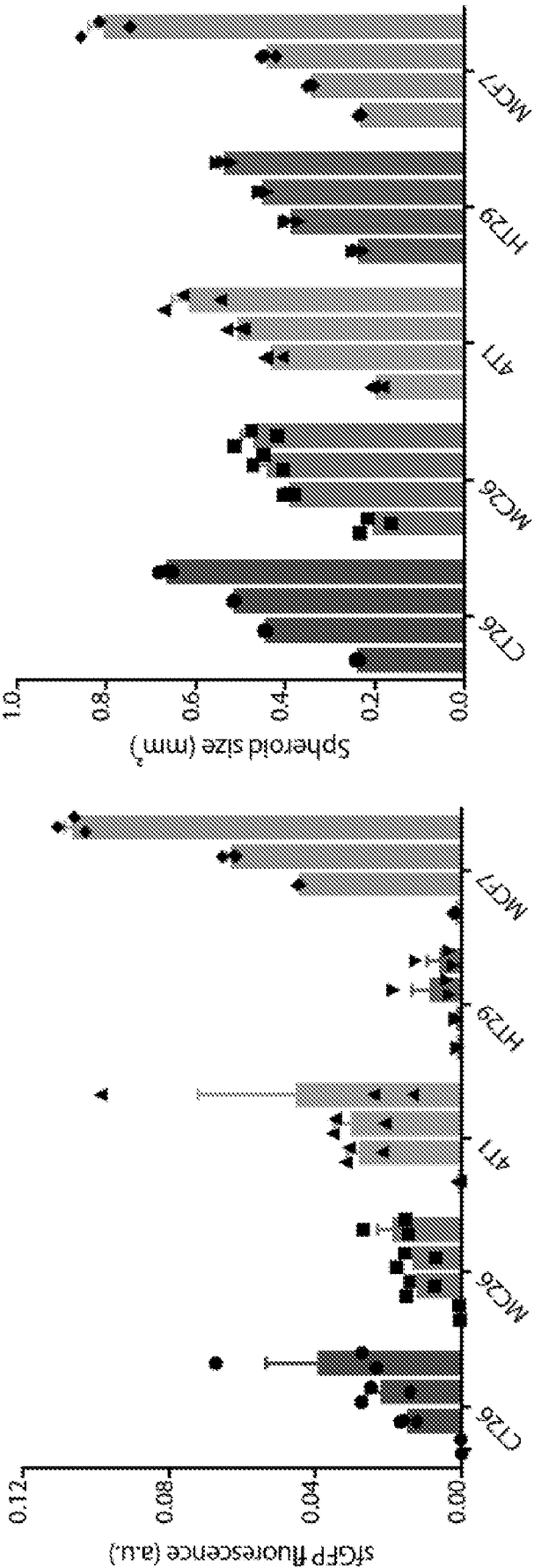


Fig. 1E

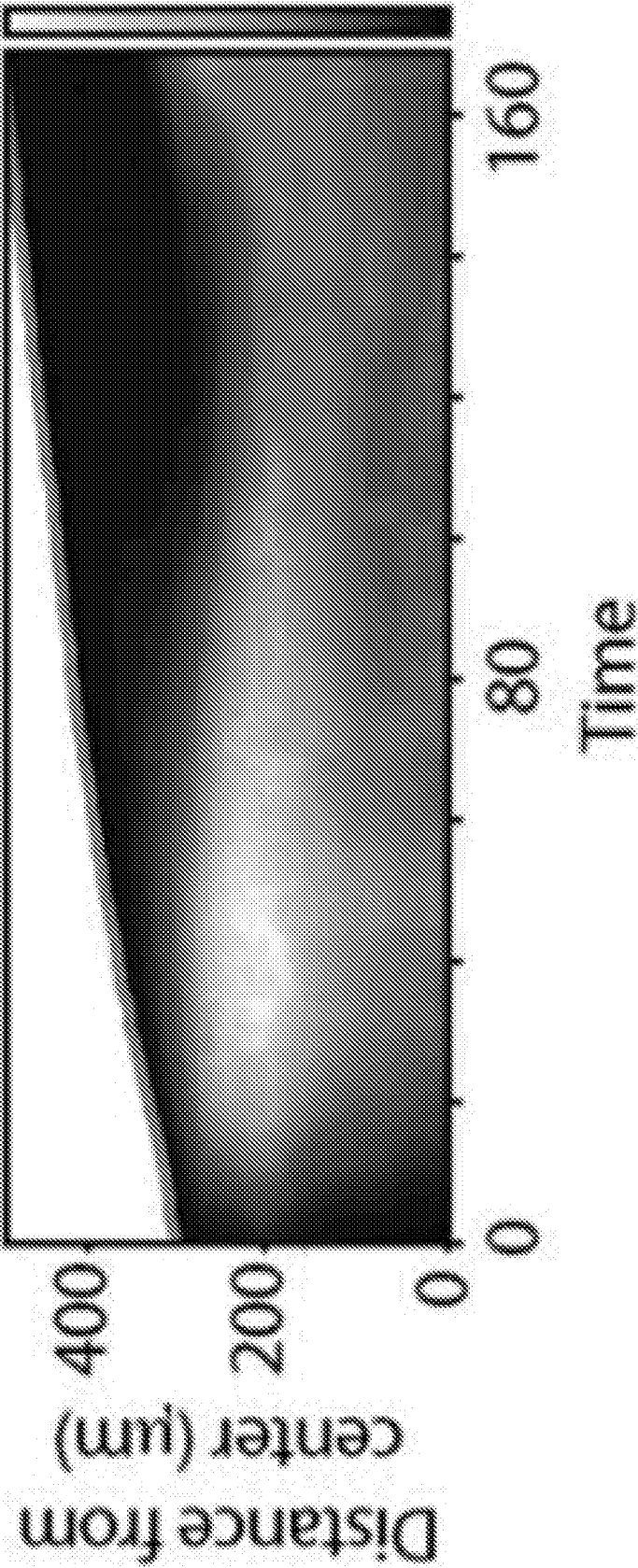


Fig. 1F

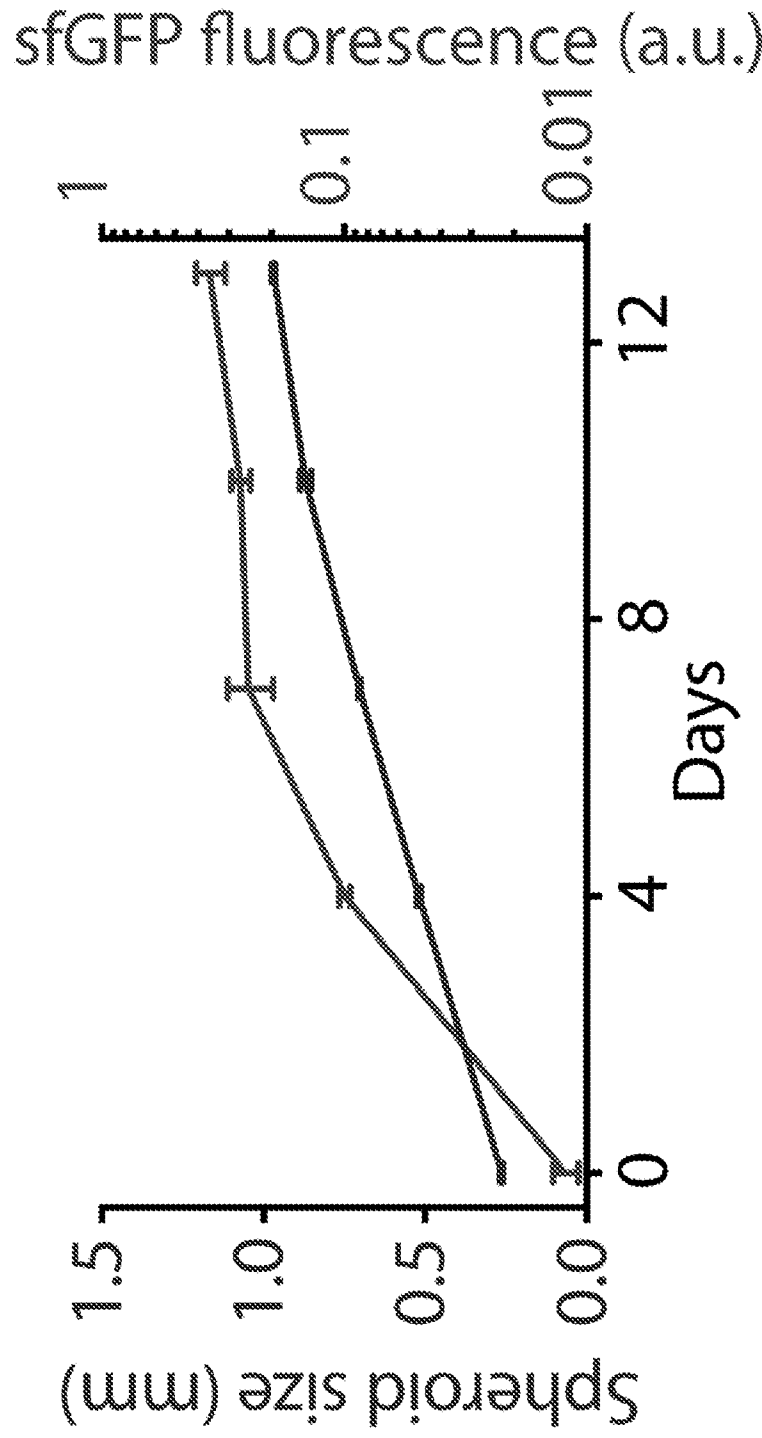


Fig. 1G

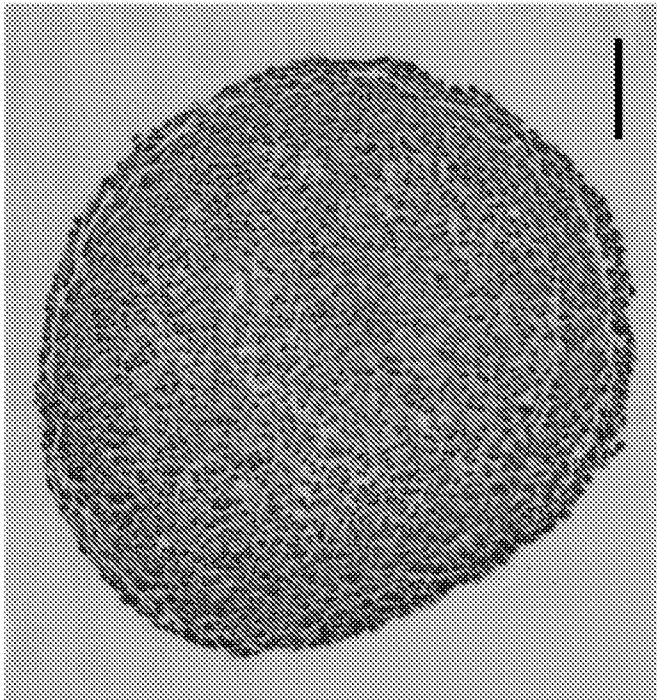
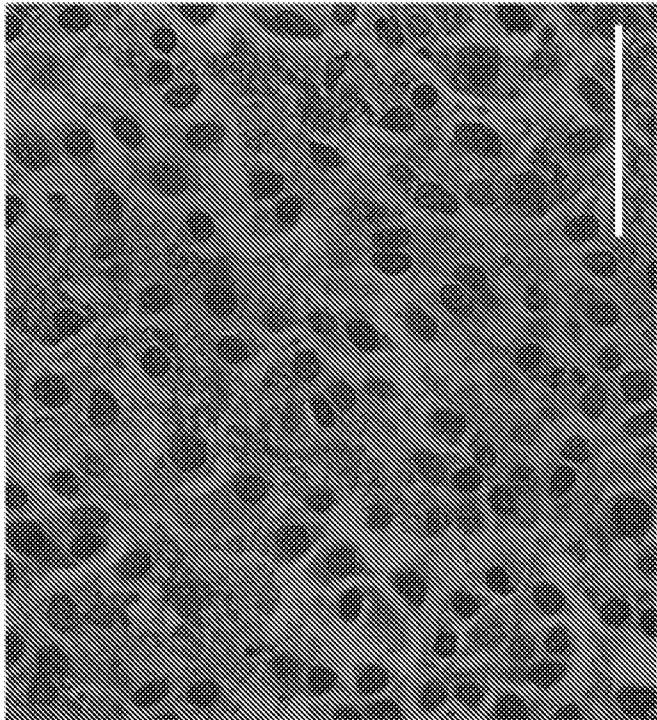


Fig. 1H

Fig. 2

Fig. 2A

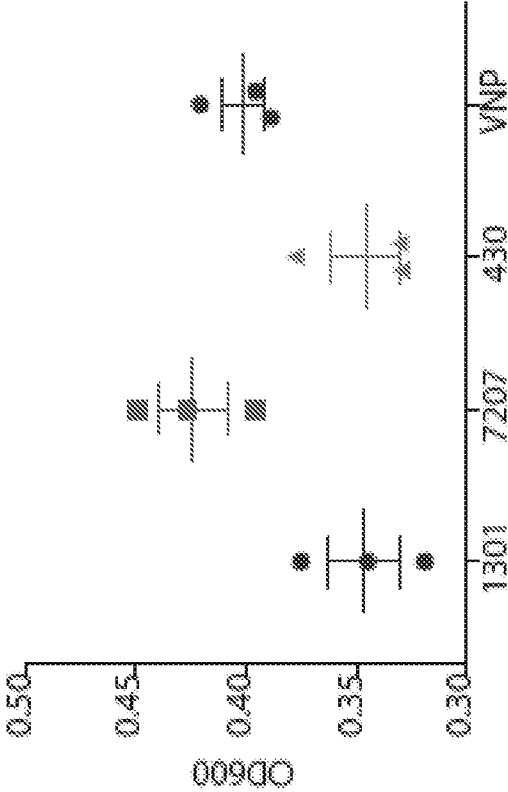


Fig. 2B

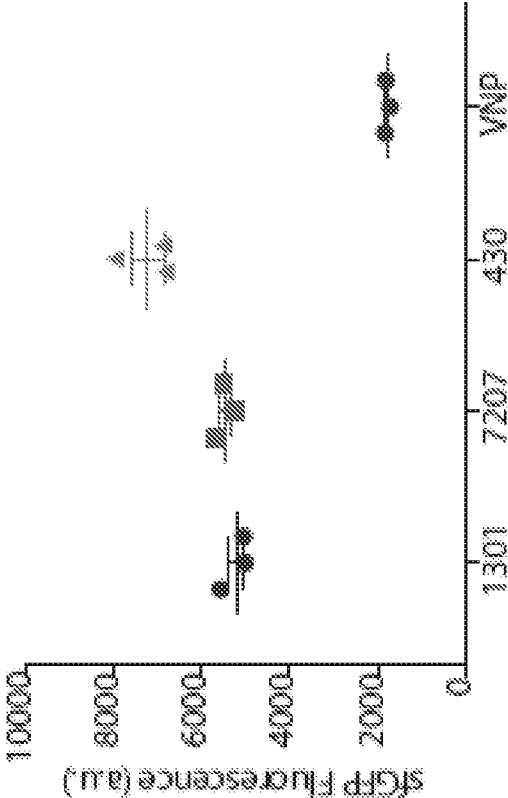


Fig. 2D

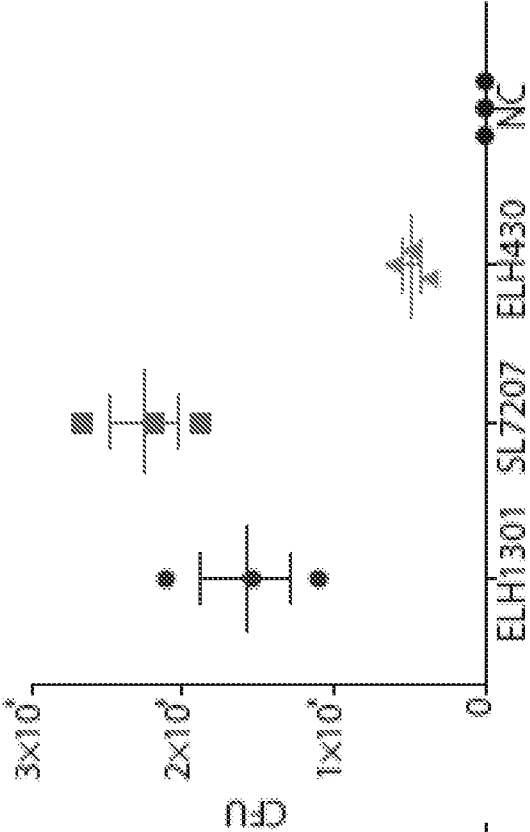


Fig. 2C



Fig. 2E

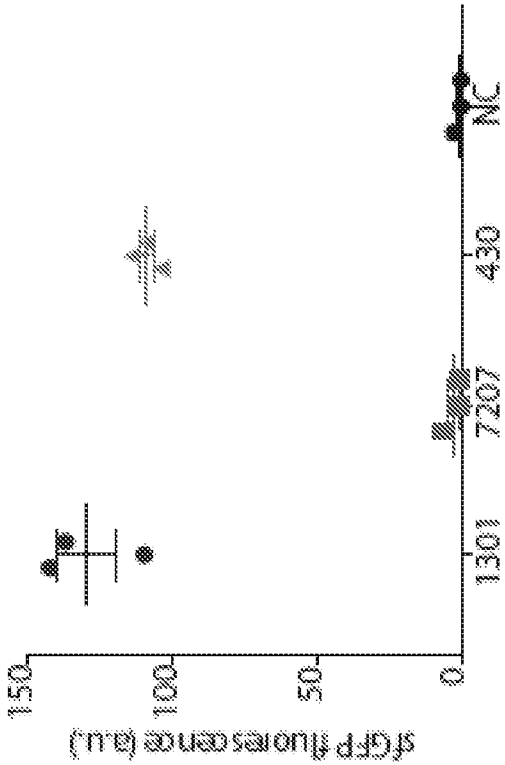


Fig. 2F

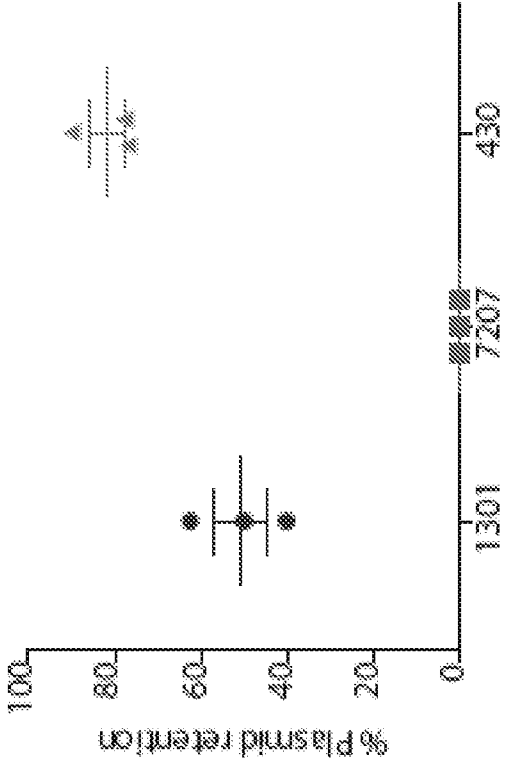


Fig. 2H

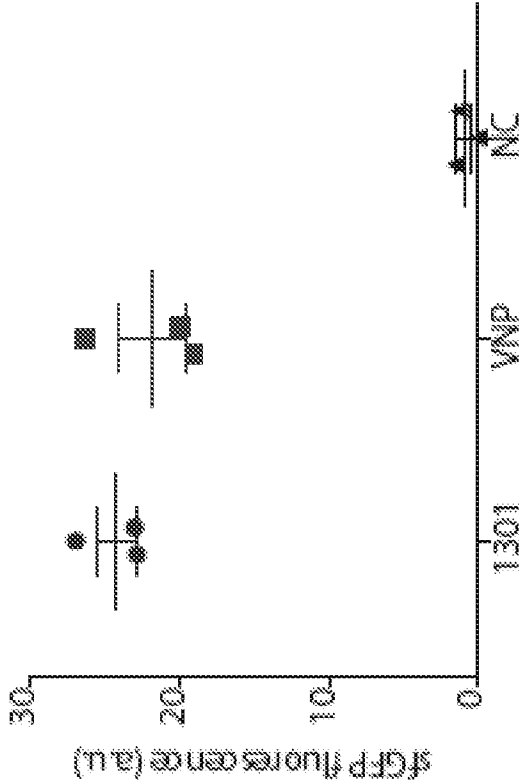


Fig. 2G

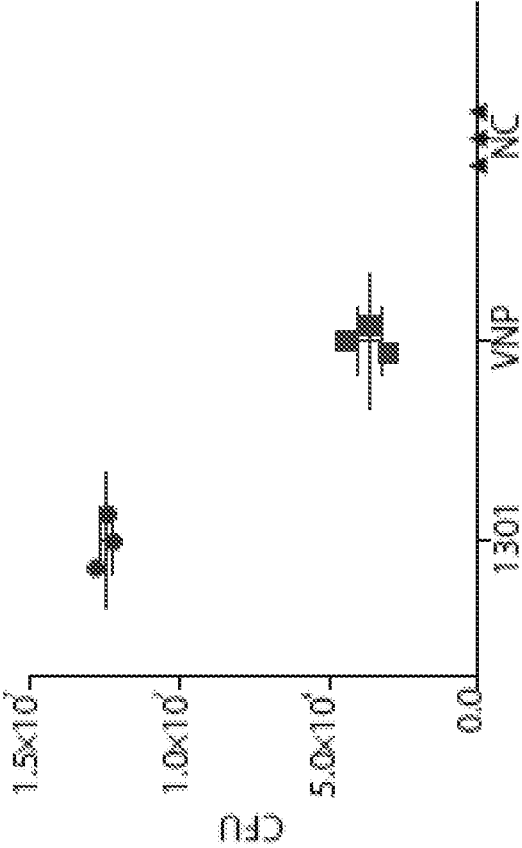


Fig. 2I

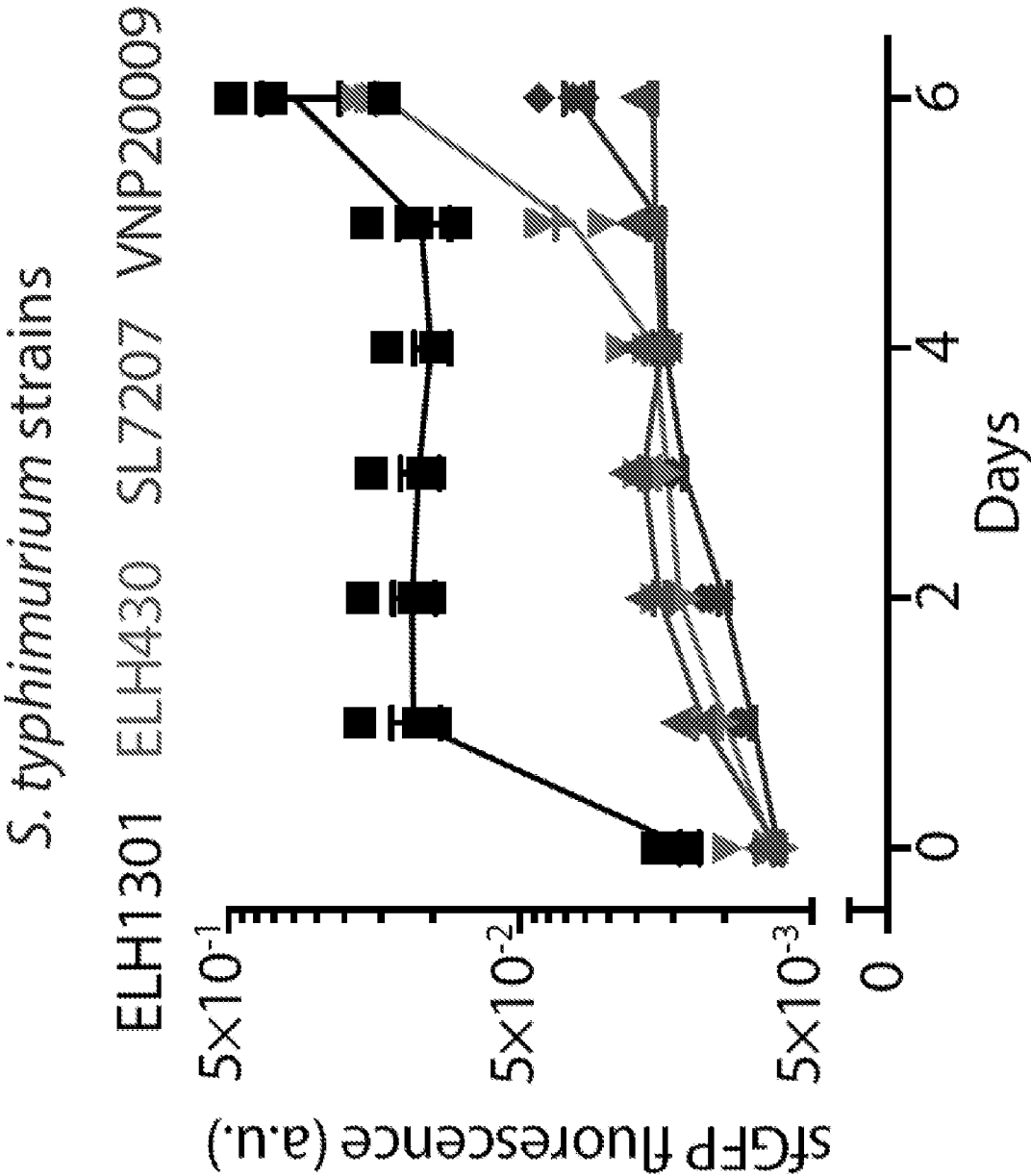


Fig. 3

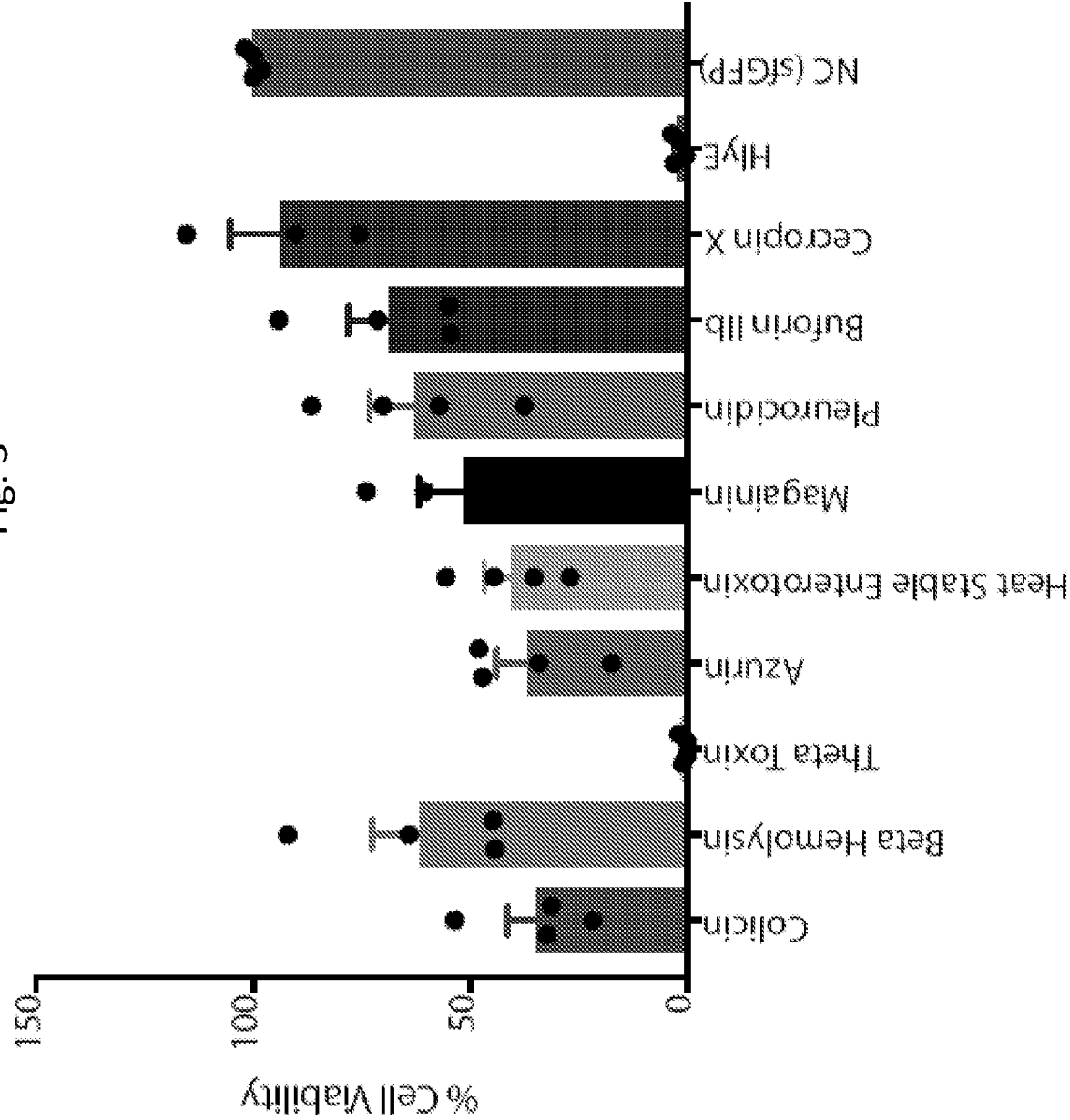


Fig. 4

Fig. 4A

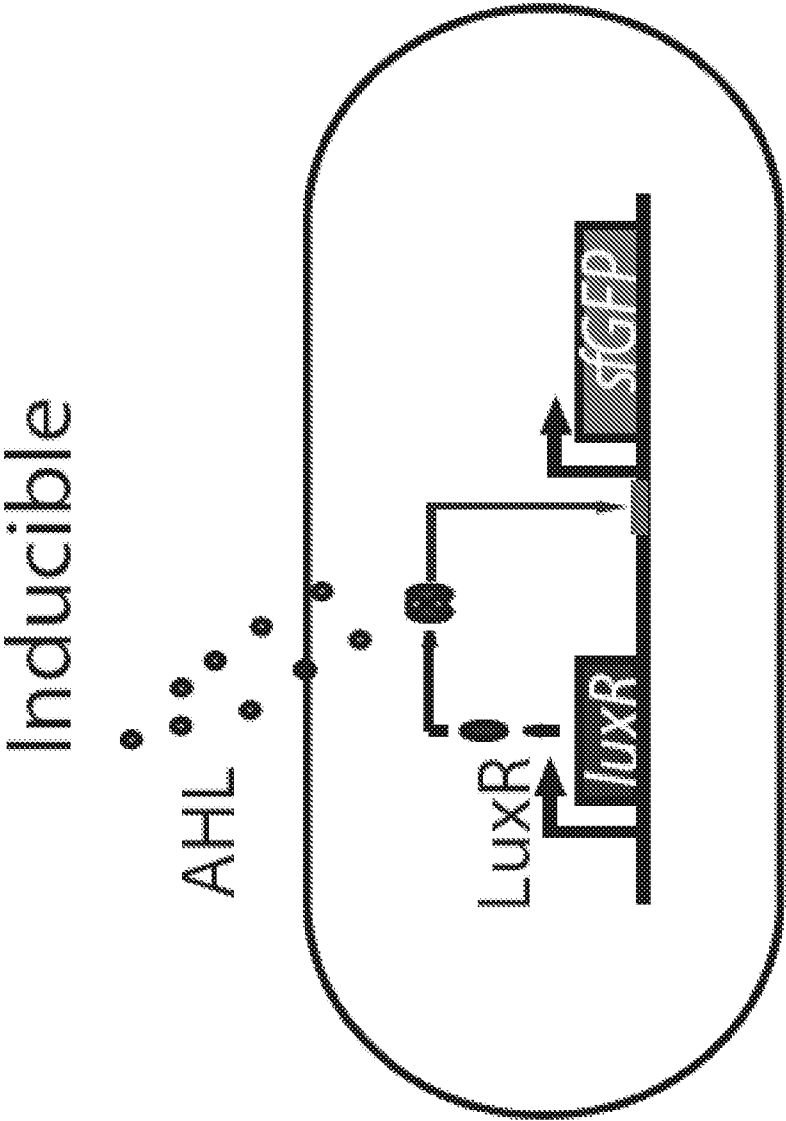


Fig. 4B

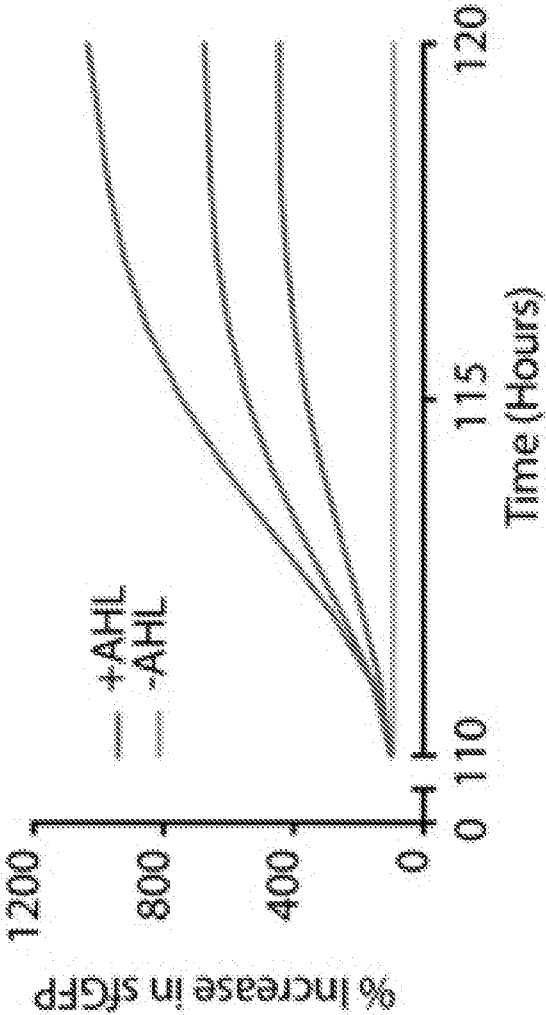
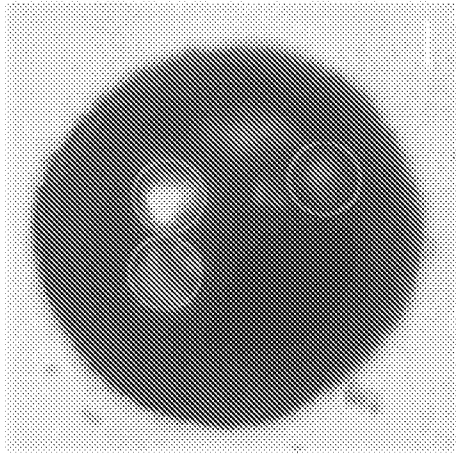


Fig. 4C

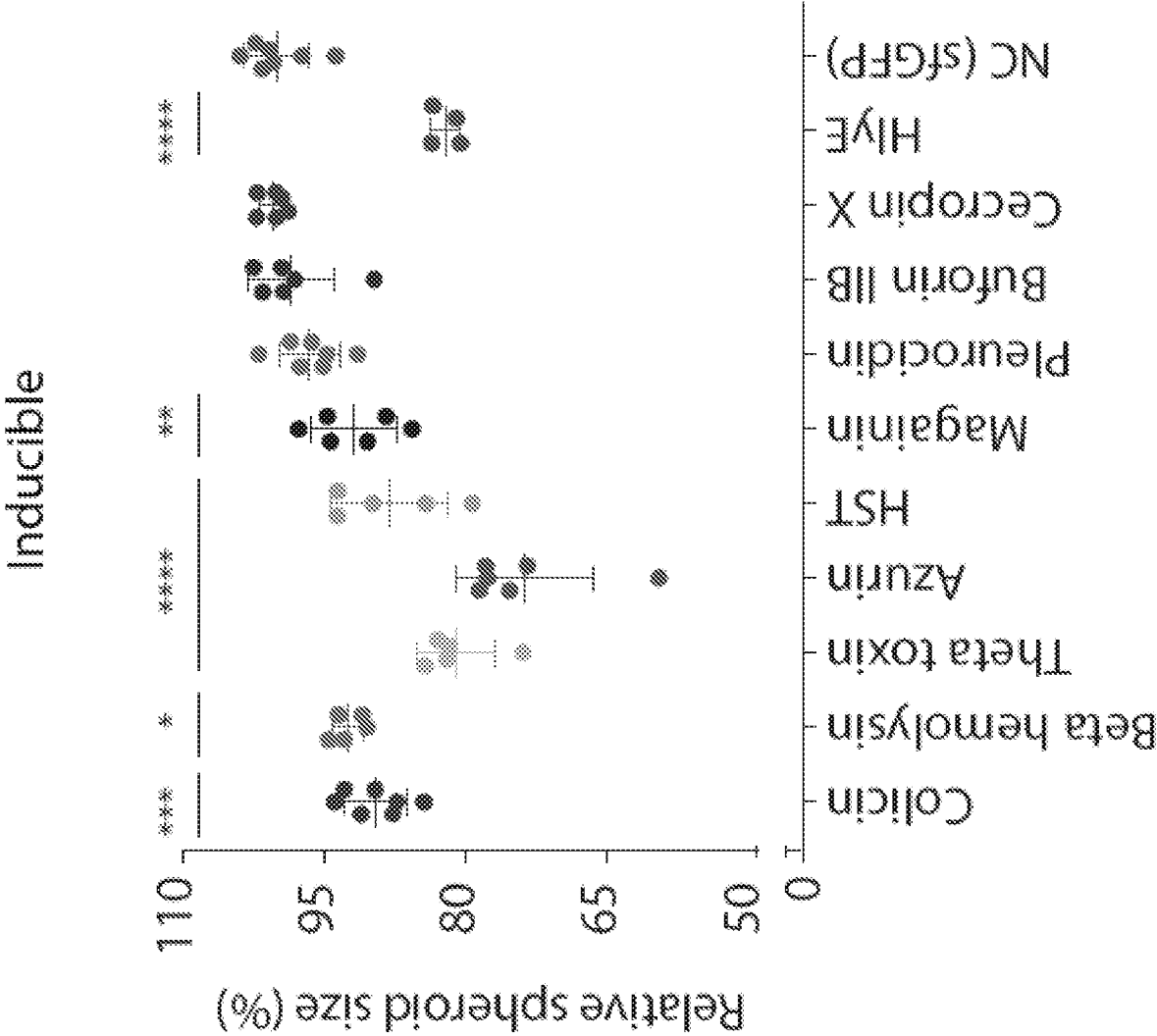


Fig. 4D

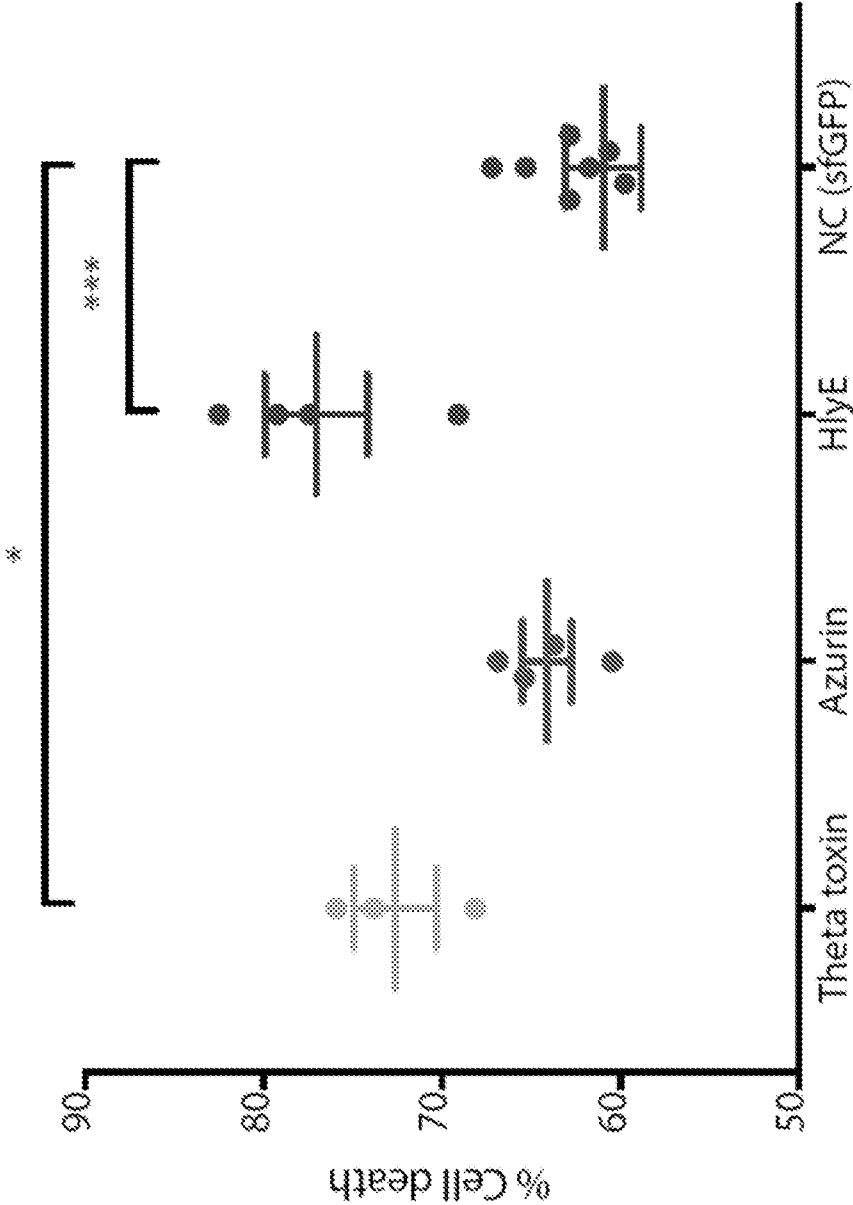


Fig. 5

Fig. 5A

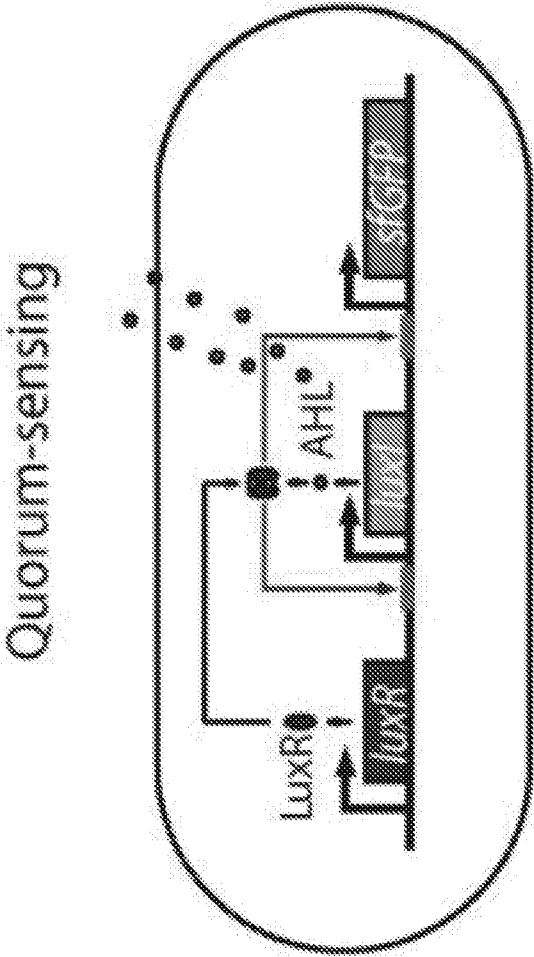


Fig. 5B

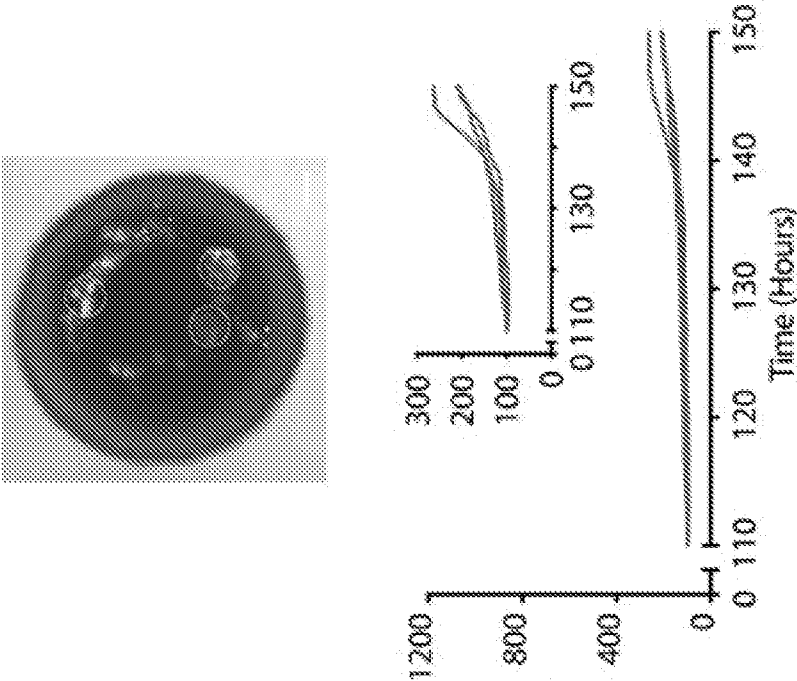


Fig. 5C

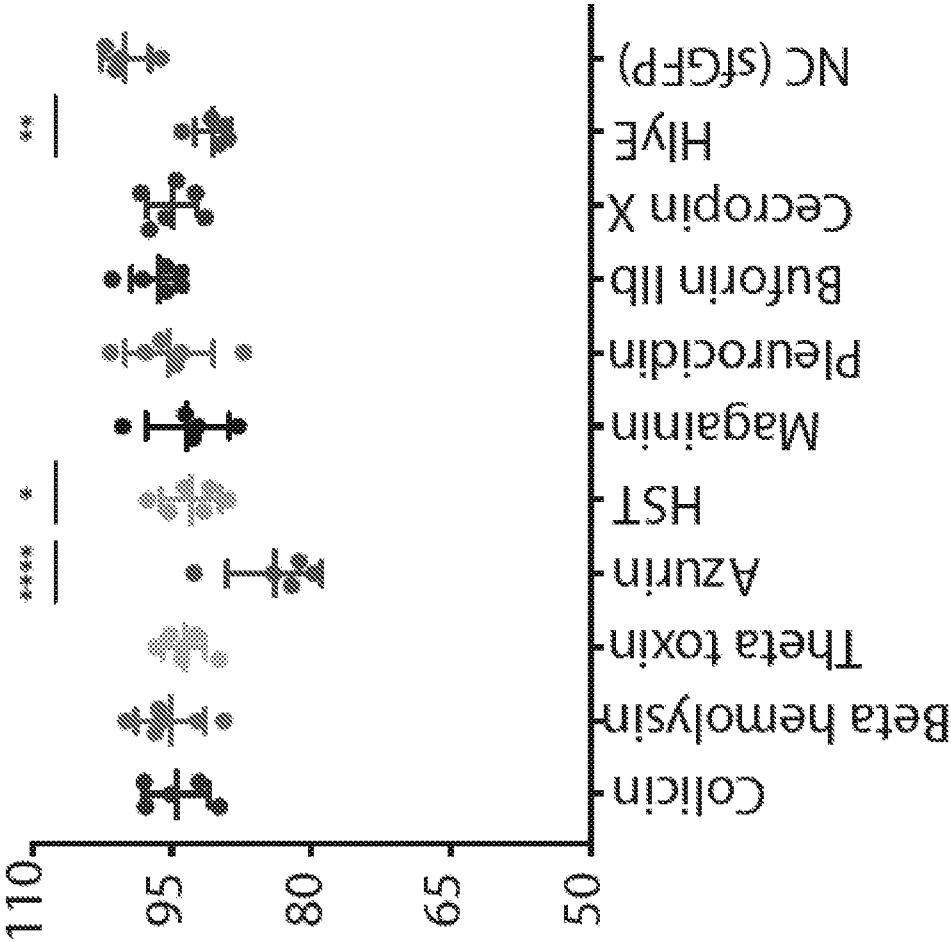


Fig. 5E

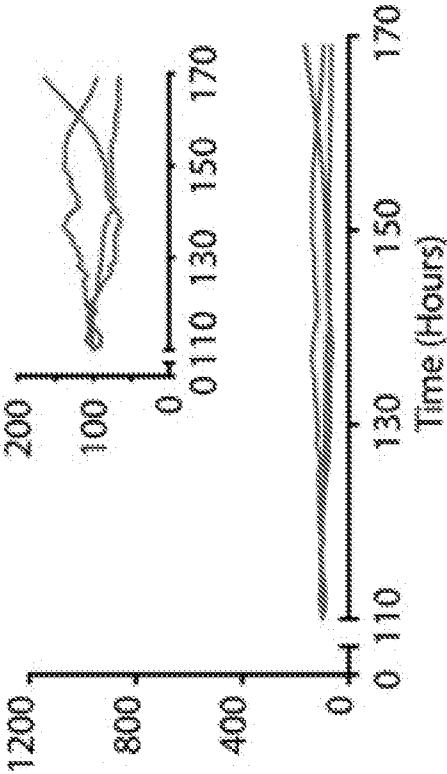
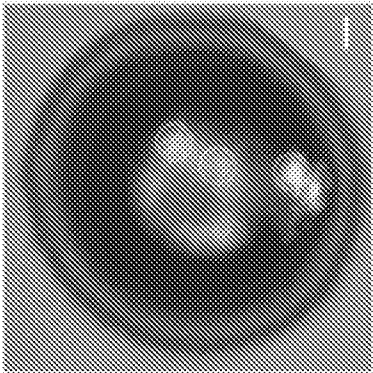


Fig. 5D

Synchronized lysis

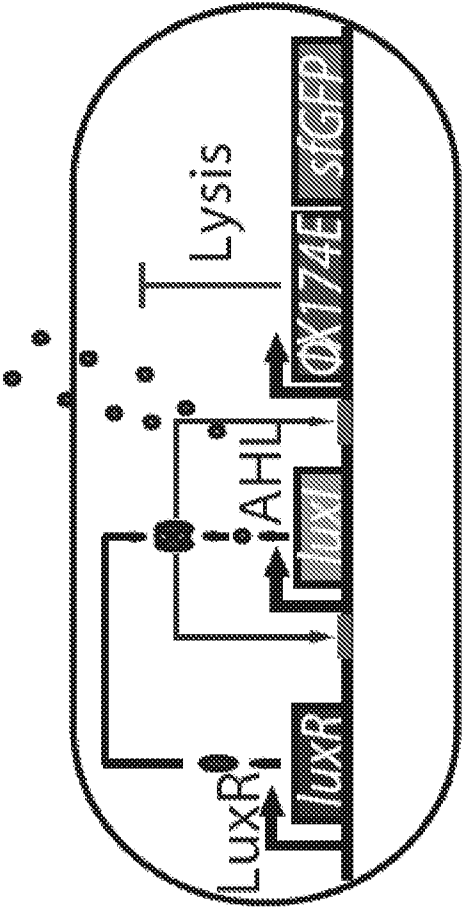


Fig. 5F

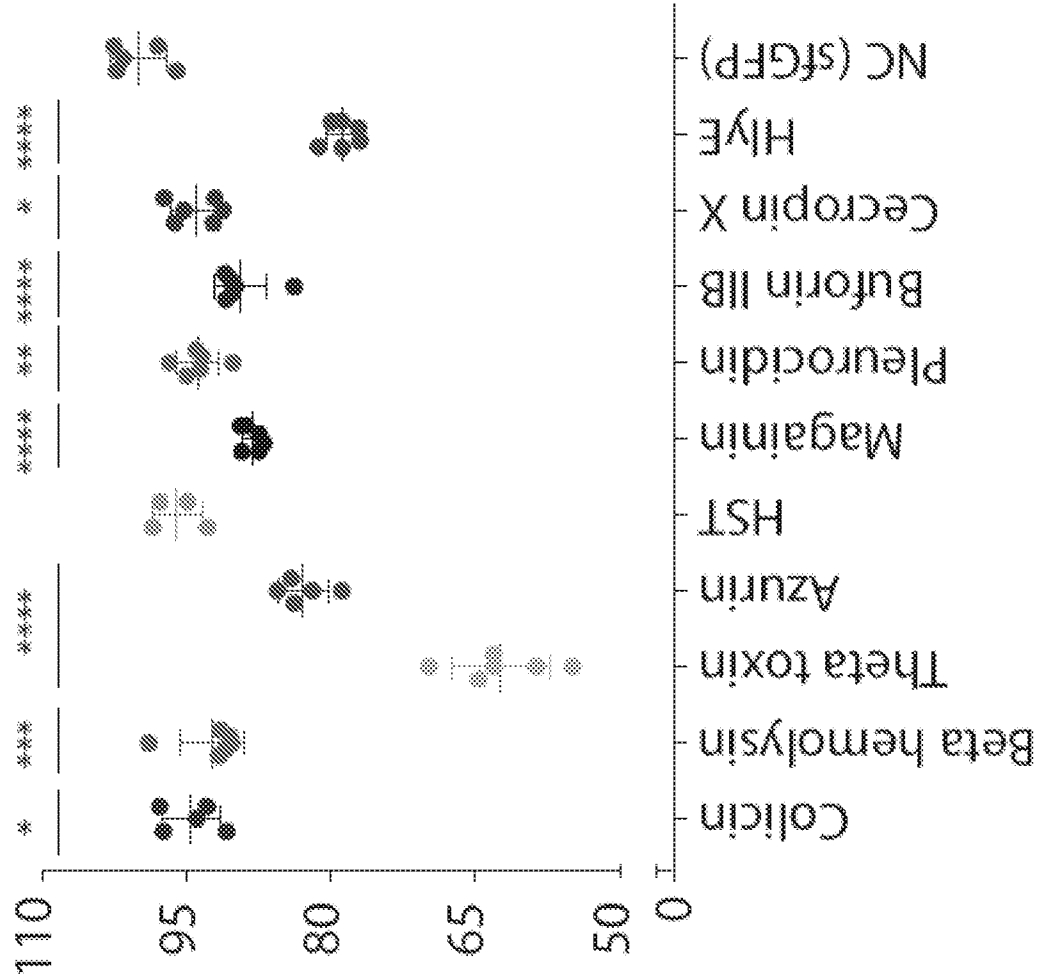


Fig. 5G

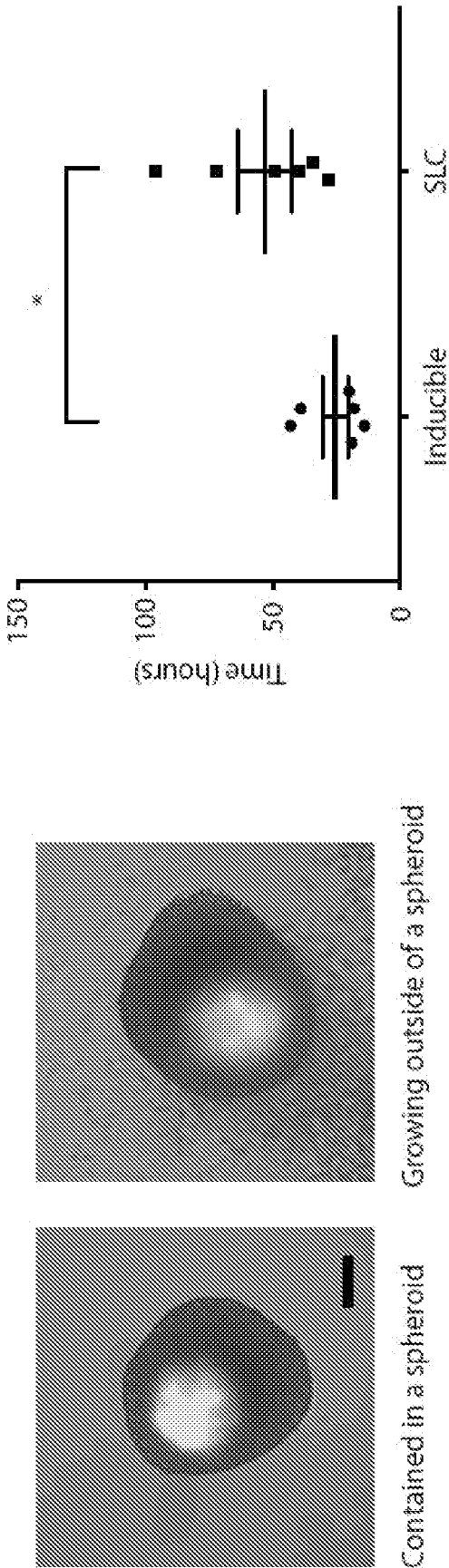


Fig. 6

Fig. 6A

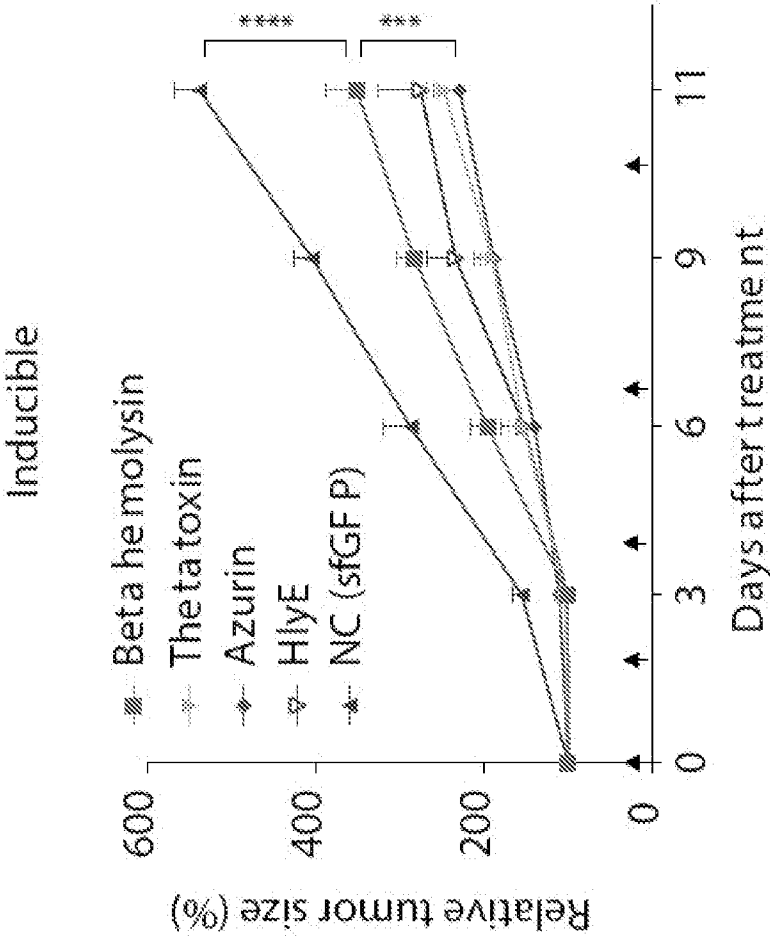


Fig. 6B

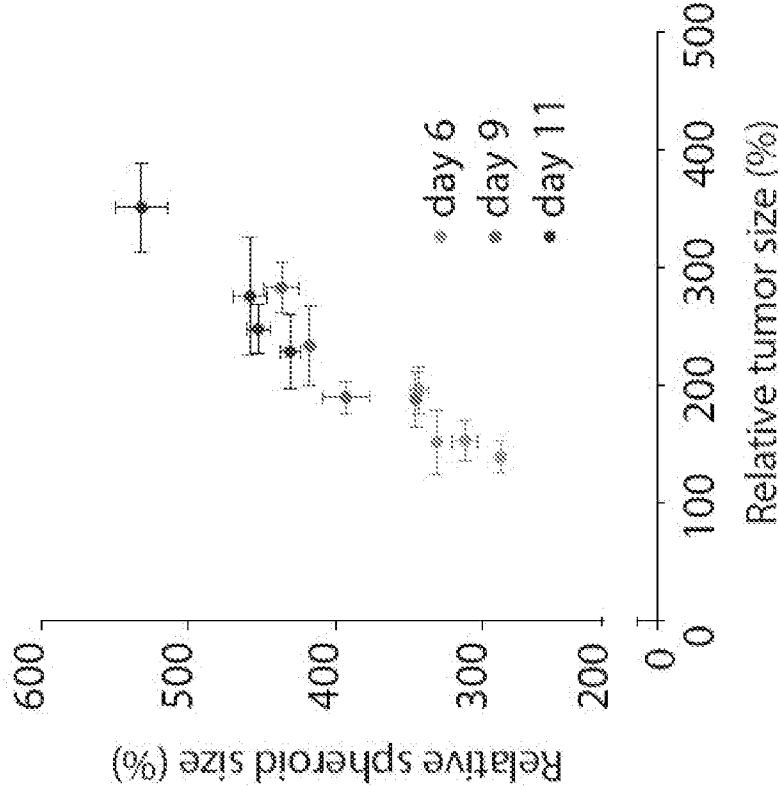


Fig. 6C

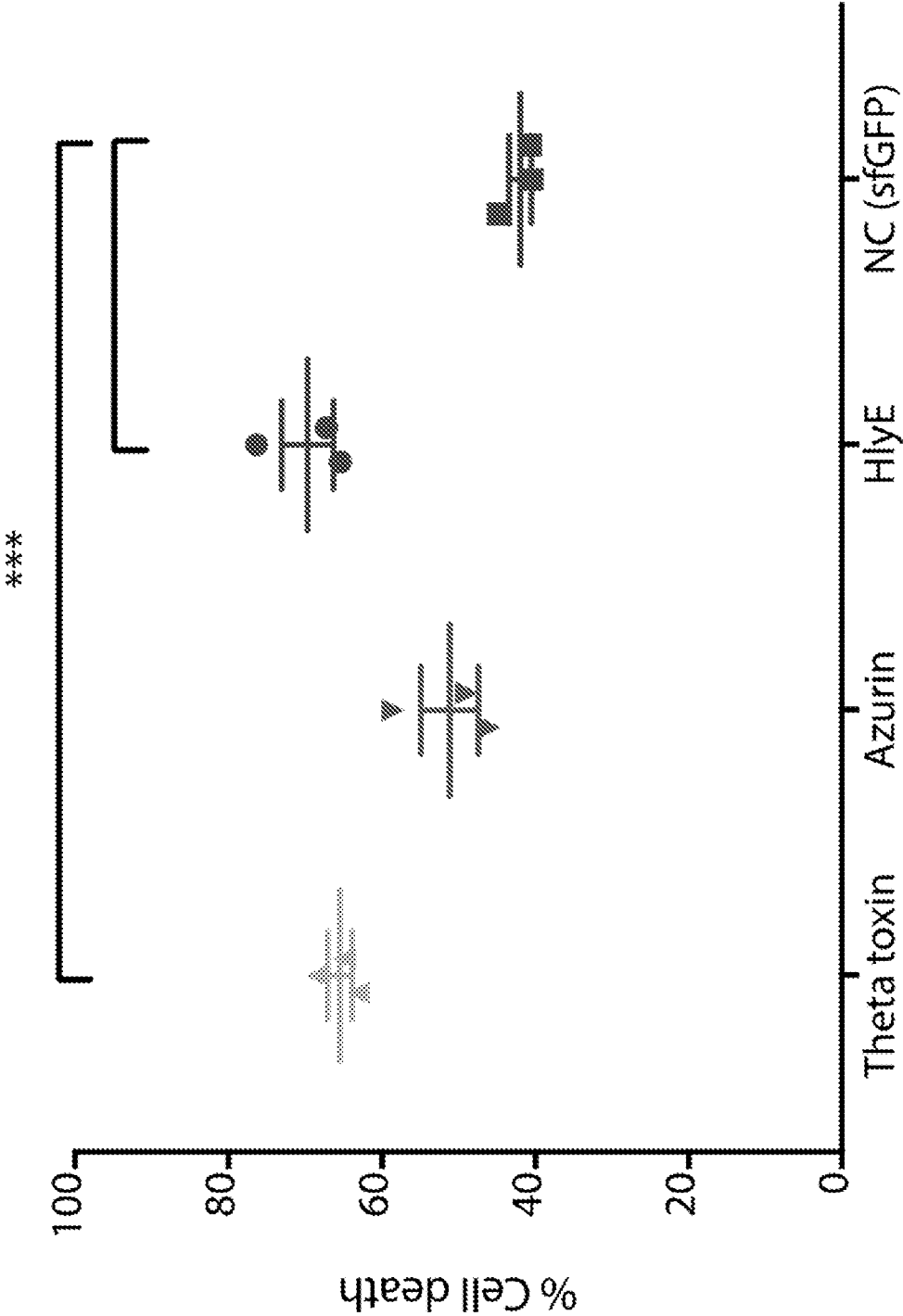


Fig. 6D

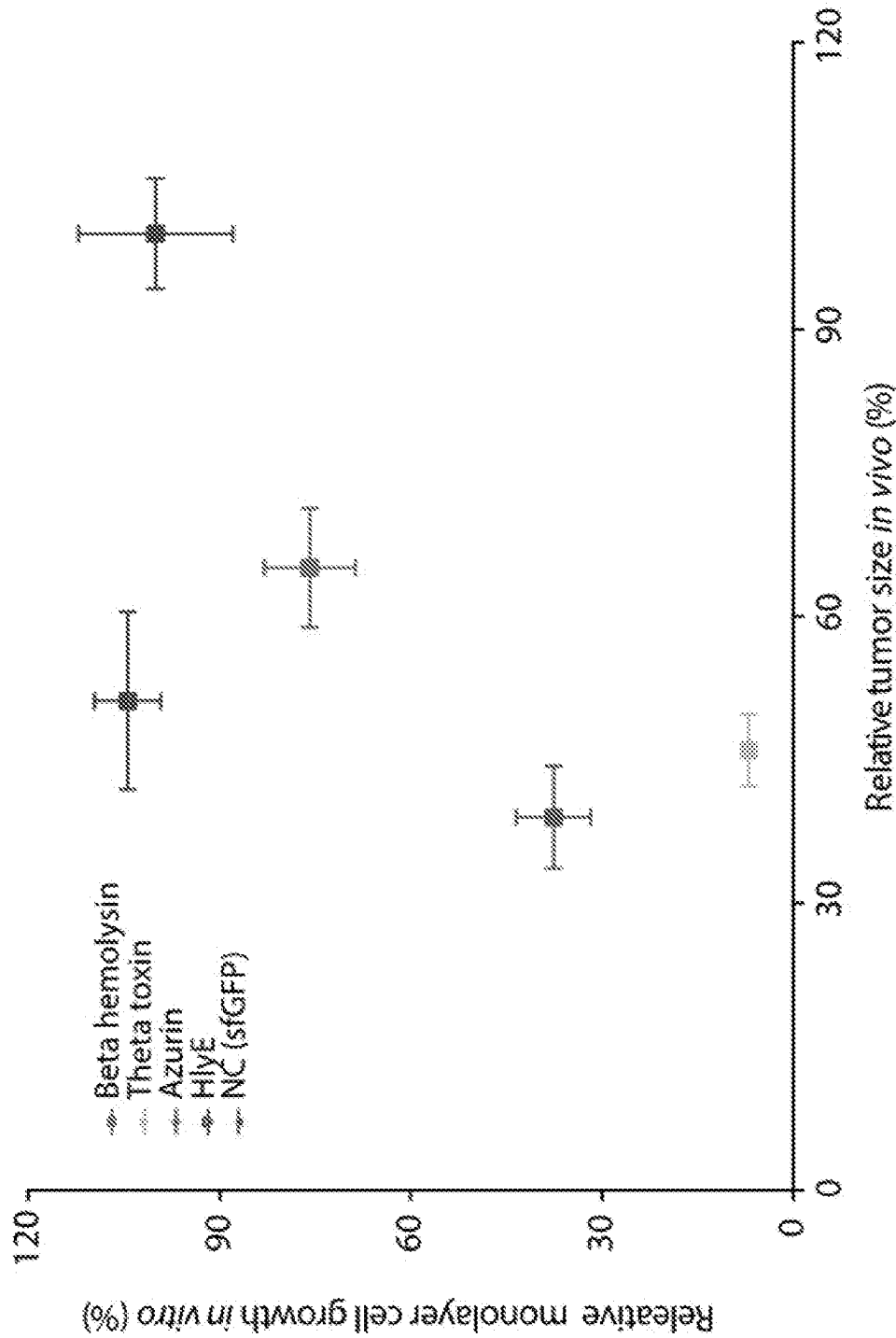


Fig. 7

Fig. 7A

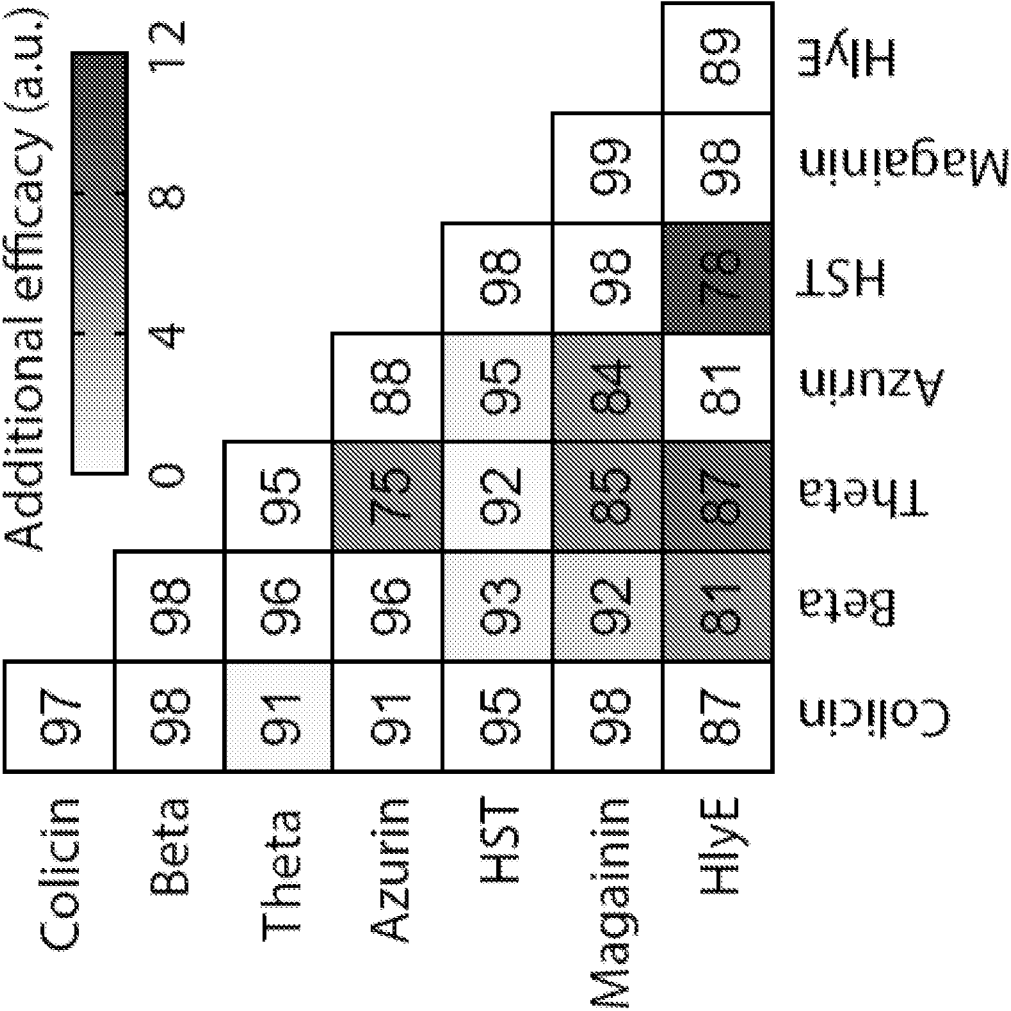


Fig. 7C

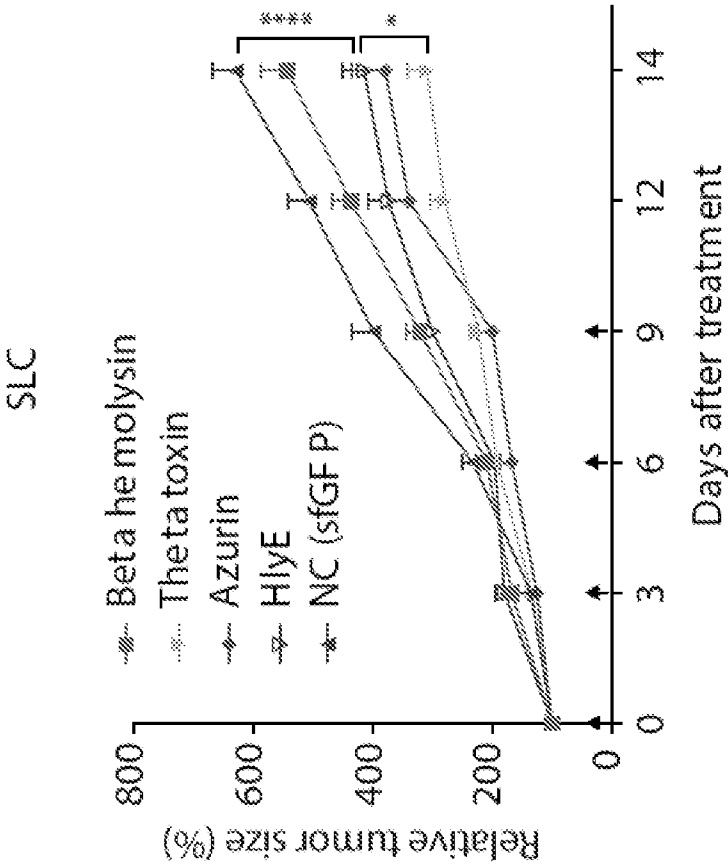


Fig. 7B

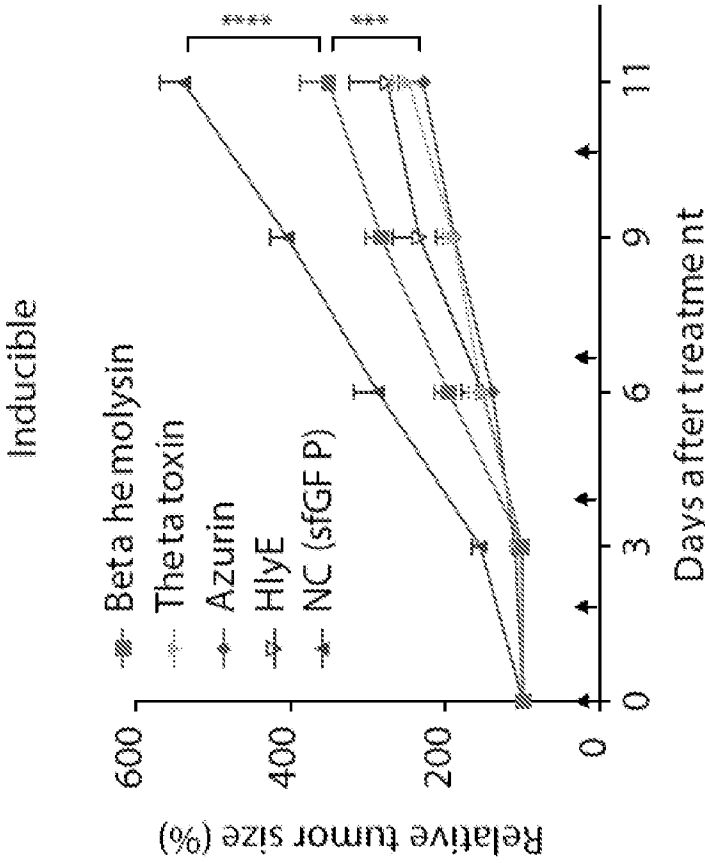


Fig. 7E

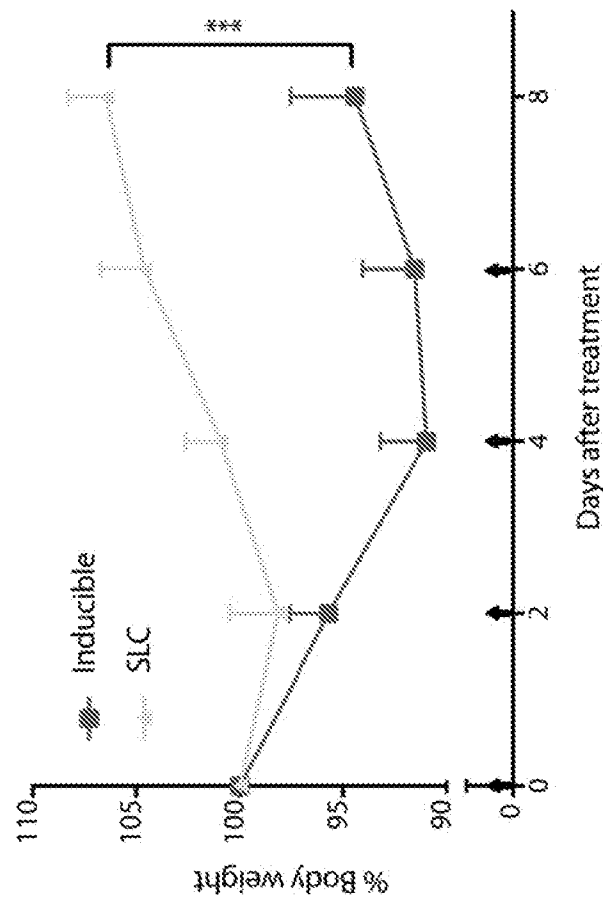
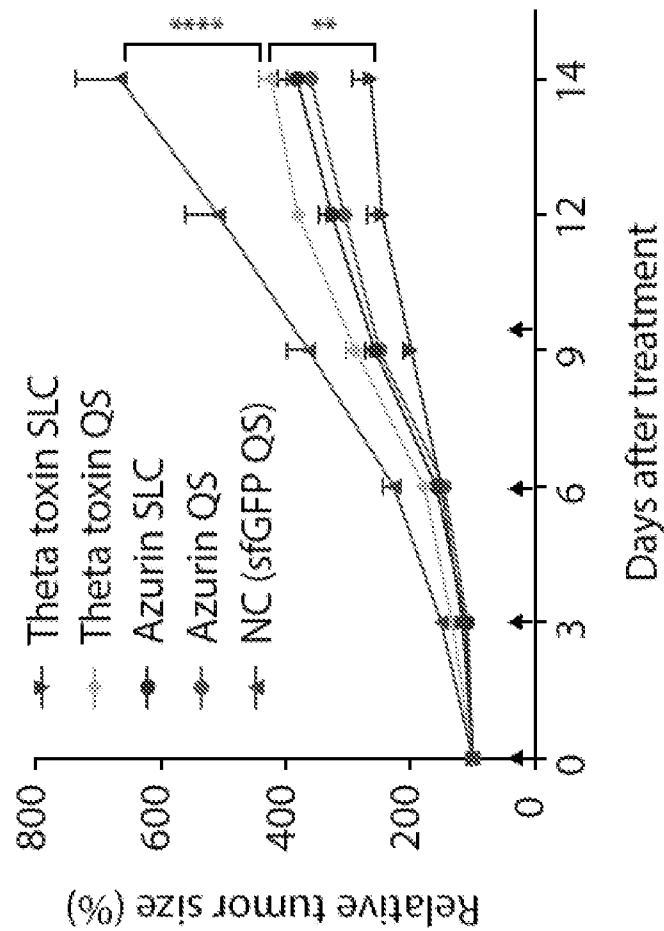


Fig. 7D

SLC and QS



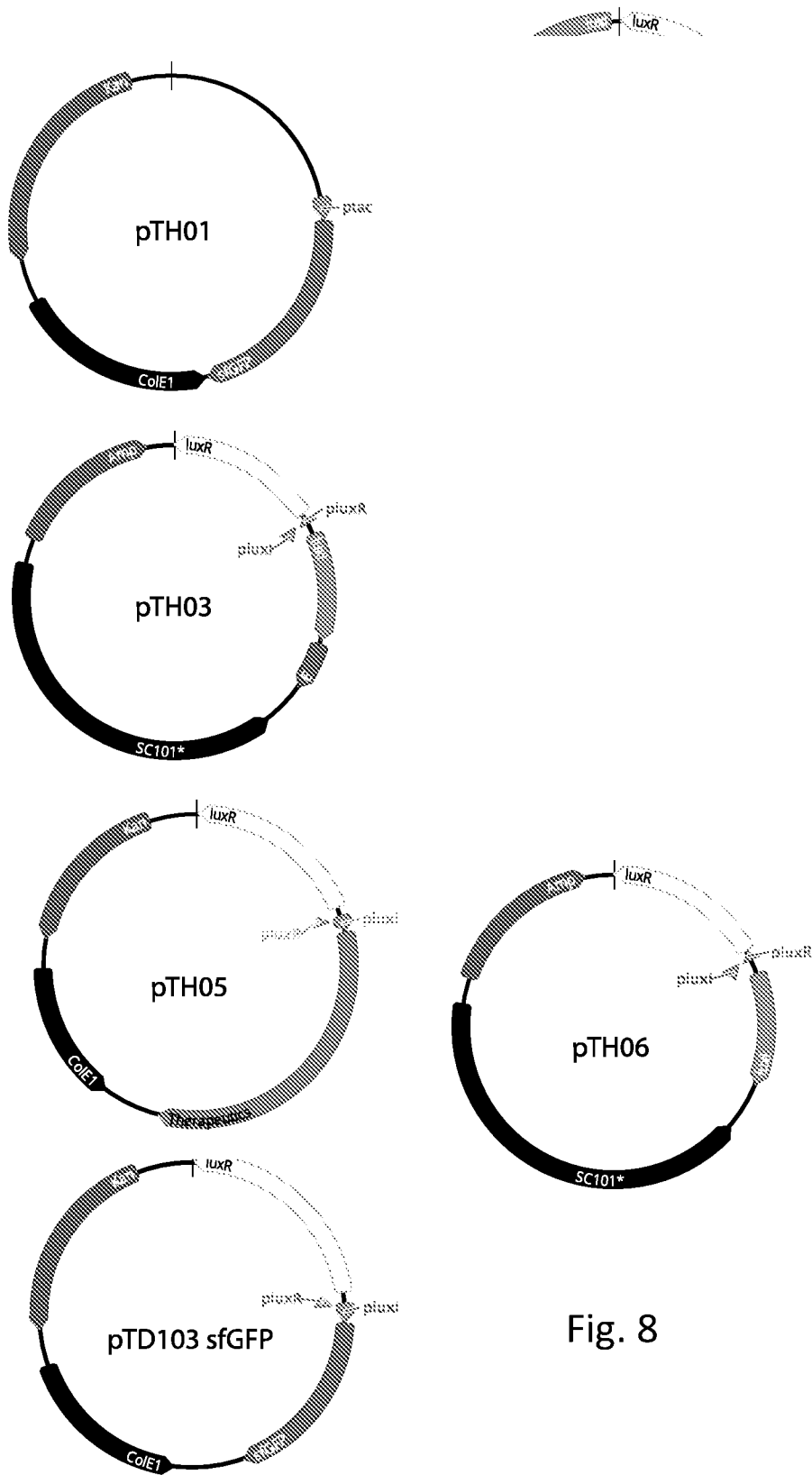


Fig. 8

Fig. 9

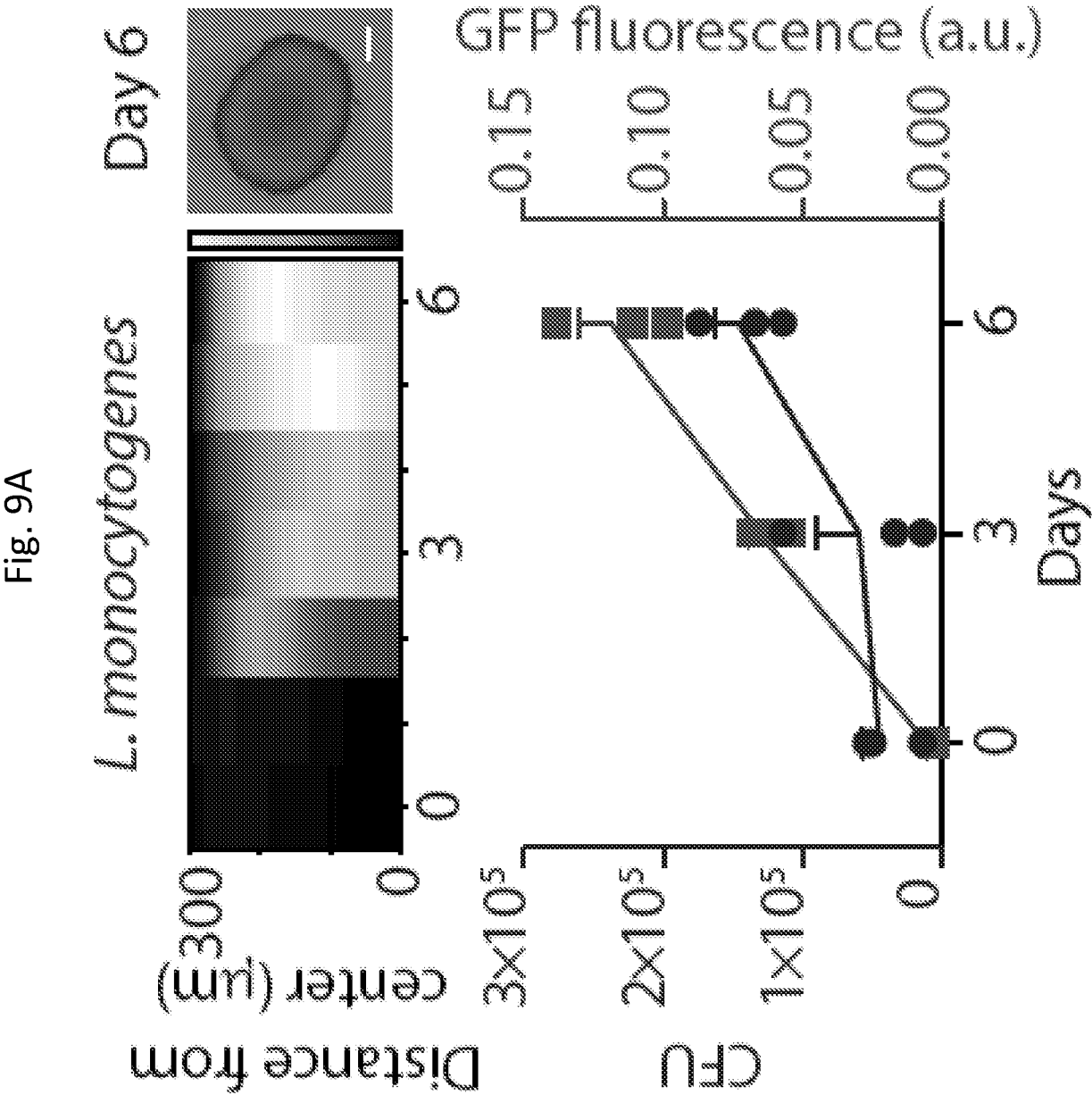


Fig. 9B

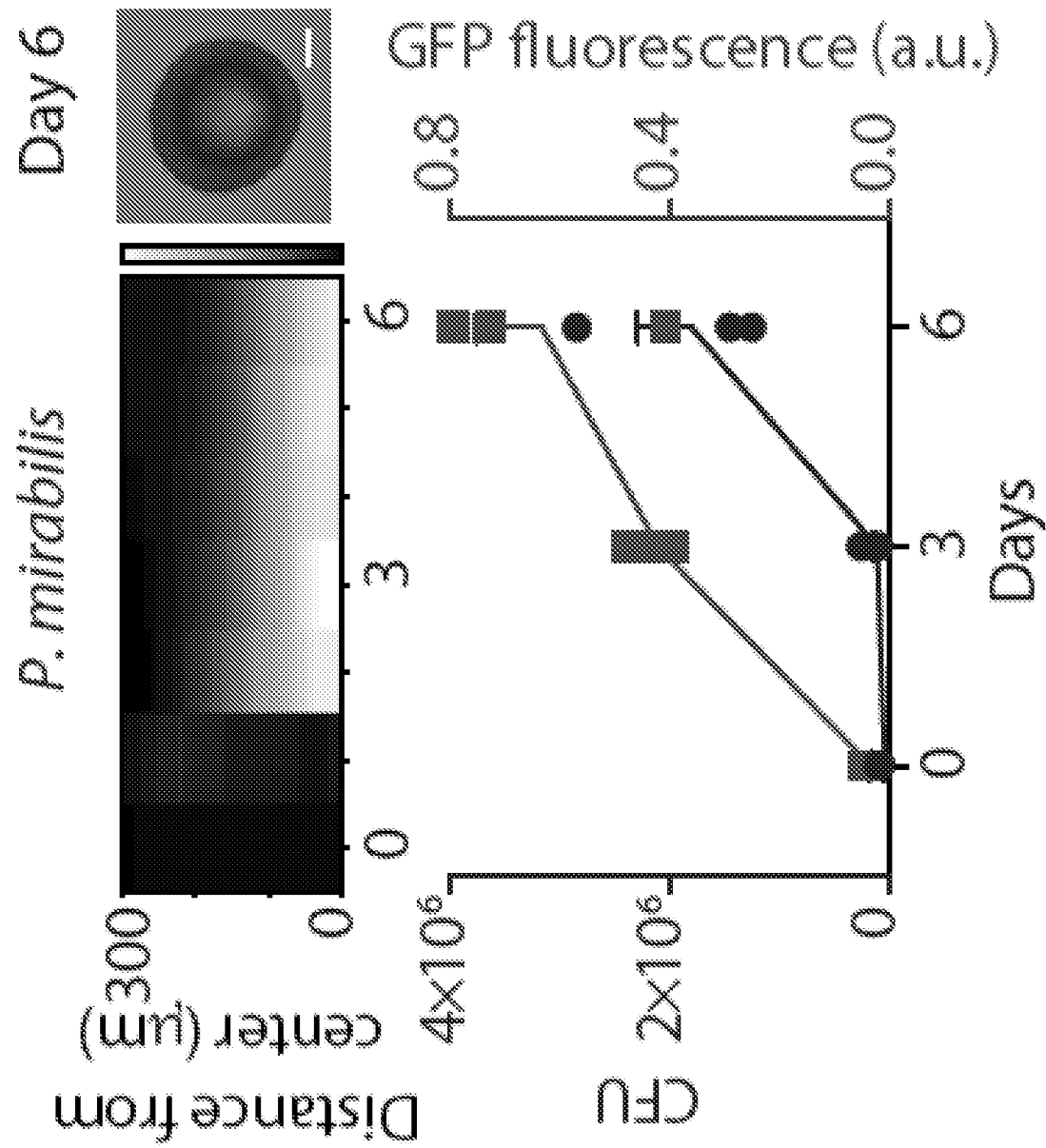


Fig. 9C

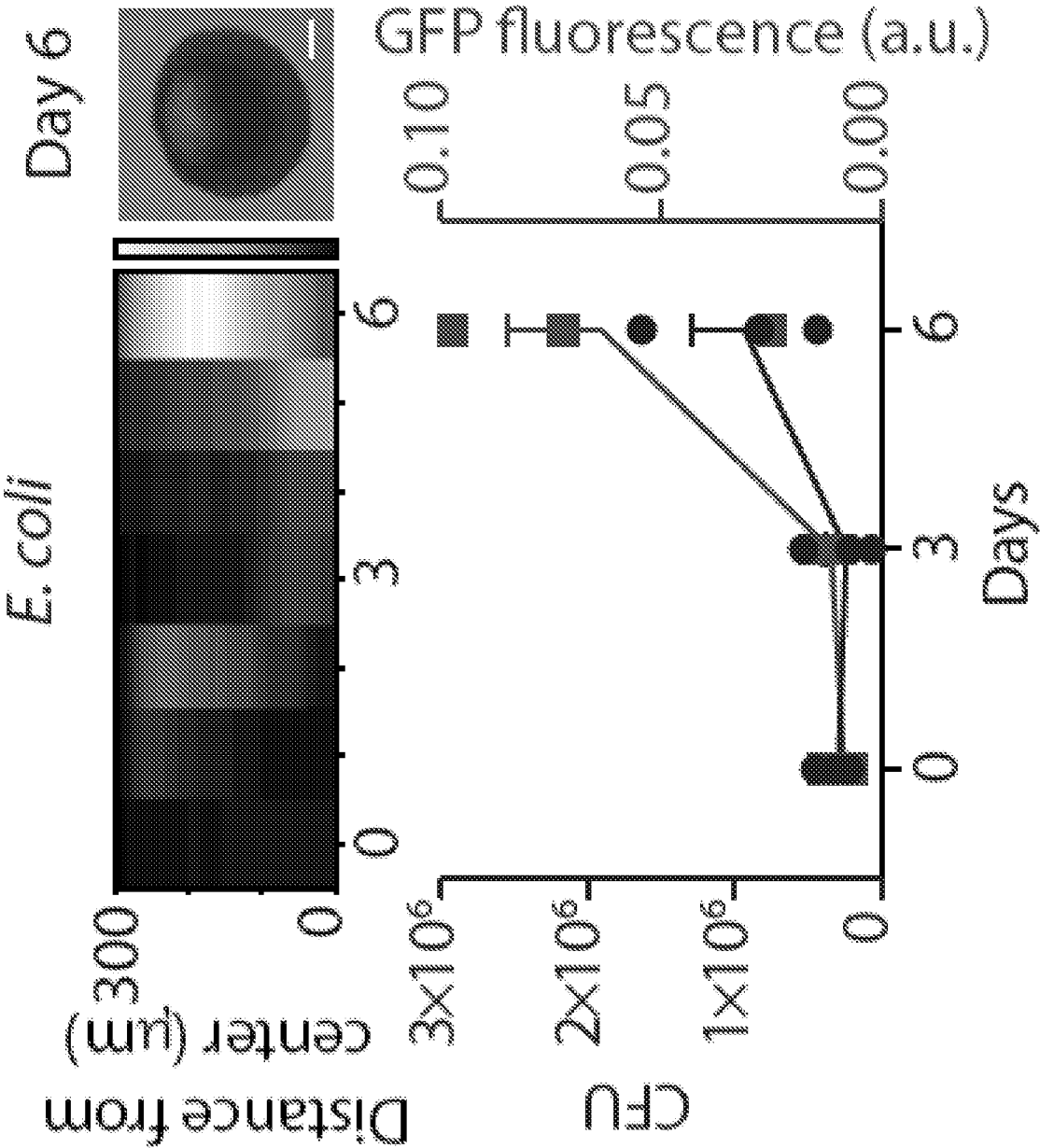
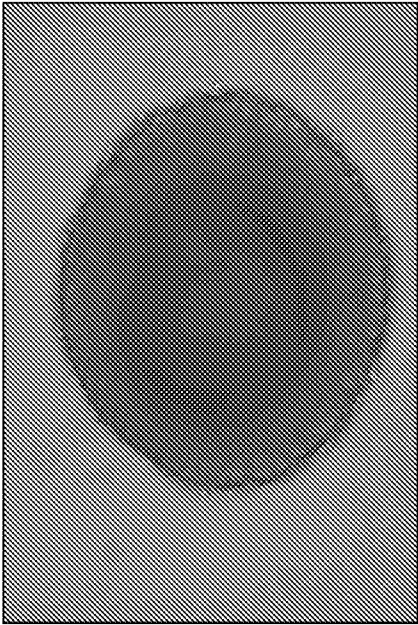
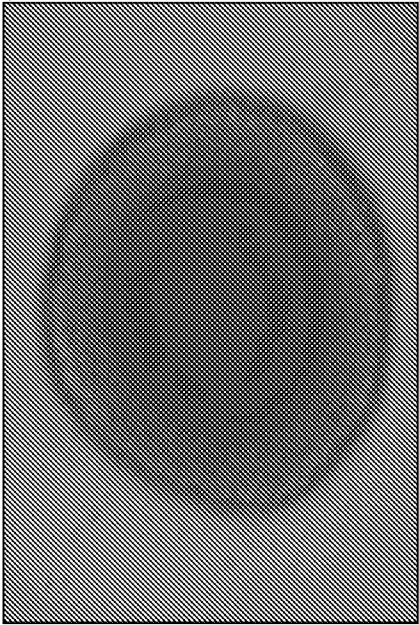


Fig. 9D

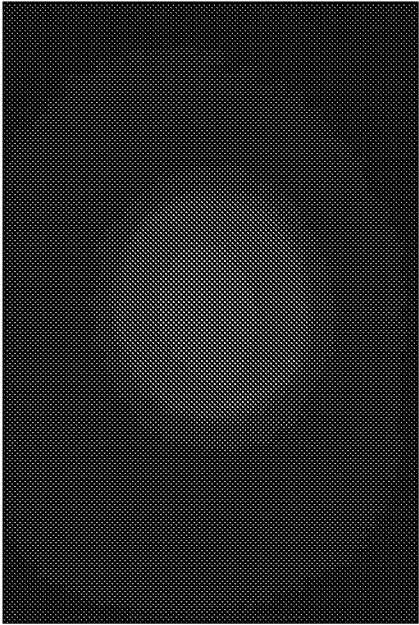
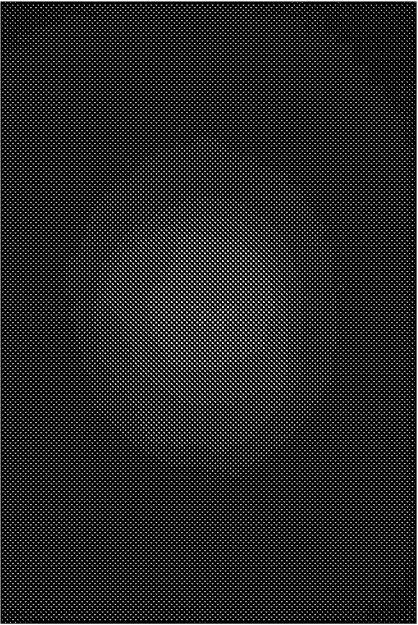
Day 9



1 mg/ml
Gentamicin



2 mg/ml
Gentamicin



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US19/53996

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 5/09, 5/0783; C12M 1/34; G01N 33/574; C07K 14/705 (2020.01)

CPC - C12N 5/0693, 5/0671, 5/0637, 5/0638; C12M 1/34; G01N 33/574; C07K 14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2017/201126 A1 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 23 November 2017; paragraphs [0038], [0041], [0046], [0048]	1-5
X	WO 2017/075389 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 04 May 2017; abstract; paragraphs [0005], [0024], [0049], [0065], [0068], [0095], [0152], [0173], [0210], [0236], [0238]-[0239], [0245], [0328]; claims 1, 47, 113	1, 6-14, 15/1, 15/11, 16/15/1, 16/15/11, 18/15/1, 18/15/11, 19-23
Y	(SHERMAN, H et al.) A Novel Three-Dimensional Immune Oncology Model for High-Throughput Testing of Tumoricidal Activity. <i>Frontiers in Immunology</i> . 23 April 2018; Vol. 9, No. 857; pages 1-6; figures 3A-B, 5; page 4, column 2, paragraph 2 – page 5, column 1, paragraph 1; DOI: 10.3389/fimmu.2018.00857	17/16/15/1, 17/16/15/11
Y	US 2017/0198261 A1 (RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY) 13 July 2017; paragraph [0016]	15/1, 15/11, 16/15/1, 16/15/11, 18/15/1, 18/15/11
Y		17/16/15/1, 17/16/15/11



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"D" document cited by the applicant in the international application	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"E" earlier application or patent but published on or after the international filing date	"&" document member of the same patent family
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 25 April 2020 (25.04.2020)	Date of mailing of the international search report 13 MAY 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300