UNIVERSITY OF CALIFORNIA SAN DIEGO

Development of an AND-gated ultrasound-controllable gene circuit for use in immunotherapy

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by

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The Thesis of Alexa Emilie Lewis is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

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Material in this thesis is co-authored with Dr. Chi Woo Yoon. The thesis author was the primary author of this material.

ABSTRACT OF THE THESIS

Development of an AND-gated ultrasound-controllable
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Master of Science in Bioengineering

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Professor Yingxiao Wang, Chair

In recent years, chimeric antigen receptor (CAR) T-cell therapy has become increasingly important in the field of cancer immunotherapy. However, many challenges continue to face CAR T-cell therapy, including on-target off-tumor toxicity, and proposed improvements still exhibit poor spatial control. To address this issue, efforts have been focused on engineering gene circuits that could be implemented either in T cells or in cancer cells to provide more control over which cells are being targeted. One proposed method for

controlling these gene circuits is focused ultrasound (FUS). FUS has been proven to be a safe means of delivering energy into tissue and may offer a way to exert high spatiotemporal control over cell function. In this project, we develop an AND-gated gene circuit under the control of both ultrasound (which induces a calcium response) and a small molecule drug. By testing our circuit using luminescent and fluorescent reporter genes, we show that our system effectively limits background activation in HEK cells, while maintaining high fold-changes between treated and untreated groups. Upon further optimization, this system could be activated by either ultrasound-mediated mechanical perturbation or heat production. These results demonstrate our system's ability to exert tight spatiotemporal control over reporter gene activation. In practice, use of this gene circuit could result in tumor cells presenting specific unique surface antigens, thereby becoming vulnerable to CAR T-cell attack, or T cells expressing particular CAR receptors, becoming more targeted and effective attackers.

1. Introduction

The proposed system incorporates several different concepts and components, each of which will be described below.

1.1 Chimeric antigen receptor T-cell therapy

Although chimeric antigen receptor (CAR) T cells were first produced in the 1980s, the technique only became widely accepted after about 20 years, and just in the past decade has much focus been concentrated on improving the therapy.[1] The inclusion of CARs is essential to increase the effectiveness of adoptive T-cell therapy, a form of personalized medicine involving the transfusion of lymphocytes for the purpose of enhancing antitumor immunity, augmenting vaccine affinity, or limiting graft-versus-host disease.[2] Early forms of adoptive T-cell therapy, developed by Rosenberg *et al*., involved harvesting tumor-infiltrating lymphocytes (TILs), expanding the cells *ex vivo*, and infusing the cells back into the patient.[3] In about 20% of patients with metastatic melanoma, this therapy resulted in complete responses, thus revealing the potential of T-cell therapies.[3] To adapt this therapy more specifically to fighting tumors, attention was turned to the T-cell receptors (TCRs) that are naturally expressed on TILs and recognize antigens exhibited by tumor cells.[3] A CAR is created by fusing the heavy and light chain variable regions of a monoclonal antibody to the constant regions of a TCR and adding costimulatory domains.[3] This process creates a simplified receptor that recognizes tumor antigens and acts with increased potency, persistence, and specificity to tumor cells.[2,3] Once the CAR T cells bind to the tumor antigens, the cells initiate antitumor cytokine production and target tumor cell killing.[4] Furthermore, noninvasive gene activation can be used to produce a precise and controlled response.[4]

Although the use of CAR T cells has shown great promise in promoting anti-tumor activity, certain complications also arise. One of the greatest risks of T-cell therapy is on-target, off-tumor toxicity, in which the CAR T cells bind to healthy cells expressing the same antigens as the tumor. This complication can be lethal when the antigens are expressed in the heart, liver, or lungs.[4] Cytokine release syndrome (or a cytokine storm) is another common side effect, in which CARs stimulate such a strong cytokine release that the immune response is elevated to fatal levels.[5] Additional obstacles include tumor lysis syndrome; variations in response, persistence, and side effects; disruption of immune system regulatory checks; heterogeneity of tumor antigen expression; and the emergence of tumors with the loss or downregulation of the target antigen (unlike TCRs, CARs require high levels of antigen expression for effective recognition and targeting).[3-5] In solid tumors, in particular, antigens are more likely to be expressed heterogeneously and at lower levels, making CAR T-cell therapy difficult.[3] Thus, this technique has shown more success in hematologic malignancies, where antigens are expressed at higher levels.[3] Focusing in particular on increasing the specificity of CAR T cells, a number of solutions have been proposed.

Methods that have been introduced to increase the specificity and controllability of CAR T cells include suicide or protein-based switches; split CARs, which assemble *in vivo* in the presence of a small molecule drug; synthetic receptors; inducible/conditional gene expression; and targeting of multiple antigens.[3-6] Unfortunately, drawbacks of these solutions include permanent down-regulation (instead of reversible control), confined tissue specificity, limited temporal resolution, “leaky” activation, low control kinetics, and toxic side effects.[4,6,7] Optogenetics, the method of using light to regulate gene expression, has been shown to be particularly effective in controlling specific molecular events.[7] Previous studies have used

light to control calcium channels, thus modulating calcium-specific biological events, and to induce nuclear translocation and dimerization for the purpose of gene regulation related to CAR T-cell activation.[4,8] However, although light has good spatiotemporal resolution, the penetration depth is limited and inadequate for deep tissue stimulation.[7,8]

1.2 Controllable gene circuits

One proposed method for exerting tighter control over CAR T-cell therapy is controllable gene circuits, which are becoming an increasingly prevalent method for modulating the spatiotemporal pattern of gene expression.[9] In order for the system to be useful, expression must be regulated in a predictable and effective manner.[10] Two of the main considerations for this method are whether reversible activation is possible and whether regulation is tight enough to allow clear distinction between activated and background expression levels.[9,11] If these two conditions are met, it is possible to precisely control gene expression both spatially and temporally. Of particular interest are gene circuits under the control of a calcium-sensitive transcription factor: nuclear factor of activated T cells (NFAT). When NFAT-response elements (NFAT-REs) are inserted upstream of a minimum promoter, calcium-induced activity of NFAT can drive activation of a reporter gene. A system such as this puts gene transcription under the control of a regulatable factor, in this case calcium concentration. However, the ubiquity of calcium in the cell environment and a certain level of constitutive NFAT nuclear localization introduce non-negligible levels of background activation in systems utilizing calcium-inducible circuits.[12] In order to combat this leakage, AND-gate circuits can be designed in which transcription is under the control of two independent inputs.

A possible use of controllable gene circuits is in gene transfer therapy. One version of this method involves delivering synthetic gene circuits to the cancer cells themselves *in vivo* using viral vectors.[13,14] This therapy could act as a stand-alone intervention or in combination with CAR T-cell therapy or other treatment methods. In practice, transfer of a particular gene circuit could result in tumor cells presenting specific unique surface antigens, thereby becoming vulnerable to CAR T-cell attack. Alternatively, gene circuits could be inserted into T cells, to allow them to express particular CAR receptors.

1.3 Calcium and NFAT

Calcium is an important signaling molecule involved in regulating many cellular functions, and modulating calcium dynamics can lead to controlled gene transcription and expression.[8,15] NFAT is one transcription factor that shows dependence on calcium levels and is of particular interest due to its role in the development and function of the immune system.[16] For example, NFAT proteins contribute to the regulation of T-cell activation; affect the transcription of the genes encoding cytokines, chemokines, and cell-surface receptors; and are involved in controlling thymocyte development, T-cell differentiation, and self-tolerance.[16,17] NFAT is not only found in T cells, however, but also has regulatory roles in the CNS, blood vessels, heart, kidney, bone, skeletal muscles, and hematopoietic stem cells.[16,18] Of the five NFAT family members, four are under the control of calcium, three are expressed in T cells, and all have highly conserved DNA-binding domains.[16] In resting T cells, the many serine residues in the regulatory domain of NFAT are phosphorylated, and the transcription factor is inhibited.[16,19] This phosphorylation level is maintained or altered by the activity of NFAT kinases and calcineurin, both of which have docking sites on NFAT.[16,19] Engagement of receptors coupled to the calcium-signaling pathway leads to the release of calcium from

intracellular stores, which in turn causes an influx of extracellular calcium, so that high levels of internal calcium are maintained (T-cell activation depends on sustained calcium signaling).[16,17] Calcium molecules inside the cell bind calmodulin, which activates the phosphatase calcineurin, which in turn binds to NFAT and dephosphorylates the serine residues, exposing the nuclear localization signal.[16,18] The activated NFAT translocates into the nucleus and induces NFAT-mediated gene transcription.[16,19] NFAT may also interact with activator proteins, such as AP1, which results in somewhat altered gene expression patterns.[16,19] Due to its importance in transcriptional control and its dependence on calcineurin (which is quickly and reversibly activated or deactivated), NFAT is a promising activator for controllable gene circuits.[17]

1.4 Focused Ultrasound

One of the most effective ways to tightly regulate a calcium response in cells is with an external modulator, such as focused ultrasound (FUS). FUS has been proposed as a means to safely and noninvasively deliver energy into deep tissues.[7] In contrast to light, which can only penetrate on the order of hundreds of micrometers from the surface of the skin, FUS can penetrate on the order of centimeters, allowing control of cells that light cannot reach.[7] FUS also has fine spatiotemporal resolution, allowing specific activation in the tumor area and mitigation of off-tumor effects.[7,11] FUS can be used as either a mechanical trigger or as a means to generate heat (as the oscillating pressure of ultrasound [US] results in cycles of mechanical loading and unloading).[7,11] In previous studies, US has been used to ablate tumors; control drug delivery, vasodilation, neuromodulation, and transgene expression; and activate transcription factors and gene circuits.[7] Applications activated by the mechanical trigger rely on the presence of naturally occurring mechanosensitive ion channels, which are stimulated

by the mechanical forces of the acoustic pressure generated by US.[7,20] Although in the past, microbubbles have been coupled to the surface of the cell to enhance the effects of FUS stimulation on mechanically sensitive ion channels, our ability to utilize this system *in vivo* is limited, due to the large size (which prohibits penetration through endothelial cells) and short circulation time of microbubbles.[7,21-23] Fortunately, there exists a great diversity of mechanosensitive proteins to explore, and previous work has shown the feasibility of using FUS to generate a calcium response in multiple cell types.[24,25] Similarly, thermosensitive ion channels can also be activated by FUS stimulation.[26,27] For example, the transient receptor potential channel TRPV1 transitions from closed to open in response to heat.[27] The development of US-sensitive gene circuits that take advantage of the functioning of these mechano- or thermo- sensitive proteins could provide a method for precise spatiotemporal control of cellular activities.

1.5 Tetracycline-inducible systems

Another tool which can be incorporated into controllable gene circuits is tetracycline-controlled expression.[28] Tetracycline-inducible systems have been shown to result in stringent, reversible, quantitative, spatiotemporal transcriptional regulation; however, the original Tet-on and Tet-off systems (in which the tetracycline repressor [TetR] is fused to a transcriptional modifier protein) demonstrate an inherent leakiness and are most effective when paired with additional regulation.[9,10] In the Tet repressor system, on the other hand, with proper positioning of the tetracycline operator (tetO) near the TATA box, gene expression is regulated by TetR alone in response to tetracycline.[10] In the absence of tetracycline, TetR binds to tetO and acts as a potent repressor by sterically interfering with the interaction between the TATA element and TATA-binding proteins (TBP).[10,29] However, when present,

tetracycline binds to TetR with high affinity, preventing TetR from binding to tetO and allowing uninhibited binding of TBPs and gene transcription.[10,29] Further work has shown that TetR can also be fused to the KRAB repressor domain of the human Kox1 zinc finger protein to create an even more potent and longer-lasting repressor.[30] Therefore, particularly when coupled with an additional regulation system, tetracycline-inducible systems allow for effective temporal control of gene expression according to the levels of tetracycline in the cellular environment. Notably, most of the work done in assessing the performance of the Tet repressor system has been tested on constitutive reporters.[10] Our proposed system instead pairs the Tet repressor with an inducible promoter, to provide AND-gated control.

1.6 System Design

This project focuses on the development of a gene circuit that utilizes the strategy of an AND logic gate, to minimize background activation and exert high spatial and temporal control. In this case, the circuit is under the control of both ultrasound (which triggers calcium release through thermal or mechanical pathway activation) and the small molecule drug doxycycline, thus providing two levels of control. In practice, use of this gene circuit could result in tumor cells presenting specific unique surface antigens, thereby becoming vulnerable to CAR T-cell attack, or T cells expressing particular CAR receptors, becoming more targeted and effective attackers.

2. Materials and Methods

2.1 Cell Culture

HEK, HEK293T, and PC-3 cells were provided by Dr. Chi Woo Yoon (UCSD Bioengineering). All HEK cell lines were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher Scientific, Waltham, MA) supplemented with 10% (v/v) fetal bovine serum (FBS) (ThermoFisher Scientific, Waltham, MA) and 100 U/mL PenicillinStreptomycin (PenStrep) (ThermoFisher Scientific, Waltham, MA). PC-3 cell lines were cultured in either high glucose DMEM supplemented with 10% (v/v) FBS and 100 U/mL PenStrep or Roswell Park Memorial Institute (RPMI) medium (ThermoFisher Scientific, Waltham, MA) supplemented with 10% (v/v) FBS and 100 U/mL PenStrep. All cells were maintained at 37 °C and 5% CO_2 in a humidified environment. Cell lines expressing blasticidin (BSD) resistance genes were cultured in medium containing 10 mg/mL BSD (Invivogen, Inc., San Diego, CA) during selection and 5 mg/mL BSD after selection.

2.2 Plasmid Construction

For each construct, the first step in plasmid construction was Polymerase Chain Reaction (PCR). Primers were designed to utilize existing template plasmids in the lab. The only template plasmids from outside sources were the pNL3.2[*NlucP*/9XIL8 NFAT-RE/minP] construct, which was generously gifted by the Shibata Lab (Nagoya University), and the WT NFAT1 plasmid gifted by Dr. Anjana Rao (Addgene #11100). PCR was performed using Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. PCR products were verified by gel electrophoresis and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). If required, PCR products were digested overnight with restriction enzymes and

CutSmart® Buffer (New England Biolabs, Ipswich, MA). Construct components were then assembled using either T4 DNA Ligase (New England Biolabs, Ipswich, MA) or Gibson Assembly® (New England Biolabs, Ipswich, MA), according to the manufacturer's instructions.

2.3 Transient Transfection

Transient transfection was performed using the Lipofectamine™ 3000 Transfection Kit (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. The day before transfection, HEK or PC-3 cells were seeded in 12-well or 24-well plates (0.4-0.5 x 10^6 cells/well or 0.2-0.3 x 10^6 cells/well, respectively). About six hours after transfection, cells were passed into a 24-well or 96-well plate or fibronectin-coated glass-bottom dishes. The next day, cells were treated and assayed as described below.

2.4 Virus and Cell Line Production

Cell lines containing the designed circuits were created using the ProFection® Mammalian Transfection System (Promega, Madison, WI). Three million Lenti-X cells (provided by Dr. Chi Woo Yoon, UCSD Bioengineering) were seeded in a 10 cm cell culture dish containing 10 mL DMEM supplemented with 10% FBS (v/v) and 100 U/mL PenStrep the day before transfection. Transfection was performed according to the manufacturer's instructions, using 10 μg of the plasmid of interest, 5 μg VSVG (envelope vector), and 5 μg ΔR (packaging vector). Two days after transfection, the supernatant was filtered through a 0.45 μm filter, transferred to a 15 mL centrifuge tube, combined with 2.5 mL cold PEG-it Virus Precipitation Solution (System Biosciences, Palo Alto, CA), and refrigerated. The next day, the supernatant/PEG-it solution was centrifuged at 1500 x *g* for 30 minutes at 4 °C. After

centrifugation, the supernatant was aspirated, and the virus pellet was resuspended in 40-100 μL PBS.

The day before infection, HEK or PC-3 cell lines were seeded in 12-well plates (0.1 x 10^6 cells/well). The day of infection, 1-15 μL of virus was added to each well. After two days, cells were passed into 6-well plates. Cells expressing BSD resistance genes were cultured in medium containing 10 mg/mL BSD during selection. From that point forward, cell lines were considered stable, and cells were cultured as usual.

2.5 Luciferase Assay

For testing purposes ionomycin (VWR International, Radnor, PA) or ATP (New England Biolabs, Ipswich, MA) were used as calcium-inducing agents. HEK or PC-3 cell lines or transiently transfected cells were seeded the day before each assay in 96-well plates (0.04-0.05 x 10^6 HEK cells/well, 0.02-0.03 x 10^6 PC-3 cells/well) in 100 μL medium without BSD. About twenty-four hours after seeding, 50 μL medium were added to each well: blank medium, medium + 3X ionomycin/ATP (final concentrations of 1 μg/mL and 30 μM, respectively), medium + 3X doxycycline (MiliporeSigma, St. Louis, MO) (final concentration 333.33 nM), medium + 3X ionomycin/ATP + 3X doxycycline. After one hour, the medium was aspirated and replaced with 100 μL blank medium. After nine hours, the Nano-Glo® Luciferase Assay System (Promega, Madison, WI) was used according to the manufacturer's instructions. Luminescence was measured using the Tecan Infinite M1000 Pro plate reader (ThermoFisher Scientific, Waltham, MA).

2.6 Flow Cytometry Measurements

HEK or PC-3 cell lines or transiently transfected cells were seeded the day before each measurement into 24-well plates (0.1-0.2 x 10^6 cells/well) in 500 μL medium without BSD.

About twenty-four hours after seeding, 250 µL medium were added to each well: blank medium, medium + 3X ionomycin/ATP (final concentrations of 1 µg/mL and 30 µM, respectively), medium + 3X doxycycline (final concentration 333.33 nM), medium + 3X ionomycin/ATP + 3X doxycycline. After one hour, the medium was aspirated and replaced with 500 µL blank medium. After nine hours, the medium was aspirated, the cells were harvested with TrypLE Express Enzyme (ThermoFisher Scientific, Waltham, MA), spun down, washed with cold phosphate buffer saline (PBS) (Sigma-Aldrich, St. Louis, MO), and resuspended in 500 µL PBS. Fluorescent signal was measured using a BD Accuri™ C6 Flow Cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ).

2.7 Microscopy

To visualize NFAT translocation in cells expressing TRPV1 channels, we transiently transfected cells with exogenous NFAT fused to an EGFP reporter. Cells seeded in a glass-bottom dish were heated using a heating stage (Instec) integrated with a Nikon Eclipse Ti inverted microscope. In this way, the temperature could be adjusted, and the effects could be visualized and imaged in real-time.

2.8 Thermal Activation Measurements

Cells expressing the TRPV1 channel (either stably or transiently) were subjected to heat to assess their activation potential. Cells were grown in a 60 mm cell culture dish to confluency. On the day of the experiment, 0.5 x 10^6 cells/treatment group in 40 µL medium were passed to PCR tubes. Ten µL blank medium or medium containing doxycycline and/or ATP were added to each tube. The samples were run in a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA) at 35 °C for 30 seconds, a higher temperature (40-42 °C) for 1-5 minutes, and returned to 35 °C. The samples were then placed in the cell incubator for 30 minutes

before being transferred to microcentrifuge tubes (with additional medium), centrifuged, resuspended in fresh medium, and plated in a 96-well plate (0.04-0.05 x 10^6 cells/well). After nine hours, the luciferase assay was run.

2.9 Naming Conventions

The construct gifted by the Shibata Lab and used as a non-AND-gated comparison, originally called pNL3.2[*NlucP*/9XIL8 NFAT-RE/minP], will hereafter be referred to as 9xRE/Nluc. Our constructs will follow similar naming conventions, with components of a single construct separated by forward slashes. In cell lines stably expressing a construct or multiple constructs, the cell type and constructs will be separated with hyphens, e.g., HEK-TetR. In the case of transient transfection, the cell type or cell line will be separated from transfected constructs by a plus sign, e.g., HEK-TetR-9xRE/2xtetO/Nluc + TRPV1.

3. Results

3.1 System design

Although calcium-driven gene circuits have proven effective in inducing gene expression, the importance of calcium as a signaling molecule in myriad natural pathways results in high levels of background activation. To combat these leakages, we developed an AND-gated system under the control of both calcium and the small molecule drug doxycycline (a tetracycline analog). In practice, calcium levels would be elevated by ultrasound, which triggers calcium release through the activation of mechano- or thermo-sensitive pathways. The circuit consists of nine repeats of an NFAT response element (NFAT-RE), an inducible minimum promoter, two tetO sequences, and a reporter gene (Figure 1a). The NFAT-RE was taken from the *IL8* promoter, rather than the *IL2* promoter (which is often used in calcium response reporter systems), since the *IL8* response element is dependent solely on NFAT, rather than two separate transcription factors (Figure 1b).[18] The tetO sequences serve as binding locations for TetR, which, when bound, will sterically inhibit transcription. In the presence of doxycycline, however, TetR will preferentially bind to the drug, thereby allowing transcription to proceed (Figure 1c). We expect to see high levels of reporter gene activation only when the cells are treated with both a calcium-inducing agent (such as ionomycin or ATP) and doxycycline. Untreated cells or cells treated with only one drug will show minimal levels of reporter gene activation. In practice, this system could be delivered to tumor cells themselves, to induce expression of particular surface antigens, or to T cells, to induce expression of particular CAR receptors (Figure 1d).

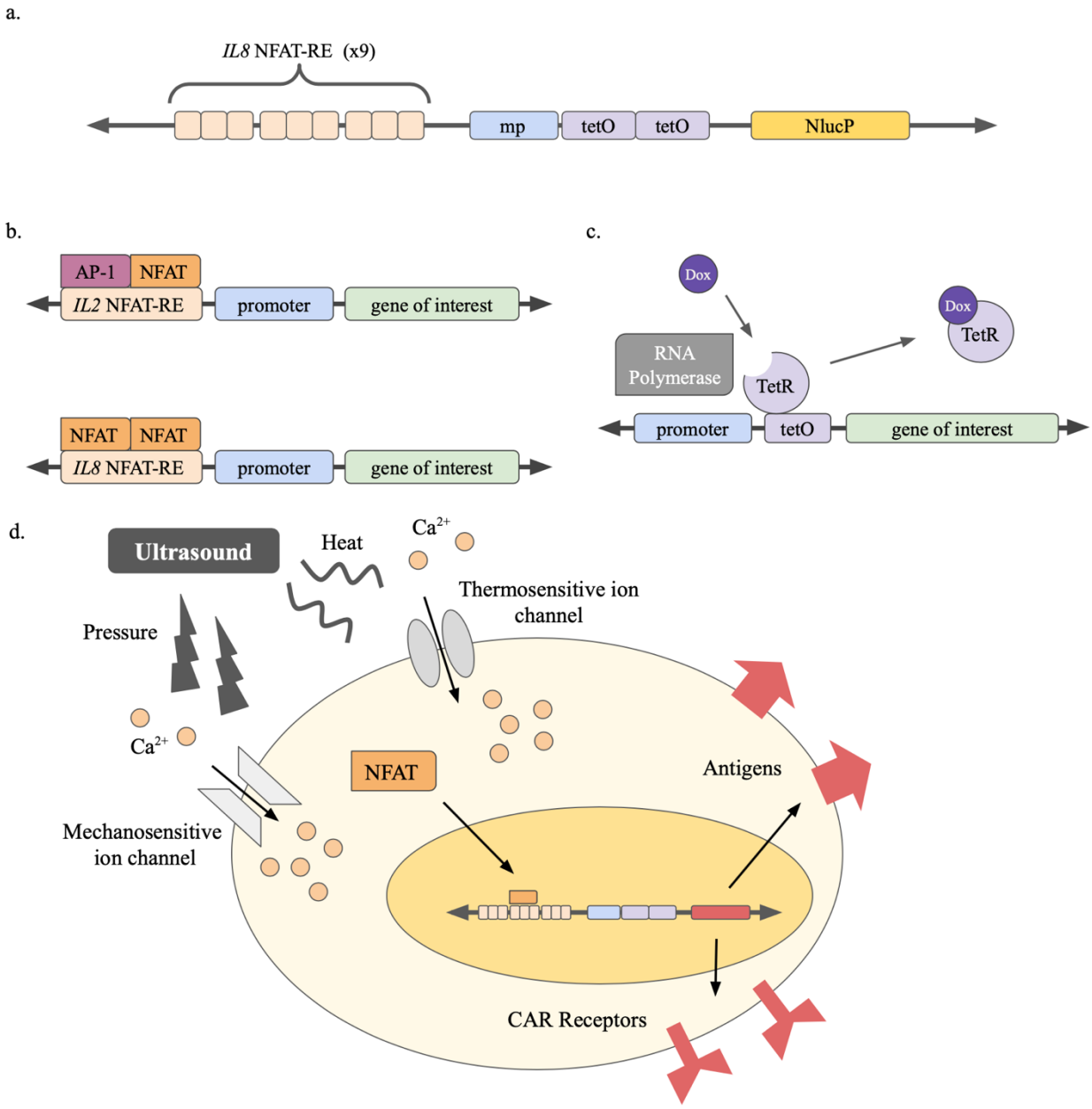


Figure 1: System design. (a) Our system design consists of nine NFAT response elements, a minimum promoter (mp), two tetO sequences, and a nanoluciferase reporter gene (NlucP). The two levels of control conveyed by the NFAT-REs and the tetO sequences prevent the reporter gene from being expressed unless both calcium and doxycycline are present. (b) Although the NFAT response element from *IL2* is often used as a calcium response reporter, our system uses the NFAT response element from *IL8*, since this response element only depends on NFAT, rather than two separate components. (c) In the Tet repressor system, in the absence of doxycycline (Dox), TetR binds to the tetO sequence, sterically inhibiting transcription. In the presence of Dox, TetR preferentially binds to Dox, allowing transcription to proceed. (d) In practice, this system could be used in multiple ways. In certain cancer cells, the system could be delivered directly to the tumor cells themselves and activated by US-mediated mechanical stimulation. In this case, the gene of interest would encode for a specific surface antigen that would make the tumor cells vulnerable to CAR T-cell attack. Alternatively, the system could be incorporated into T cells. US-mediated thermal activation would result in the cell expressing CAR receptors specific to certain tumor antigens. In either case, US stimulation would result in calcium influx into the cell, activating NFAT and inducing expression of the gene of interest.

3.2 US-mediated activation methods

As described, US can activate both mechanosensitive and thermosensitive pathways. Preliminary work for this project, as well as previous work in the lab, has offered two methods by which we can use US to induce our system.

Previous work by Dr. Chi Woo Yoon has shown that PC-3 cells, a prostate cancer cell line, are particularly susceptible to mechanical perturbation. Repeated application of FUS to PC-3 cells results in a calcium response that fluctuates in time with the FUS stimulation (Figure 2a). Expressing our construct in PC-3 cells would allow us to take advantage of this natural mechanosensitivity. FUS stimulation would trigger an influx of calcium into the cell, resulting in the dephosphorylation of NFAT. The dephosphorylated NFAT would enter the nucleus and bind to the NFAT-REs in our construct, thus inducing expression of our gene of interest when doxycycline is present.

An alternative activation method would utilize thermosensitive TRPV1 channels. To test the feasibility of this strategy, we developed a HEK cell line stably expressing TRPV1 and transiently transfected the cells with exogenous NFAT bound to a fluorescent reporter. When we monitored these cells under a microscope rigged with a heating stage, we were able to see translocation of the fluorescently labeled NFAT from the cytoplasm to the nucleus upon the application of heat (Figure 2b). This translocation could be reversed by turning off the heat and re-induced by reapplying the heat. Similar experiments completed previously have shown that this translocation only occurs in cells expressing TRPV1 channels; in cells without TRPV1 channels, no translocation occurs. These results indicate the possibility of using US-mediated heating to trigger a calcium response and induce our system in cells expressing TRPV1.

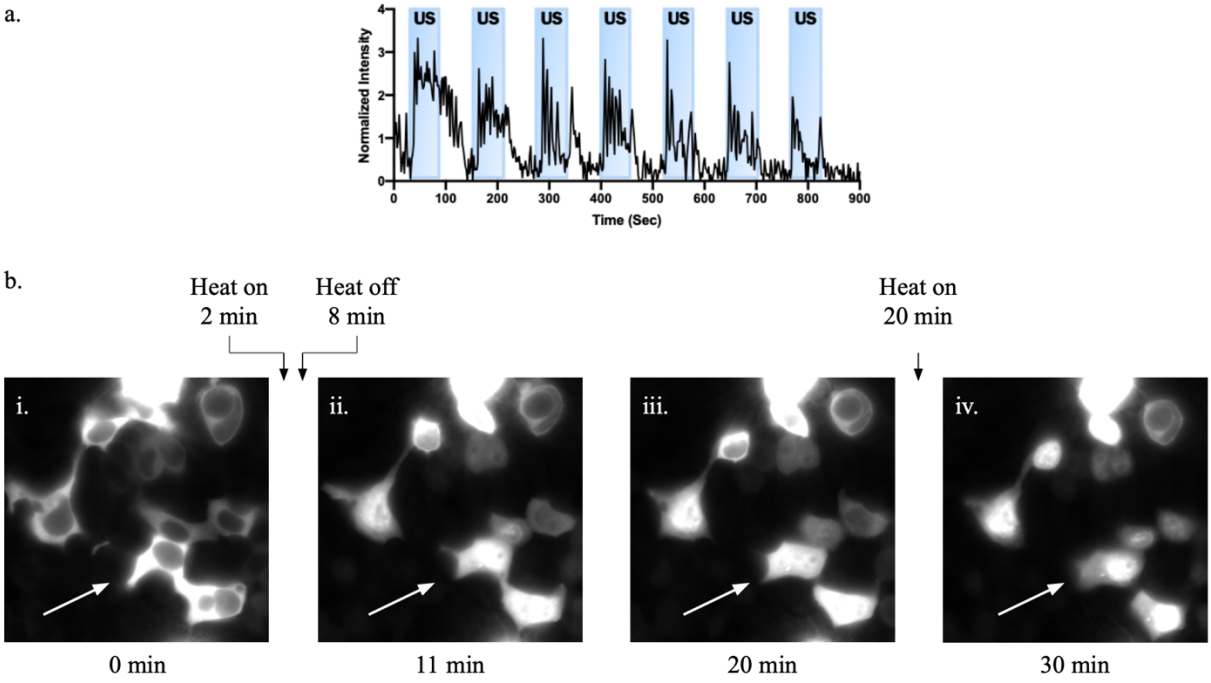


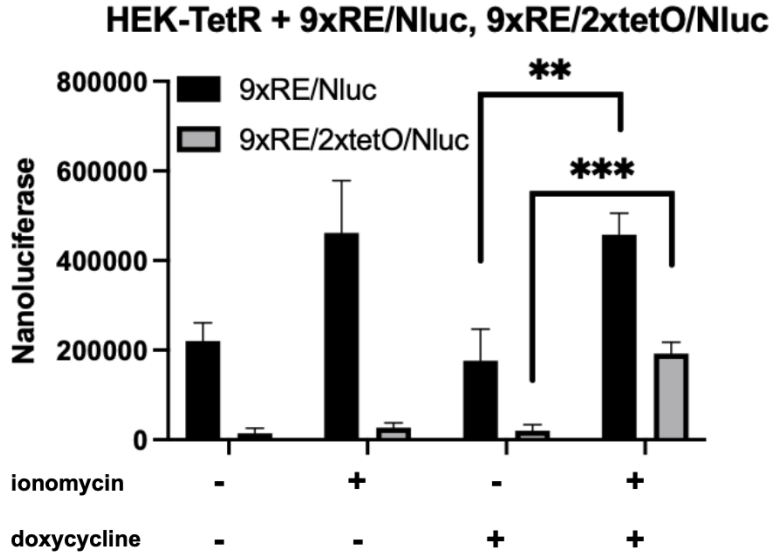
Figure 2: US-mediated activation methods. (a) Previous work has shown that PC-3 cells are particularly susceptible to mechanical perturbation. Repeated application of FUS to PC-3 cells results in a calcium response that fluctuates in time with the FUS stimulation. Fluorescently labeled calcium is measured and normalized F/F0. (b) In HEK cells expressing TRPV1, application of heat induces translocation of fluorescently labeled NFAT from the cytoplasm to the nucleus. (i) Prior to heating, NFAT is localized in the cytoplasm. (ii) Application of heat results in the influx of NFAT into the nucleus. (iii) When heat application is ceased, after an initial lag, NFAT begins to be transported out of the nucleus. (iv) Reapplication of heat resumes the process of nuclear localization.

3.3 Development of an AND-gated system

As discussed, we were interested in developing an AND-gated gene circuit, to reduce background activation and provide increased spatial and temporal control. To assess the efficiency of this AND-gated system, we began with a construct under the control of only a single component: 9xRE/Nluc. This construct contains nine tandem repeats of the *IL8* NFAT-RE and a nanoluciferase reporter under the control of a minimum promoter. Although this construct had been shown to be inducible by calcium-dependent transcription factor activation, we hypothesized that the incorporation of another layer of control would reduce background activation and provide the ability to regulate the system more tightly. For this

reason, a new construct, identical to 9xRE/Nluc except for the addition of two tetO sequences, was developed: 9xRE/2xtetO/Nluc.

a.



b.

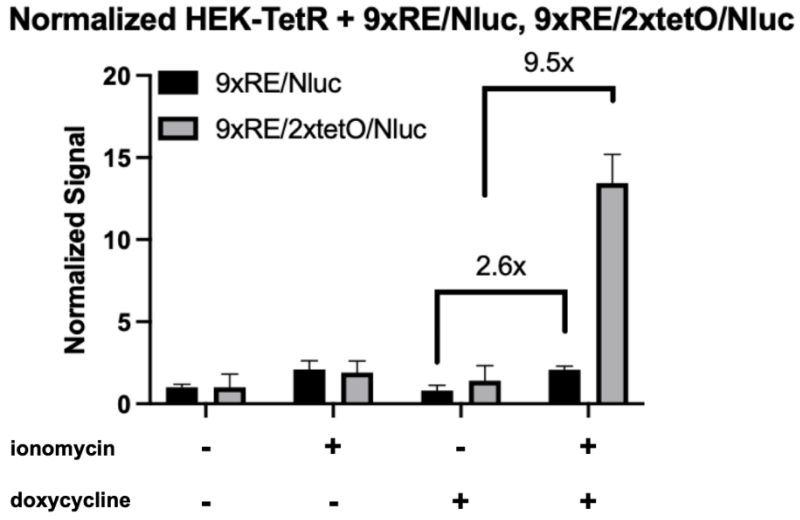


Figure 3: Development of an AND-gated system. (a) The addition of two tetO sequences significantly reduces the reporter gene activation levels. Overall activation levels are likely reduced due to incomplete dissociation between TetR and tetO. Statistical significance by Tukey's test is indicated by asterisks (**, $p \leq 0.01$; ***, $p \leq 0.001$). (b) When readings are normalized to the mean of the double negative groups, we can clearly see that despite overall reduced signal, the fold-change between the double positive group and the doxycycline-only group is much greater in the presence of the tetO sequences, revealing the efficiency of the Tet repressor system in minimizing background activation.

As seen in Figure 3a-b, cells expressing 9xRE/Nluc showed a 2.6-fold induction of signal with the addition of ionomycin but no sensitivity to doxycycline. Cells expressing 9xRE/2xtetO/Nluc, however, showed greatly reduced activation compared to cells expressing the non-AND-gated system in all treatment groups, indicating the effectiveness of the Tet repressor system in reducing undesired transcription. Notably, even though the AND-gated double positive treatment group showed reduced activation levels compared to cells without Tet repression, this group still showed 9.5-fold induction compared to the doxycycline-only group, ensuring the possibility of distinguishing between treated and untreated groups. In fact, cells transfected with 9xRE/2xtetO/Nluc showed greater fold changes in the presence of both drugs than cells transfected with 9xRE/Nluc. Since overall activation levels are less significant than fold changes, we do not expect the decreased signal to present any limitations to the system.

3.4 System characterization in HEK cells

To further test the efficacy of the AND-gated luciferase-based system in HEK cells, we developed a HEK cell line stably expressing both TetR and 9xRE/2xtetO/Nluc and repeated the nanoluciferase assay (Figure A1a). Once again, we saw significantly increased activation in the double positive group. The low levels of activation in the doxycycline-negative groups further supported the effectiveness of the Tet repressor system, and although the doxycycline-only group showed increased activation compared to the doxycycline-negative groups, the activation levels were still far below that of the double positive group, indicating the importance of calcium and NFAT for induction of the system.

As seen in the initial comparison, the addition of the tetO sequences resulted in overall reduction in signal, even in the presence of doxycycline. To assess the dependence of

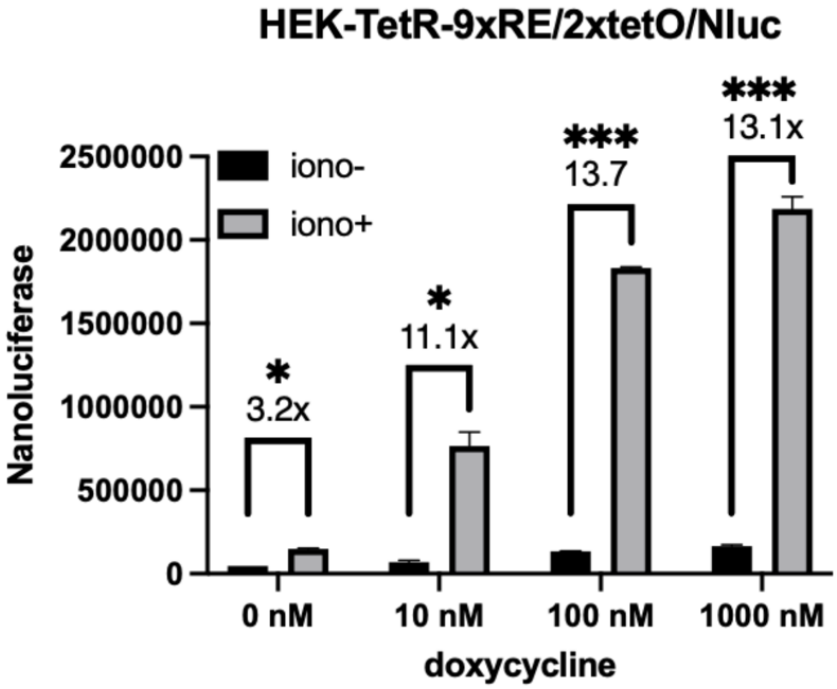


Figure 4: System Characterization in HEK Cells. In the absence of ionomycin (iono), activation levels are low for all concentrations of doxycycline. Increased levels of doxycycline lead to increased activation. However, the fold-change between the signal from the double positive group and the doxycycline-only group level out with higher concentrations of doxycycline (about 13-fold induction with both 100 nM and 1000 nM). Statistical significance by Tukey's test is indicated by asterisks (*, $p \leq 0.05$; ***, $p \leq 0.001$).

activation level on doxycycline concentration, we treated HEK cells stably expressing TetR and 9xRE/2xtetO/Nluc with a consistent amount of ionomycin (either 0 or 1 μg/mL) and varying concentrations of doxycycline. The results from the nanoluciferase assay are in Figure 4. As anticipated, in the absence of ionomycin, activation levels were low for all concentrations of doxycycline. Furthermore, increased levels of doxycycline led to increased activation. However, since the ratio between the signal from the double positive group and the doxycycline-only group leveled out with higher levels of doxycycline (about 13-fold induction with both 100 nM and 1000 nM), the previously used concentration of 333 nM is reasonable. Since these results suggest that doxycycline is working effectively, the overall

decrease in activation caused by the addition of the tetO sequences (even in the presence of doxycycline) is likely due to incomplete dissociation of the TetR component from the tetO sequences. Even as the doxycycline binds to TetR, allowing dissociation from the tetO sequences, an excess of both TetR and tetO components may be enough to ensure continued binding (albeit at lower levels) and thus decreased activation.

Finally, to test our system using a fluorescent reporter, the nanoluciferase gene was replaced with an EGFP reporter gene: 9xRE/2xtetO/EGFP. A new cell line stably expressing TetR and the construct containing the fluorescent reporter was developed. As with the nanoluciferase reporter, the HEK cell lines showed low signal in the absence of either ionomycin/ATP or doxycycline but significant increase in signal in the presence of both drugs when compared to the doxycycline-only group (Figure A1b-c). These findings further underline the efficacy of this system in HEK cells.

3.5 Adaptation in PC-3 cells for mechanical inducibility

Since the gene circuit was working effectively in HEK cells, we advanced to testing in PC-3 cells, where US-mediated mechanical perturbation can lead to a calcium response. As with the HEK cells, viral transfection was used to create a cell line stably expressing both TetR and 9xRE/2xtetO/Nluc. Unfortunately, instead of seeing the same pattern among treatment groups as seen in HEK cells, there was no significant difference in expression level observed between the double positive group and the doxycycline-only group (Figure 5). Notably, the reporter expression level in PC-3 cells overall was much higher than that in HEK cells: the maximum reading in HEK cells was on the order of 1-2 x 10^6, while the maximum reading in PC-3 cells was closer to 5 x 10^7. To ensure that the high readings were not

intrinsically associated with the nanoluciferase reporter, we moved on to utilizing a fluorescent reporter.

When the construct containing the fluorescent reporter was expressed in PC-3 cells, we again saw greatly increased signal compared to HEK cells. In all treatment groups, we saw expression from greater than 90% of cells, prohibiting any assessment of the system's functionality.

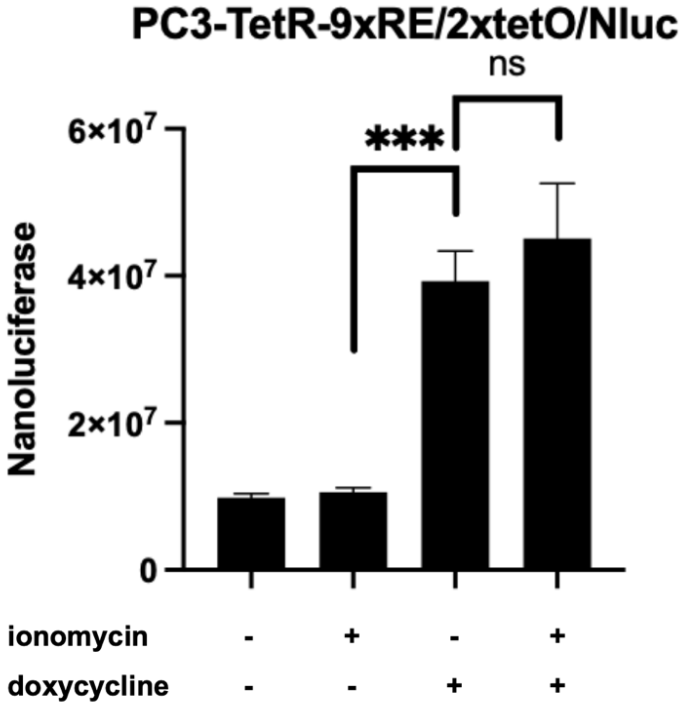


Figure 5: Adaptation in PC-3 Cells. In PC-3 cells, higher background levels of NFAT result in increased system leakage. Although the system is still sensitive to doxycycline, the presence of ionomycin results in no further induction. Furthermore, the measured nanoluciferase signal from PC-3 cells was around 50 times higher than that from HEK cells, resulting in automatic signal attenuation by the plate reader. Statistical significance by Tukey's test is indicated by asterisks (***, $p \leq 0.001$; ns, no significance).

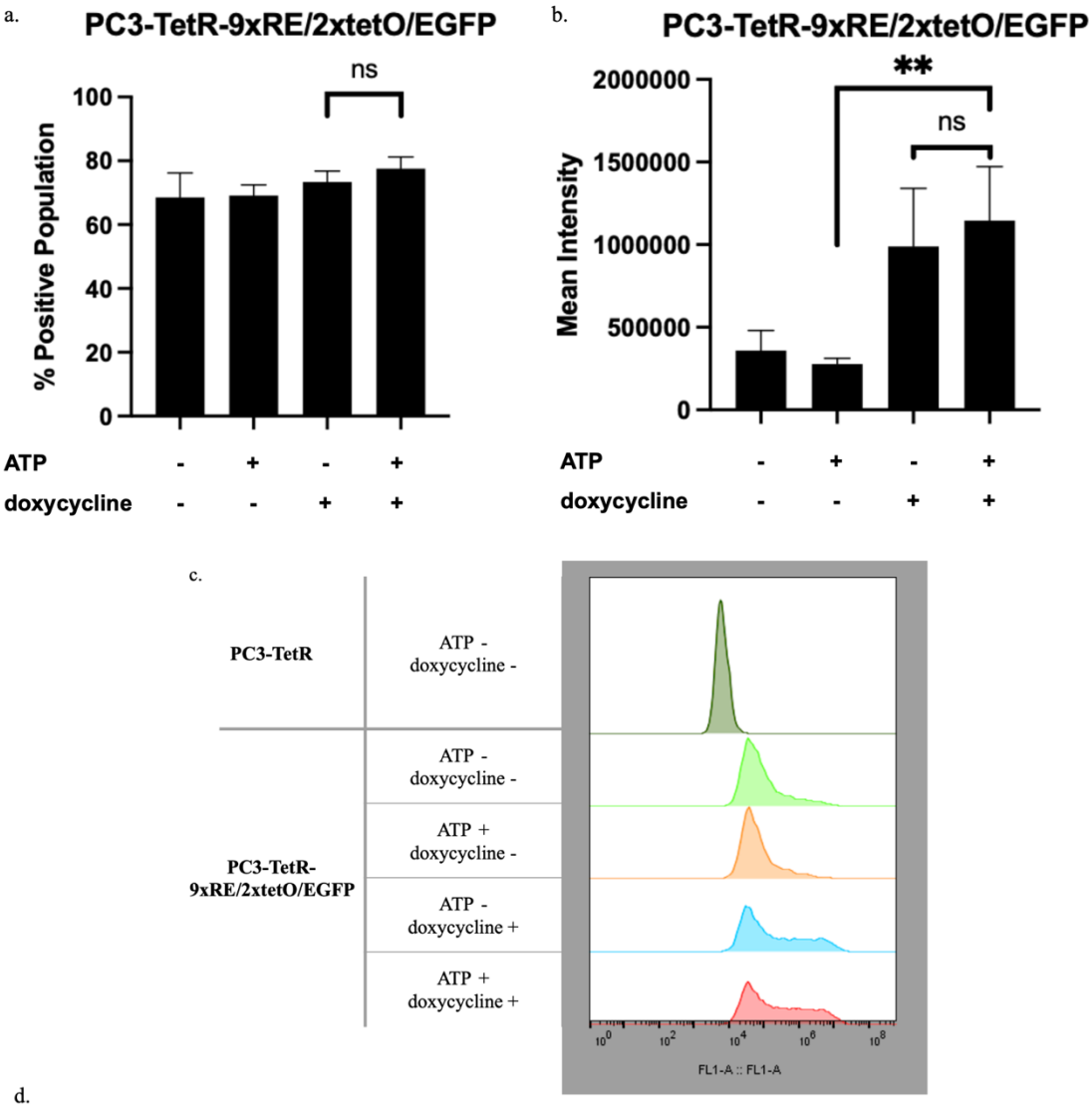
3.6 System optimization in PC-3 cells

To optimize the system in PC-3 cells and account for an increased level of background NFAT expression, we implemented several adjustments. We first sorted the cells and selected

the 20% of the population showing the weakest signal in the absence of treatment and changed the cell culture medium from DMEM to RPMI (which has a lower calcium concentration). At this point, we were able to see some shift in the mean signal intensity after treatment. Although the percentage of cells showing signal was still high across all treatment groups, the mean signal intensity was clearly higher in doxycycline-positive groups compared to doxycycline-negative groups (Figure 6a-c). Unfortunately, as had been the case when using the nanoluciferase reporter, both doxycycline-positive groups were near the saturation point, and it was not possible to make a distinction between the activation levels.

In an additional effort to reduce the baseline activation in PC-3 cells, we used TetR-KRAB instead of TetR as the inhibitor for more robust repression. A new cell line was created to stably express TetR-KRAB and 9xRE/2xtetO/EGFP. The cell line expressing TetR and 9xRE/2xtetO/EGFP was also recreated using a reduced amount of 9xRE/2xtetO/EGFP virus. The results in Figure 6d-g show that although TetR-KRAB is effective in reducing overall activation, we are still not able to clearly distinguish between the two doxycycline-positive groups. Although TetR or TetR-KRAB acts as an effective inhibitor in the absence of doxycycline, once doxycycline is introduced, expression appears to be saturated such that the addition of ionomycin/ATP is not able to further induce the system. Furthermore, the slow kinetics of TetR-KRAB would make the repressor ineffective in our system.

Figure 6: System optimization in PC-3 cells (a) Even after optimization steps (sorting, changing media, etc.), when fluorescent reporter levels in PC-3 cells were measured by flow cytometry, the percent positive population was consistently high across all treatment groups. Statistical significance by Tukey's test is indicated by asterisks (ns, no significance). (b) Despite these high signal levels, we were able to see a shift in mean intensity in the presence of doxycycline, revealing functionality of the system but suggesting high basal levels of NFAT. Statistical significance by Tukey's test is indicated by asterisks (**, $p \leq 0.01$; ns, no significance). (c) The shift in mean intensity can be seen in the raw data, as well. (d) Replacing TetR with TetR-KRAB resulted in significant repression of signal under all conditions. Statistical significance by Tukey's test is indicated by asterisks (***, $p \leq 0.001$). (e) However, even in cells expressing TetR-KRAB, there is no clear induction from ATP and (f) no shift in mean intensity across treatment groups. Furthermore, the kinetics of TetR-KRAB make it unsuitable for use in our application. Statistical significance by Tukey's test is indicated by asterisks (ns, no significance). (g) The lack of a shift in the mean intensity can be seen in the raw data, as well.


a.
PC3-TetR-9xRE/2xtetO/EGFP
ns
% Positive Population
100
80
60
40
20
0
ATP - + - +
doxycycline - - + +
b.
PC3-TetR-9xRE/2xtetO/EGFP
**
ns
Mean Intensity
2000000
1500000
1000000
500000
0
ATP - + - +
doxycycline - - + +
c.
PC3-TetR
ATP -
doxycycline -
PC3-TetR-9xRE/2xtetO/EGFP
ATP -
doxycycline -
ATP +
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doxycycline +
FL1-A :: FL1-A
d.
PC3-TetR-9xRE/2xtetO/EGFP vs. PC3-TetRKRAB-9xRE/2xtetO/EGFP
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TetR
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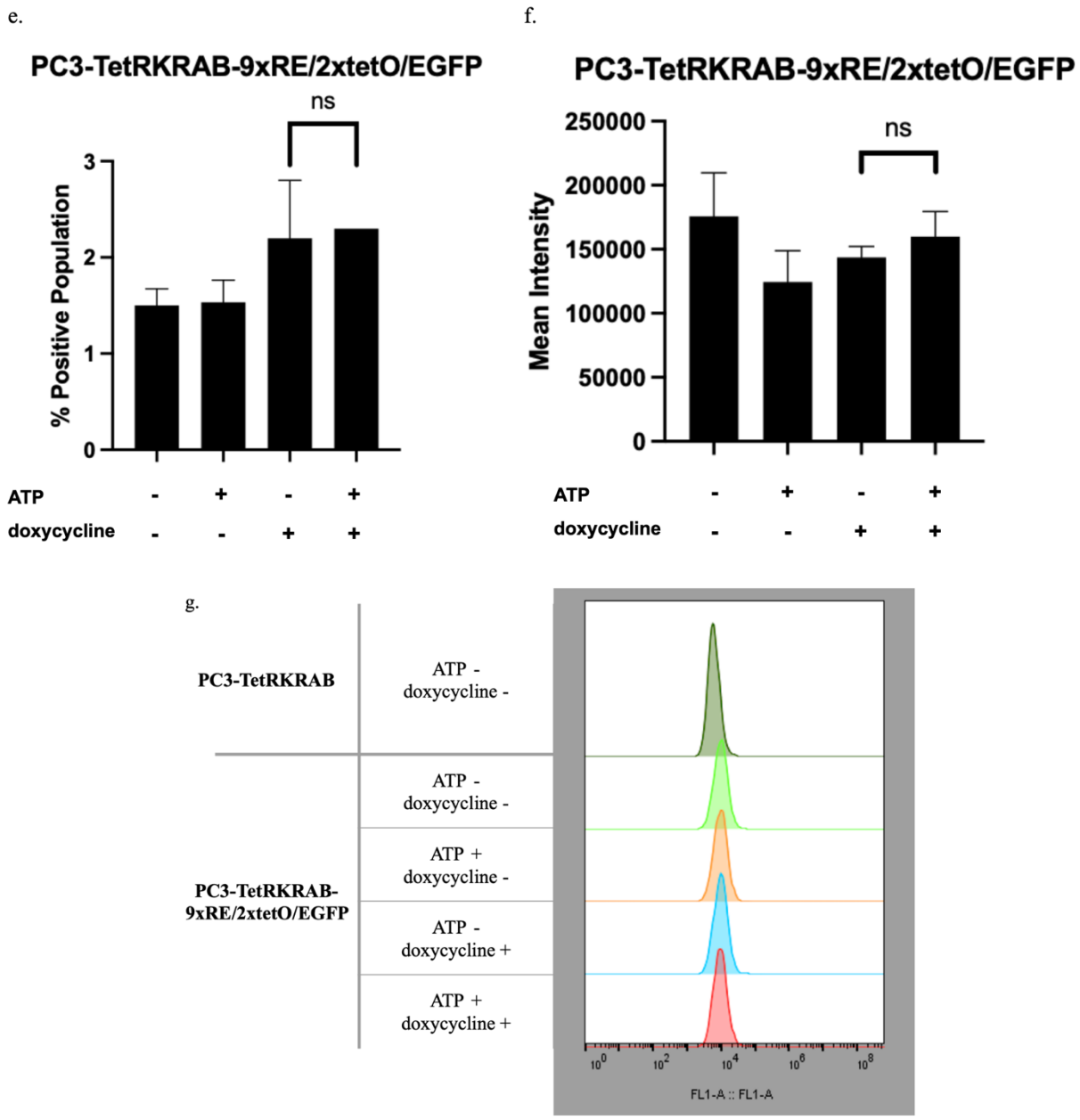

Figure 6: System optimization in PC-3 cells, Continued (a) Even after optimization steps (sorting, changing media, etc.), when fluorescent reporter levels in PC-3 cells were measured by flow cytometry, the percent positive population was consistently high across all treatment groups. Statistical significance by Tukey's test is indicated by asterisks (ns, no significance). (b) Despite these high signal levels, we were able to see a shift in mean intensity in the presence of doxycycline, revealing functionality of the system but suggesting high basal levels of NFAT. Statistical significance by Tukey's test is indicated by asterisks (**, $p \leq 0.01$; ns, no significance). (c) The shift in mean intensity can be seen in the raw data, as well. (d) Replacing TetR with TetR-KRAB resulted in significant repression of signal under all conditions. Statistical significance by Tukey's test is indicated by asterisks (***, $p \leq 0.001$). (e) However, even in cells expressing TetR-KRAB, there is no clear induction from ATP and (f) no shift in mean intensity across treatment groups. Furthermore, the kinetics of TetR-KRAB make it unsuitable for use in our application. Statistical significance by Tukey's test is indicated by asterisks (ns, no significance). (g) The lack of a shift in the mean intensity can be seen in the raw data, as well.

3.7 Integration with TRPV1 for heat inducibility

While working to optimize our system in PC-3 cells, we also began to develop our system such that it could be activated by US-mediated heating. Since we had been able to induce NFAT translocation in TRPV1-expressing HEK cells previously, we created a HEK cell line stably expressing TetR and 9xRE/2xtetO/Nluc and transiently expressing TRPV1. We began by testing a range of heating conditions, varying both time and temperature. ATP was used as a positive control for calcium response induction. As shown in Figure 7, application of heat in the presence of doxycycline resulted in significant induction compared to the doxycycline-only group. However, we failed to see the high fold-changes that had

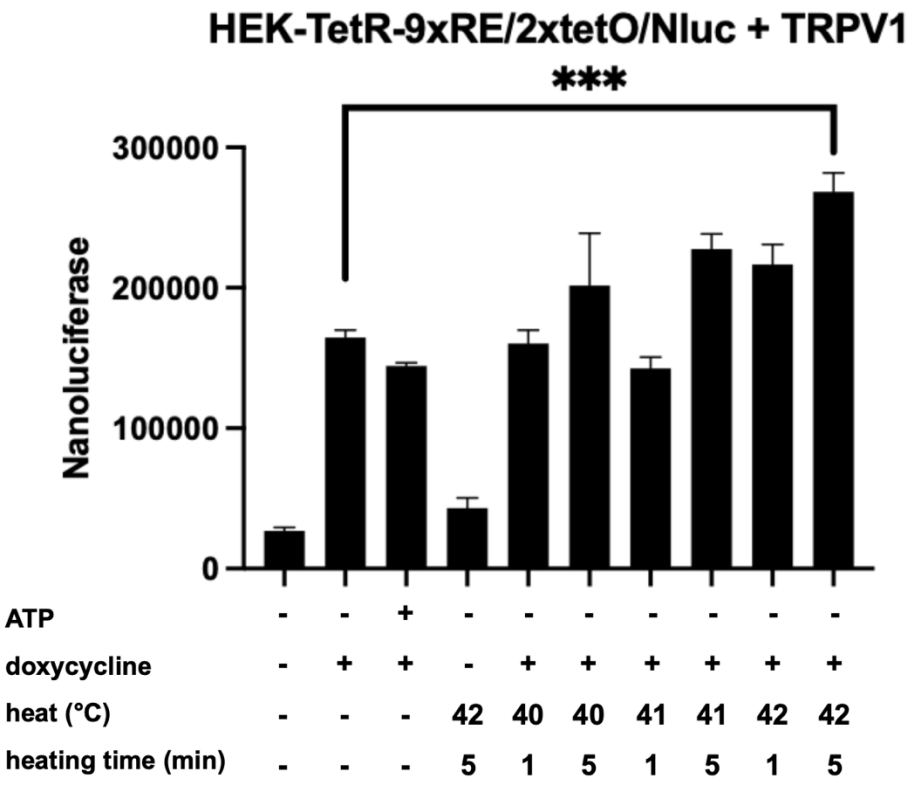

Figure 7: Integration with TRPV1. In cells transiently expressing TRPV1, although heat application results in significant induction compared to the doxycycline only group, we do not see the high fold-changes identified during the initial system development. These results may be explained by overactive TRPV1 channels, poor cell condition due to the presence of TRPV1, or insufficient removal of doxycycline. Statistical significance by Tukey's test is indicated by asterisks (***, $p \leq 0.001$).

occurred in our initial system development. One possible explanation for these observations is that overactive TRPV1 channels or an overabundance of TRPV1 channels may have allowed an influx of calcium even in the absence of heat. Another possibility is that poor cell condition or stress from the treatment or the presence of TRPV1 resulted in cell death, which also would trigger calcium influx. In any case, these results indicated the potential of the system to be induced by heat after optimization.

Discussion

The growth of the field of CAR T-cell therapy has highlighted the need for tightly regulated controllable systems. Although the treatment has shown promise, the risk of off-target effects is still high, and current solutions do not sufficiently address the issue. US offers a method of ensuring tight spatiotemporal control, but since the calcium dynamics in cells are affected by many factors other than US, this solution is best utilized when paired with an additional level of regulation. A tetracycline-inducible system can supply this control by repressing any background activation by calcium in the absence of doxycycline, a synthetic tetracycline antibiotic. This method further introduces a level of novelty to our system in that Tet repression has traditionally been used on constitutive promoters; our system incorporates additional control by combining Tet repression with an inducible promoter.

The system we developed shows how an AND-gated system can effectively regulate activation of the target gene, thereby offering a means of providing both spatial and temporal control. By combining nine repeats of the *IL8* NFAT response element with a minimum promoter and two tetO sequences, we created a gene circuit that activates only in the presence of both calcium and doxycycline. In HEK cells expressing our construct and TetR, we saw 9.5-fold induction of activation between cells incubated with both ionomycin and doxycycline and cells incubated with only doxycycline.

In PC-3 cells, we did not see such clear induction. Prostate cancer is the most common noncutaneous human malignancy and the second most lethal cancer among men.[31] Previous work in the lab has shown that PC-3 cells are particularly susceptible to mechanical perturbation by US, so they serve as an appropriate cancer model in which to test our system. However, despite achieving some minor decreases in activation, we continue to see

consistently high levels of leakage compared to HEK cells. The use of TetR-KRAB instead of TetR dramatically repressed activation, however, the kinetics of TetR-KRAB make it unrealistic for use in our system (previous work has shown that TetR-KRAB may irreversibly silence up to 50% of the population in which it is expressed).[32] In any case, although TetR-KRAB reduces background activation, there is still no significant difference between cells incubated with both ionomycin/ATP and doxycycline and cells incubated with only doxycycline. Based on these results, it seems likely that PC-3 cells exhibit higher basal levels of NFAT than those for which our system is designed. Previously published studies have found evidence of overactivation, or even constitutive activation, of NFAT in various cancers, including prostate cancer.[31,33-36] Although the reasons for this overactivation are not well understood, NFAT proteins have been shown to regulate cellular processes such as proliferation, migration, and apoptosis, so highly active NFAT proteins may increase the malignant potential of tumor cells.[33-35] These findings indicate that NFAT is present at a higher basal level in PC-3 cells than HEK cells. However, the fact that we saw a shift in mean intensity level of expression in PC-3 cells indicates that the system is still working to a certain extent. Since our system was originally designed in HEK cells, we may simply be required to optimize the system to accommodate the increased levels of NFAT. One possible direction would be to decrease the number of NFAT-RE repeats in the gene circuit. With fewer binding locations available to the NFAT, the transcription factor may not be able to activate expression as readily.

Similarly, the possibility of using our system in cells expressing TRPV1 channels shows promise but requires further optimization. We demonstrated clear NFAT translocation to the nucleus in TRPV1-expressing cells upon the application of heat. However, when testing

this method in cells expressing our construct, we were unable to see high fold-changes between the double positive group and the doxycycline-only group. Comparing cells infected with different amounts of TRPV1 virus or sorting cells based on TRPV1 expression levels would allow identification of cells expressing an appropriate amount of TRPV1 channels. Further comparison of heating times and temperatures would also determine the ideal conditions for inducing a response while minimally impairing cell condition.

Conclusions and Future Work

This project has demonstrated the feasibility of an AND-gated US-controlled gene circuit. Previous work has shown the promise of inducing gene expression by taking advantage of cellular calcium dynamics, and US has been proven to be a safe and effective means of controlling these dynamics. However, the addition of Tet repression allows us to significantly reduce background activation compared to a system relying on calcium induction alone. This system works robustly in HEK cells using calcium-inducing agents but will need to be further optimized to function in PC-3 cells (due to the increased basal levels of NFAT) or to function under thermal control.

Since we show how effectively this system can function in HEK cells, the high background activation found in PC-3 cells is likely caused by excessive levels of NFAT in the cells. To account for this, we may tune our gene circuit by either decreasing the number of NFAT-RE repeats, thereby limiting induction, or increasing the number of tetO sequences, thereby further enabling repression. These options would help adapt our system to a cellular environment containing high levels of NFAT.

Another direction would be to take advantage of the system's functionality in HEK cells and optimize the methodology of heat activation. While PC-3 cells have been shown to be sensitive to mechanical perturbation, US-mediated heating remains a viable option. We have demonstrated that in cells expressing TRPV1, application of heat results in an influx of NFAT into the nucleus in HEK cells. By using heat as a means of activation, we could further optimize the system in HEK cells, where we already see dramatic background suppression. Optimization steps may include adjusting the level of TRPV1 in cells, experimenting with

different temperatures and heating times, and more closely examining the kinetics of the heat application and doxycycline dosing.

In summary, we have shown the potential of an AND-gated system for use in immunotherapy. The effectiveness of the current system in HEK cells demonstrates the increased control that can be achieved when a second level of regulation is included in the system. Although further work is required to determine the best way to incorporate this system into a feasible therapy, this project has laid the groundwork for a means to exert tight spatial and temporal control over immunotherapy treatments.

Material in this thesis is co-authored with Dr. Chi Woo Yoon. The thesis author was the primary author of this material.

Appendix

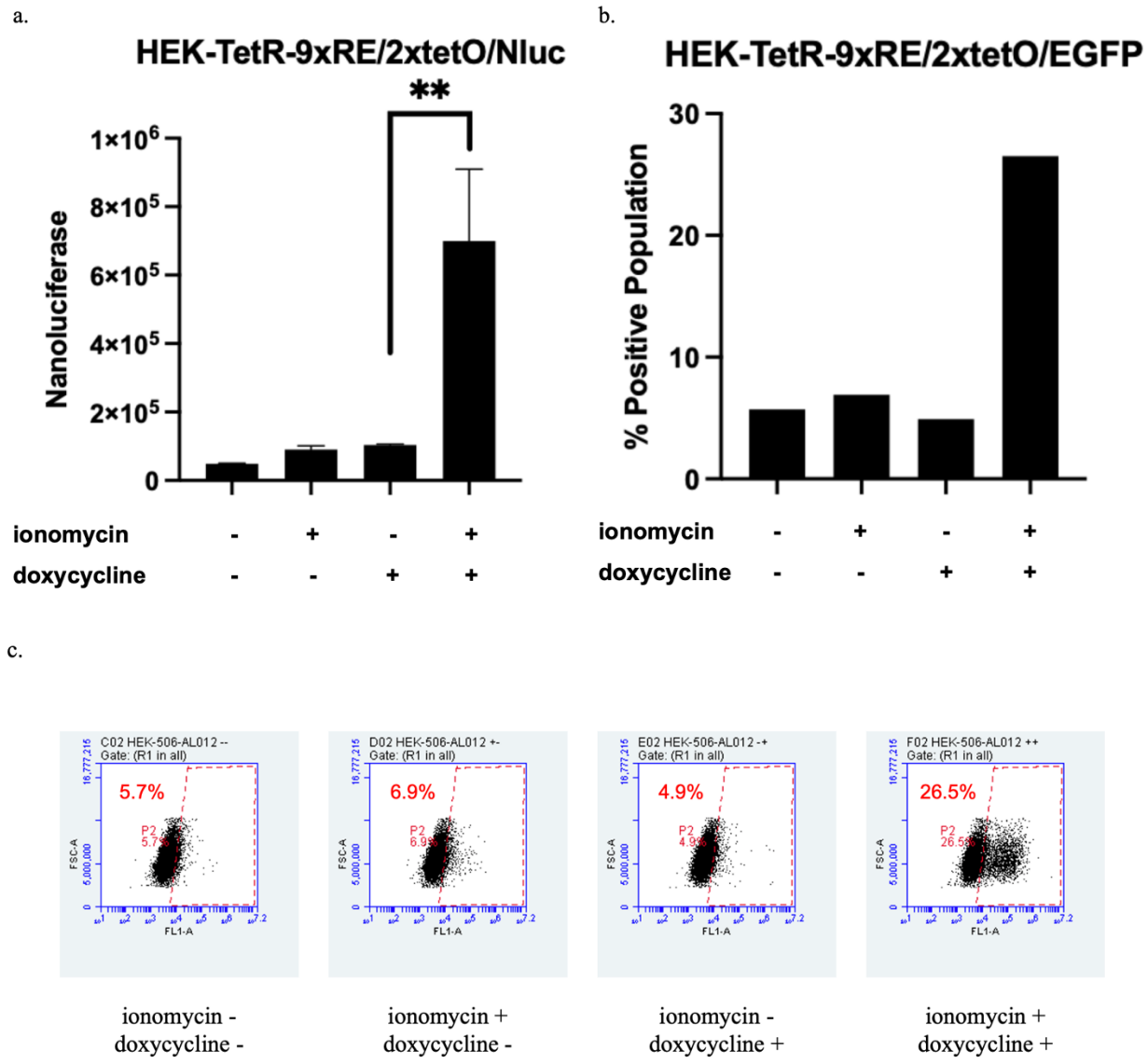

Figure A1: System characterization in HEK cells. (a) HEK cells stably expressing TetR and 9xRE/2xtetO/Nluc reveal the importance of both control factors in the system. Activation levels are low unless both doxycycline and ionomycin/ATP are present. Statistical significance by Tukey's test is indicated by asterisks (**, $p \leq 0.01$). (b) As seen when using a nanoluciferase reporter, in HEK cells with a fluorescent reporter, the Tet repressor system effectively minimizes background activation, and the addition of ionomycin further induces activation in the double positive group. (c) This pattern can be seen in the raw data, as well.

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