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				19b. TELEPHONE NUMBER 617-253-8966	

RPPR Final Report

as of 01-May-2020

Agency Code:

Proposal Number: 71194LSDRP

Agreement Number: W911NF-17-2-0098

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Final Report for Period Beginning 16-May-2017 and Ending 15-May-2019

Title: FACETS: Fabrication of Autonomously Constructed Engineered Three-dimensional Shapes

Begin Performance Period: 16-May-2017

End Performance Period: 15-May-2019

Report Term: 0-Other

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Distribution Statement: 1-Approved for public release; distribution is unlimited.

STEM Degrees: 0

STEM Participants: 4

Major Goals: The project consists of two phases. In Phase 1, the objective was to explore design approaches for genetically programming a single cell line to give rise to a multicellular system with a predetermined three-dimensional form. In Phase 2 was to design and build tunable control of three-dimensional form in a clonal cell population, with differentiated surfaces.

Phase 1 objectives included modeling and simulation, circuit design and construction, experiments, and a demo of three-dimensional sizes and shapes that are stable for at least 1 week under optimal growth/differentiation. Phase 1 simulation objectives included development of theoretical models that bridge developmental biology and synthetic biology objectives to achieve programmed development of morphology in an expanding clonal cell population. Potential issues in design were identified (see discussion below) and simulations were adjusted based on experimental data. Phase 1 objectives also included circuit design, construction, and experiments for interior / exterior circuits and other morphology circuits.

Phase 2 objectives included modeling and simulation, circuit design and construction, experiments, and a demonstration of programmed forms that are stable in size and shape over a minimum period of 1 week under optimal growth/differentiation conditions. Simulations and modeling objectives included creating of a 'shape compiler' for fast and accurate modeling. Phase 2 theoretical modeling objects were to predict points and potential mechanisms of control of morphology development. Simulations were Adjusted based on experimental data to refine predictions, including new objectives for cell-sorting based shape and image analysis. Phase 2 circuit objectives included design, construction, and experiments of both shape selection and facet selection circuits.

Constructing genetic oscillators to provide clocked inputs for the circuits in both phases arose as a key challenge to be addressed during the course of the project and our oscillator solutions are outlined below. In addition, optimization of 3D synNoth Sender-Receiver and the Phloretin system required additional effort.

Accomplishments: Report uploaded under Upload

Training Opportunities: Postdoctoral associates and graduate students involved with the project have exposure to technical workshops hosted by the Synthetic Biology Center. These include talks from industry professional about career opportunities and Research Ethics in Synthetic Biology Seminar. Technical writing assistance is also available from the Biological Engineering Department.

Results Dissemination: Participants traveled to conferences to share results.

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Honors and Awards: Nothing to Report

Protocol Activity Status:

Technology Transfer: Nothing to Report

PARTICIPANTS:

Participant Type: PD/PI

Participant: Ron Weiss

Person Months Worked: 2.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

Participant Type: Co-Investigator

Participant: Ed Boyden

Person Months Worked: 1.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

Participant Type: Co-Investigator

Participant: Jake Beal

Person Months Worked: 6.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

Participant Type: Co-Investigator

Participant: Stanislav Shvartsman

Person Months Worked: 1.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

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Participant: Gizem Gumuskaya

Person Months Worked: 13.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

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Participant Type: Other Professional

Participant: Jonathan Babb

Person Months Worked: 5.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

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Person Months Worked: 12.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

Participant Type: Postdoctoral (scholar, fellow or other postdoctoral position)

Participant: Katherine Kiwimagi

Person Months Worked: 10.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

Participant Type: Technician

Participant: Matthew Lima

Person Months Worked: 6.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

Participant Type: Technician

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Person Months Worked: 6.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

Participant Type: Postdoctoral (scholar, fellow or other postdoctoral position)

Participant: Shiva Razoui

Person Months Worked: 6.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

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Participant Type: Postdoctoral (scholar, fellow or other postdoctoral position)

Participant: Lushuai Zhang

Person Months Worked: 7.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

Participant Type: Graduate Student (research assistant)

Participant: Andrew Moorman

Person Months Worked: 3.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

Participant Type: Other Professional

Participant: Dianne Bickford

Person Months Worked: 1.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

Participant Type: Postdoctoral (scholar, fellow or other postdoctoral position)

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Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

Participant Type: Graduate Student (research assistant)

Participant: Asmamaw Wassie

Person Months Worked: 2.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

RPPR Final Report
as of 01-May-2020

Fabrication of Autonomously Constructed Engineered Three-dimensional Shapes (FACETS)

Final Report

Project Title: Fabrication of Autonomously Constructed Engineered Three-dimensional Shapes (FACETS)

Prime Contractor: MIT

Contract Number: W911NF-17-2-0098

Period of Performance: 5/15/2018 – 5/15/2019

Performing Organizations: Raytheon BBN Technologies, Weiss Lab at MIT, Boyden Lab at MIT, and Shvartsman Lab at Princeton

1. TASK OBJECTIVES

The project consists of two phases. In Phase 1, the objective was to explore design approaches for genetically programming a single cell line to give rise to a multicellular system with a predetermined three-dimensional form. In Phase 2 was to design and build tunable control of three-dimensional form in a clonal cell population, with differentiated surfaces.

Phase 1 objectives included modeling and simulation, circuit design and construction, experiments, and a demo of three-dimensional sizes and shapes that are stable for at least 1 week under optimal growth/differentiation. Phase 1 simulation objectives included development of theoretical models that bridge developmental biology and synthetic biology objectives to achieve programmed development of morphology in an expanding clonal cell population. Potential issues in design were identified (see discussion below) and simulations were adjusted based on experimental data. Phase 1 objectives also included circuit design, construction, and experiments for interior / exterior circuits and other morphology circuits.

Phase 2 objectives included modeling and simulation, circuit design and construction, experiments, and a demonstration of programmed forms that are stable in size and shape over a minimum period of 1 week under optimal growth/differentiation conditions. Simulations and modeling objectives included creating of a 'shape compiler' for fast and accurate modeling. Phase 2 theoretical modeling objects were to predict points and potential mechanisms of control of morphology development. Simulations were Adjusted based on experimental data to refine predictions, including new objectives for cell-sorting based shape and image analysis. Phase 2 circuit objectives included design, construction, and experiments of both shape selection and facet selection circuits.

Constructing genetic oscillators to provide clocked inputs for the circuits in both phases arose as a key challenge to be addressed during the course of the project and our oscillator solutions are outlined below. In addition, optimization of 3D synNoth Sender-Receiver and the Phloretin system required additional effort.

The objectives in the original state of work for the proposal are listed below.

Task 1.1: Modeling and simulation:

Subtask 1.1.1: Modeling and simulation: Develop theoretical models that bridge developmental biology and synthetic biology objectives to achieve programmed development of morphology in an expanding clonal cell population. Models will include development of morphology in an expanding cell population, including the range of possible forms that can be achieved and the stability and reproducibility of selected target morphologies. We plan to simulate use of autonomous cell segregating for interior/exterior differentiation and of Turing patterning and gradients to control staged development of a defined morphology, and to run 3D simulations using Morpheus or similar tools to differentiate between design choices. We will identify potential issues in design and predict experimental results. We plan to adjust simulations based on experimental data to refine predictions. **BBN, MIT/Weiss, Princeton (Months 1-12)**

Deliverables: Reports on model implementation and simulation results.

Completion Criteria: Delivery of reports for Milestone 1.

Task 1.2: Circuit Design and Construction

Subtask 1.2.1: Develop interior / exterior circuits: Design and build genetically programmed circuits that control the autonomous segregating of interior/exterior cells within a sphere. We plan to build circuit modules for reset, a replicoscillator clock, a stochastic toggle to control genetic expression of multiple cadherins, and multiple recombinase memory elements (Step 1; Milestone 2). **MIT/Weiss (Months 1-12)**

Subtask 1.2.2: Develop morphology circuits: Design and build genetically programmed circuits that control the development of defined morphology in an expanding population of cells. We plan to build circuits for leader election and bandpass gradient algorithms based on Turing pattern formation using Phloretin and VLPs signaling molecules, and recombinase memory elements (Steps 2-4; Milestone 3). **MIT/Weiss (Months 1-12)**

Deliverables: Report on genetic circuit designs.

Completion Criteria: Delivery of report on genetic circuit designs for Milestone 2 and 3.

Task 1.3: Experiments

Subtask 1.3.1: Perform differentiation experiments: We will perform experiments to differentiate interior vs. exterior cells. We plan to use hanging drop experiments with engineered mammalian cells using genome-integrated circuits from Task 1.2 via autonomous cell segregating, execute leader election and gradient algorithms, and stabilize 3D size and shape using recombinase and growth genes. **MIT/Weiss (Months 1-12)**

Subtask 1.3.2: Support differentiation experiments: Assist in design of experiments in Subtask 1.3.1, assisting in analysis and interpretation of experimental data, and in recommending adjustments of experiment and design. **BBN, Princeton (Months 1-12)**

Subtask 1.3.3: Monitor differentiation experiments: Apply methods including Expansion Microscopy (ExM) and develop target metrics for monitoring the differentiation of cells, distinguishing the phenotypes of interior and exterior cells, and for monitoring morphology development of the clonal population for the experiments in Subtasks 1.3.1. **MIT/Boyden (Months 1-12)**

Subtask 1.3.4: Demo 1: Demonstrate that the final three-dimensional size and shape of the clonal population engineered is stable for at least 1 week under optimal growth/differentiation conditions. **MIT/Boyden, MIT/Weiss (Months 10-12)**

Deliverables: Experimental data and analyses, and images from expansion microscopy.

Completion Criteria: Delivery of experimental data, recommendations and analyses for Milestone 2, 3; Demo of a programmed 3D shape that is stable for 1 week for Milestone 4.

Task 1.4: Project Management and Reporting

Subtask 1.4.1: Project management and reporting: Manage project or subcontract timelines, finances, and deliverables, attend monthly team meetings and government meetings, provide inputs to reports, and report scientific results. **MIT, BBN, Princeton (Months 1-12)**

Deliverables: Financial reports and technical reports or report inputs.

Completion Criteria: Delivery of financial and technical report inputs or subcontractor portions.

Phase 2 Tasks: Goals: expand on work from Phase I to design and build tunable control of three-dimensional form in a clonal cell population, with differentiated surfaces.

Task 2.1: Modeling and Simulation:

Subtask 2.1.1: Modeling and simulation: Use theoretical models to predict points and potential mechanisms of control of morphology development. We plan to model methods for programming and tuning to produce multiple forms from a single progenitor line using exosomal inputs, differentiation of surface regions into multiple faces via selective growth, and production of at least three distinct three-dimensional forms with discernible faces from a single engineered progenitor cell line. We will run simulations using Morpheus or similar tools to differentiate between design choices, identify potential issues in designs, and predict experimental results. Adjust simulations based on experimental data to refine predictions. **BBN, MIT/Weiss, Princeton (Months 13-24)**

Deliverables: Reports on model implementation and simulation results.

Completion Criteria: Delivery of reports for Milestone 5.

Task 2.2 Circuit Design and Construction

Subtask 2.2.1 Develop shape selection circuits. Design and build genetic circuit modules that allow production of multiple 3D shapes based on initial external stimuli. The circuit will proceed autonomously without external cues provided by the experimenter and the resulting final size and shape will be stable. We plan to use exosomes or small molecules that direct outgrowth from facets in a 3D coordinate system on the surface of sphere. Optionally, stabilized shapes may harden into bone (Step 5, Milestone 6). **MIT/Weiss (Months 13-24)**

Subtask 2.2.2 Develop facet circuits. Design and build genetic circuits that distinguish between exterior cells on various facets of the final shape. We plan to use recombinase memory circuits to identify each facet such that the inputs in Subtask 2.2.1 will lead to expression of a different marker and differential growth on each facet (Step 5, Milestone 7). **MIT/Weiss (Months 13-24)**

Deliverables: Report on genetic circuit designs.

Completion Criteria: Delivery of report on circuit designs for Milestone 6 and 7.

Tasks 2.3 Experiments

Subtask 2.3.1: Perform shape experiments: We will perform experiments to produce at least three distinct three-dimensional forms with discernible faces from a single engineered progenitor cell line. We plan to use hanging drop experiments with engineered mammalian cells using genome-integrated circuits from Task 2.2 to establish a coordinate system on the surface of a sphere and outgrow from user-specified facets. **MIT/Weiss (Months 13-24)**

Subtask 2.3.2: Support shape experiments: Assist in design of experiments from Subtask 2.3.1, assisting in analysis and interpretation of experimental data, and in recommending adjustments of experiment and design. **BBN, Princeton (Months 13-24)**

Subtask 2.3.3: Monitor individual forms: Apply methods including Expansion Microscopy and develop target metrics to identify individual shapes in experiments from Subtask 2.3.1. **MIT/Boyden (Months 13-24)**

Subtask 2.3.4: Demo 2: Demonstrate that each programmed form is stable in size and shape over a minimum period of 1 week under optimal growth/differentiation conditions. **MIT/Weiss, MIT/Boyden (Months 22-24)**

Deliverables: Experimental data and analyses, and images from expansion microscopy.

Completion Criteria: Delivery of experimental data, recommendations and analyses for Milestone 6, 7 and demo of three individual shapes that are stable for 1 week for Milestone 8.

Task 2.4: Project Management and Reporting

Subtask 2.4.1: Project management and reporting: Same as subtask 1.4.1. **(Months 13-24)**

The remainder of the report is organized around the key challenges identified and our solutions and progress for the following project sub-teams:

1. Oscillator
2. 3D synNotch Sender-Receiver
3. Computational model
4. Shape compiler
5. Cell-sorting based shapes and image analysis

2. TECHNICAL PROBLEMS

Oscillator: Constructing genetic oscillators in mammalian cells is challenging both at the molecular and circuit level. Given a certain circuit that has the potential oscillation possibility, the oscillation window is narrow compared to the steady-state regimes, predicted by computational models. Critical criteria for driving oscillation include but are not limited to matched protein production and degradation rates, similar rates of degradation of proteins and mRNAs, enough time-delay, etc. In mammalian cells, tools to tune each of these parameters are still very limited. Further, intrinsic noises (concerning each gene) and extrinsic noises (concerning cell-wide processes) imply that such dynamic processes depart from the orthogonal assumption, that has widely been adopted by synthetic biology to manipulate exogenous gene on/off switches in host cells independent of endogenous cellular events, posing even greater difficulties. Finally, orchestrating all these molecular behaviors with temporal precision to achieve oscillation is daunting.

3D synNotch Sender-Receiver: In order to test the performance of the synNotch circuit co-culturing sender and receiver cells in informative patterns is paramount. This is better established for the standard synNotch circuit where the contact mediates expression of the circuit output (i.e. fluorescent reporter signal emerges at the sender/receiver contact site). However, to detect signal propagation, higher spatial resolution in patterning had to be achieved. This is mainly due to the fact that receiver/sender cells have to be patterned in <500 micron proximity of each other to develop contact as the two populations grow. To make sure that each population retains its pattern, the cells had to be seeded at higher densities. In such cases within the 12-30 hr mark that the circuit was expected to generate the reporter signal the cells would die and detach from the surface, making it difficult to assess propagation. Alternatively, when cells were seeded at lower confluency they would not retain a crisp-edged pattern by the time the sender/receiver contact would emerge, similarly contributing to issues with propagation assessment. Collaborators at

University of Florida who are specialized in seeding cells in 3D at high spatial resolution (<100 micron) have taken on this challenge, leading to promising preliminary propagation data.

The other technical challenge stemmed from imaging the cells with three fluorophores for over 30 hours using the plate reader. This affected cell viability mainly attributed to phototoxicity.

Circuit design and optimization of the phloretin systems: Leaky expression of the hybrid promoter design in the phloretin system needed optimization. Most of the effort on the phloretin system was in overcoming this leakiness.

Shape compiler: 3D simulation and design are computational intense processes, and require long simulation times. The major technical challenge for the shape compiler was to integrate the design and compiler features with a modeling system fast and accurate enough to provide full circle design.

Cell-sorting based shapes and image analysis: One major technical challenge was getting an accurate count of cells in the initial stages of 3D aggregates. To fix this, we designed a fluorescence cytometry-based protocol and used it to set up high-replicate 3D aggregates. FACS sorting allows for precise cell counting and therefore more reliable aggregate composition. We found that this method is more replicable and reliable than hand pipetting, and allows for exact selection of transfection bin based on transfection marker expression. Another challenge was tracking cell and aggregate movement in a 3D context. We created image analysis pipelines to solve this, but they operate mostly on a 2D slice of a 3D aggregate.

3. GENERAL METHODOLOGY

Oscillator: Our general strategy to tackle the major problems is to construct a series of negative feedback circuits with the core components comprising an activator and a repressor. The activator activates gene expression of the repressor, and the repressor in turn inhibits transcription or translation of the activator. With such a simple circuit topology, we have a large flexibility to tune the circuits and components, in terms of promoter strength, binding cooperativity, tight repression, etc. To achieve relatively short life-times of proteins and mRNAs, which we consider essential for dynamics, all proteins were PEST-tagged and 3' UTR contains AU-rich element.

3D synNotch Sender-Receiver: HEK293FT cells were either transfected with sender cell components such as hEF1a-CD19 or receiver cell components such as hEF1a-synCD19 tTA and the reporter circuit TRE-mKate and a transient transfection marker as described in the figure below. Receiver cells were then coculture in 3D spheres with either sender or wild type cells. After sphere formation cells were then brought to a single cell suspension for collection of FACs via vigorous pipetting and pooling of cells from many wells to increase the population number. This FACs data was then converted to MEFL and performance was observed.

Circuit design and generation of stable synNotch cell lines in HEK293FT cells: Design and construction of both sender and receiver lentiviral vectors was accomplished. Stable sender cells were constructed with just one lenti integration. Receiver cells needed two different virus (one for the receptors components and the other for the reporter circuit.) To make propagating receiver cells an additional third virus was produced to deliver a TRE inducible promoter driving the CD19 membrane bound ligand of the sender cells.

3D synNotch Sender-Receiver in CHO: To integrate the CHO cells with synNotch circuit we proposed the scheme in Fig. 1 and proceeded with the following steps to engineer the corresponding cell lines:

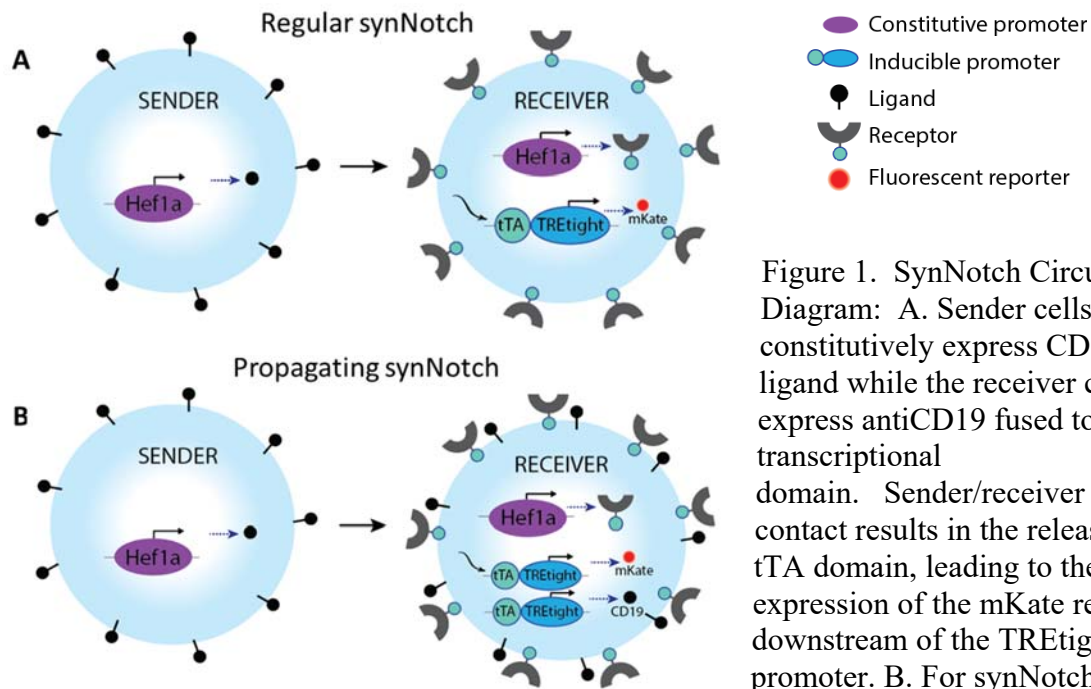


Figure 1. SynNotch Circuit Diagram: A. Sender cells constitutively express CD19 surface ligand while the receiver cells express antiCD19 fused to the tTA transcriptional domain. Sender/receiver cells' contact results in the release of the tTA domain, leading to the expression of the mKate reporter downstream of the TREtight promoter. B. For synNotch

propagation CD19 expression is regulated with the inducible TREtight promoter, thus the receivers can become potential senders.

- To engineer the basic variants of stable senders and receivers we first virally transduced CHOK1 landing pad (CHOK1-LP) cells with Hef1a-CD19 and Hef1-antiCD19 correspondingly. We sorted for stable monoclonal populations of each senders and receivers.
- To engineer more complex receiver circuitry with reporter signal and propagation we first constructed 3 viruses with HEK293FT cells each containing Hef1a-antiCD19, TREtight-mKate, or TREtight-CD19.
- We then introduced the following virus loads to the CHOK1 and stable CHO-antiCD19 receivers at four virus levels in the format presented in Table 1.

CHOK1-LP	Stable CHOK1-antiCD19 receiver
Hef1a-antiCD19 + TRE-mKate	TRE-mKate
Hef1-antiCD19 + TRE-mKate + TRE-CD19	TRE-mKate + TRE-CD19

Table 1. Viral transduction of CHOK1-LP and CHOK1 senders with additional gene circuits at equal viral load dilutions.

- For the given 4 transduction conditions at 4 virus levels (4x4) we proceeded with the highest virus load and a second load that exhibited the weakening of the mKate signal.
- To indirectly assess the MOI for each of these viruses we performed a functionality assay on the cells lines. We co-cultured each polyclonal cell line with transiently transfected HEK

senders according to the scheme presented in Fig.2. (HEK transfection has higher efficiency than the CHOK1, making it more suited for screening experiments).

- | |
|---|
| <p>1. Testing CHO synNotch cells as "receivers":
 S: HEK293 WT transient (Hef1a-EBFP + Hef1a-CD19)
 R: CHO Lenti (Hef1a-antiCD19 + TRE-mKate + TRE-CD19)</p> <p>2. Testing CHO synNotch cells as "senders":
 S: CHO Lenti (Hef1a-antiCD19 + TRE-mKate + TRE-CD19)
 R: HEK293 WT (Hef1a-antiCD19 + TRE-mKate + Hef1a-EBFP)</p> |
|---|

Figure 2. Co-culture of transient HEK sender/receiver cells with stable CHOK1 receiver/sender cells. 1. Co-culture of transient HEK senders with stable CHO-receivers to assess

the expression of TRE-mKate. 2. Co-culture of stable CHO-senders with transient HEK receivers to assess their performances as senders indicated by the reporter mKate expression.

Circuit design and optimization of the phloretin systems: We used the standard design, build and test cycle to perform several rounds of optimization of our circuit design. With each round we were able to improve system performance. A summary of our stable sender circuit performance on transiently transfected receiver cells is depicted in figure 3 below.

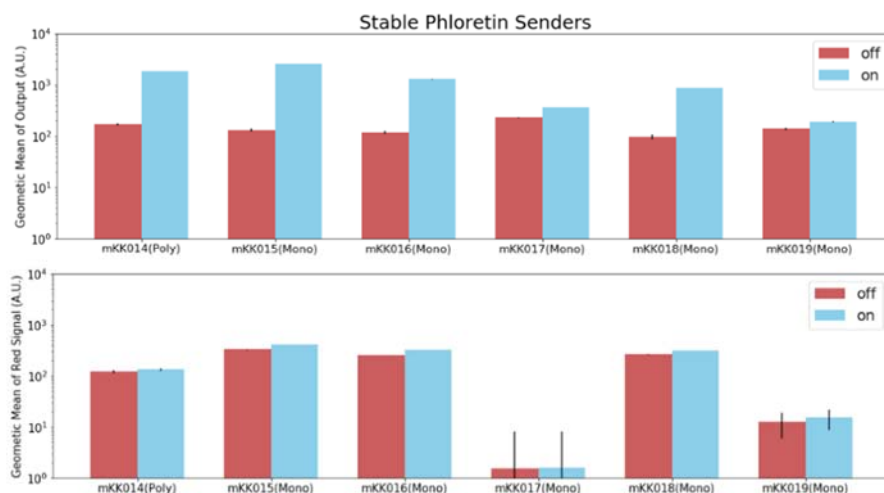


Figure 3: The top panel is a bar graph of the off (no Phloretin Acid) and on (Phloretin Acid) state for the transiently transfected receiver components in the different stable cell lines. The bottom panel is of the corresponding red signal, which can be used as a proxy for the state of silencing occurring in the stably transduced sender components. All data used in these bar graphs can from a mid range bin of the transfection marker (i.e. $10^{3.5}$ to $10^{4.5}$)

Computational model: We developed a computational model based on an extended 2D Viscek model of interacting self-propelled particles. Within this approach, cells are described as active agents that exert traction forces on the underlying substrate to propel themselves in 2D space. In turn, they interact with other cells through steric repulsion and cell-cell adhesion. The interparticle interaction also involves a stochastic part, which describes the contribution of random forces due to various stochastic processes at the subcellular level. In addition, to mimic the experimental setup, in which cells undergo their dynamics at the surface of a curved plate, we added in our

model an effective external field that constrains cell movements and bounds them to the center of the simulation domain. This captures cell movements due to gravity inside a potential well.

Shape compiler: We constructed a prototype workflow and compiler for turning a high-level shape specification written in the Protelis aggregate programming language into an implementation as a cellular shape-formation system. We further developed composable SBOL modules encapsulating design motifs, applied CompuCell3D simulations to begin parameterization of modules, and worked on integration of simulation and compiler into a complete design and simulation workflow.

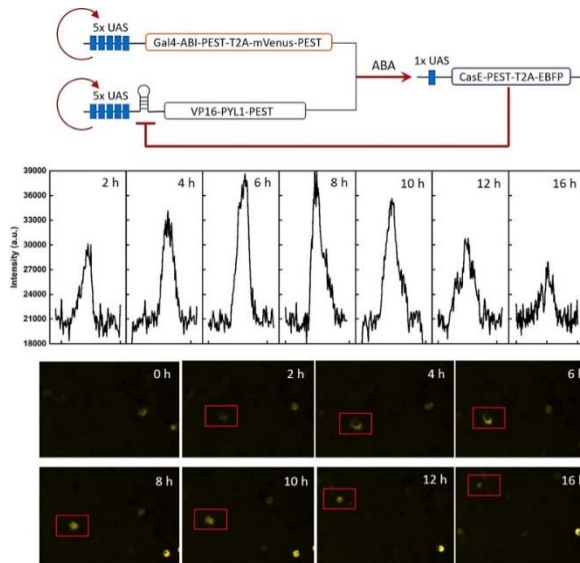
Cell-sorting based shapes and image analysis: We genetically engineered a variety of cell lines with different cadherin expression and co-cultured them in 3D aggregates. Using time lapse and confocal imaging, we tracked the formation of 3D patterns and shapes. By systematically varying different features of the aggregates including time of cadherin induction, cadherin type, ratio, and size, we established a series of rules that define the formation of self-assembling shapes. To computationally analyze these results, we built an image analysis pipeline using several tools including ImageJ and Fiji.

4. TECHNICAL RESULTS

Oscillator: For negative feedback circuits, gene expression oscillation usually occurs within a regime where gene expression level is intermediate, predicted by computational models. We use three types of activator genes/promoters to tune gene expression level over a wide range, which lead to three main phenotypes, as described below:

- DNA-launched replicon (DREP)-produced transactivator. Replicon serves as a self-amplification component. Transfected cells fall into bistable steady-state, exclusively expressing high level of one gene, either activator or repressor.
- Non-DREP self-amplification unit, such as 5x UAS_Gal4-VP16. Such self-amplification units are milder than DREP. While some cells show bistable behavior, some have both activator and repressor coexisting with intermediate levels.
- Non-amplification transactivator, such as hEF1a_Gal4-VP16. Due to tight repression, non-amplification transactivator-based circuits display low expression levels of transactivator and high levels of repressor during the observation period.

So far, most cells embedded with negative circuits fall into the abovementioned steady-states after an initial gene expression increase, yet a small population of cells with certain circuits show the damped oscillation. One example is displayed in the figure below.



3D synNotch Sender-Receiver: HEK293FT cells were either transfected with sender cell components such as hEF1a-CD19 or receiver cell components such as hEF1a-synCD19 tTA and the reporter circuit TRE-mKate and a transient transfection marker as described in the figure below. Receiver cells were then cocultured in 3D spheres with either sender or wild type cells. After sphere formation cells were then brought to a single cell suspension for collection of FACs via vigorous pipetting and pooling of cells from many wells to increase the population number. This FACs data was then converted to MEFL and performance was observed.

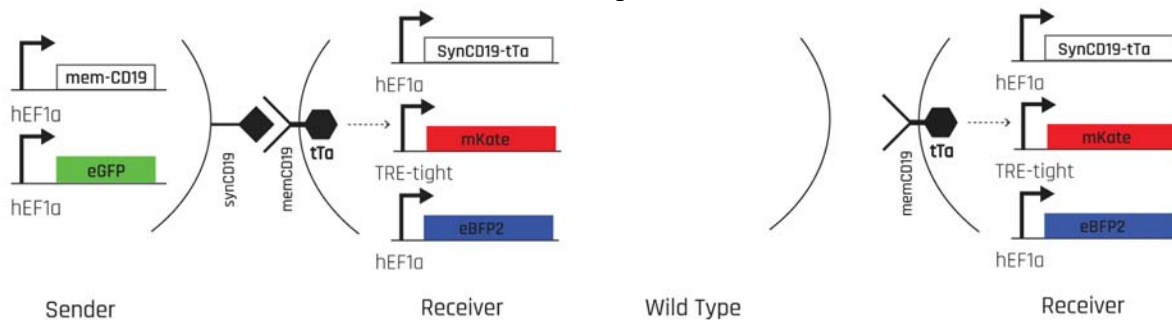


Figure: The left panel is of synCD19 sender and receiver circuits. The right panel is the experimental negative control: Wild type cells co-cultured with receiver cells.

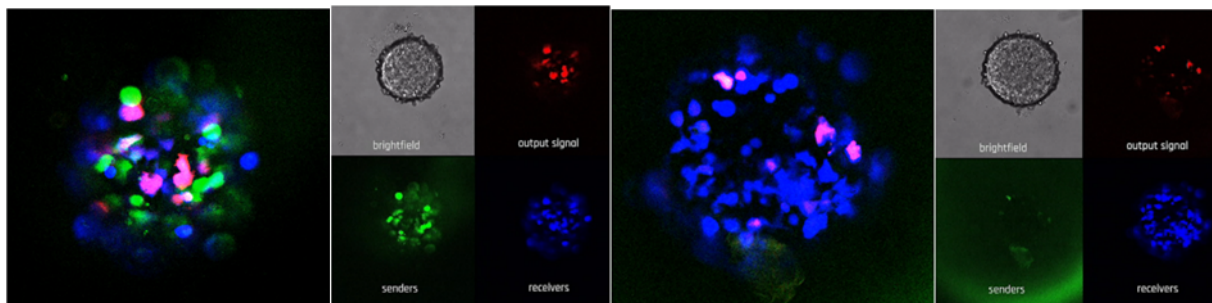


Figure: The left panel depicts the expression of 3 different color markers BFP, GFP, and mKate as a result of the co-culture of sender and receiver cells. Respectively, these markers correspond

to the receiver population, sender population, and the resulting downstream activation of the signaling output. The right panel is of the corresponding co-culture of wild type cells and receiver cells for a negative control.

3D synNotch Sender-Receiver in CHO:

FACS analysis of circuit performance:

With FACS analysis we identified the reporter signal expression for the co-culture experiments presented in **Fig. 2**, as compared to the control conditions (not shown). We next single sorted cells with reporter signal to arrive at monoclonal CHO1-LP synNotch cells. We performed this for both virus levels (high: level 1, intermediate: level 2). We next set out to measure the synNotch performance of 11 monoclonal cell populations from virus level 1, and 12 from virus level 2. To first check these cells for leaky mKate reporter expression we co-cultured them with wildtype HEK293 cells and using FACS assessed the mKate expression level. A representative FACS analysis of the mKate reporter signal for a monoclonal virus level 1 vs. virus level 2 cells (**Fig. 3**) shows that a larger population of a virus level 2 cells were turned ON (**Fig. 3B**). The positive controls also show that the reporter signal to leaky background expression was more significant for the virus level 2 monoclonal populations (**Fig. 3C**). Taken together, this data points to a larger dynamic range for the virus level 2 monoclonal cells as there is a larger intensity gap between the peak of the ON vs. OFF states.

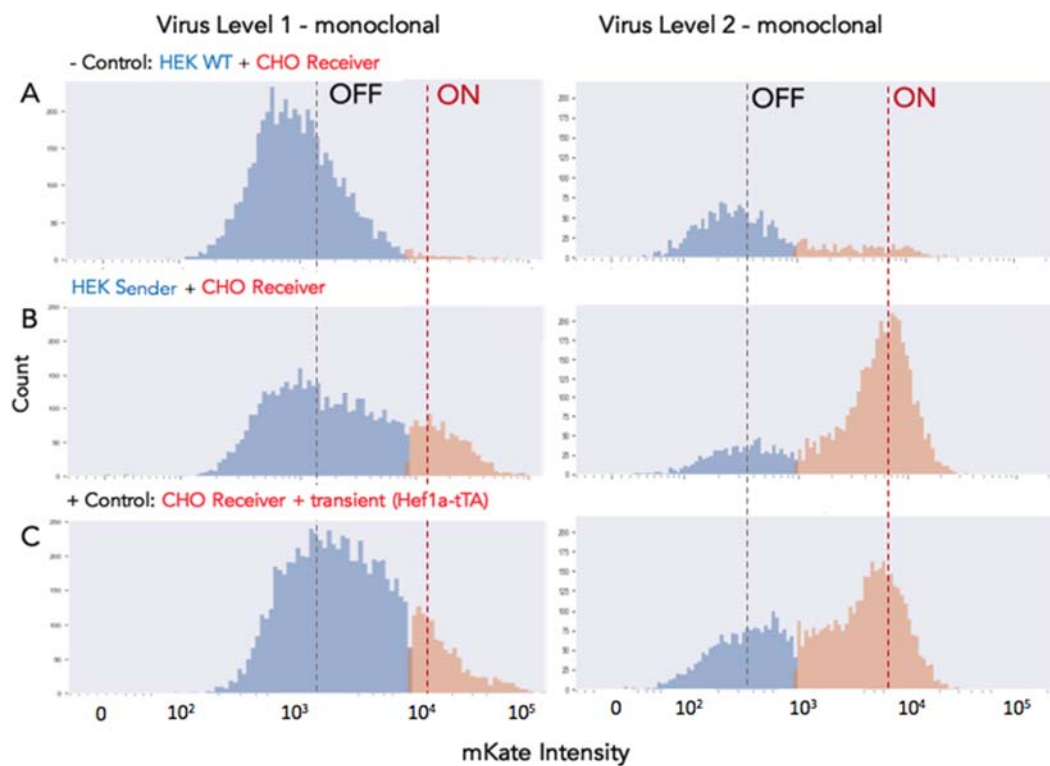


Figure 3. Monoclonal CHO1-LP synNotch assessment: **A.** Comparison of the leaky mKate expression in the CHO1-LP synNotch cells in co-culture with HEK293 WT cells shows a higher level of leakiness for the monoclonal cells infected with virus level 1. **B.** HEK293 sender cells transiently expressing Hef1a-CD19 turned on a larger population of the CHO1-LP synNotch cells towards a higher mKate expression level. **C.** CHO1-LP synNotch cells transiently

transfected with Hef1-tTA used as the positive control, also exhibited higher S/N for the virus level 2 monoclonal population.

Time-lapse imaging assessment of circuit performance:

In order to test for the integration of different components of the synNotch receiver cells we co-cultured monoclonal sender and receiver CHOK1 cells and analyzed the circuit performance via timelapse images. After testing various co-culture experiment schemes, we established a relatively robust protocol to observe the reporter signal (**Fig.4**): We first seeded sender cells inside the 6mm-diameter culture ring placed in one well of a 12-well plate. Then incubated at 37 °C for 12 hrs, followed by seeding the receiver cells outside of the ring. We then incubated the co-culture at 37 °C for an additional 20 min prior to removing the ring and subsequent imaging with Cytation 5 imaging reader.

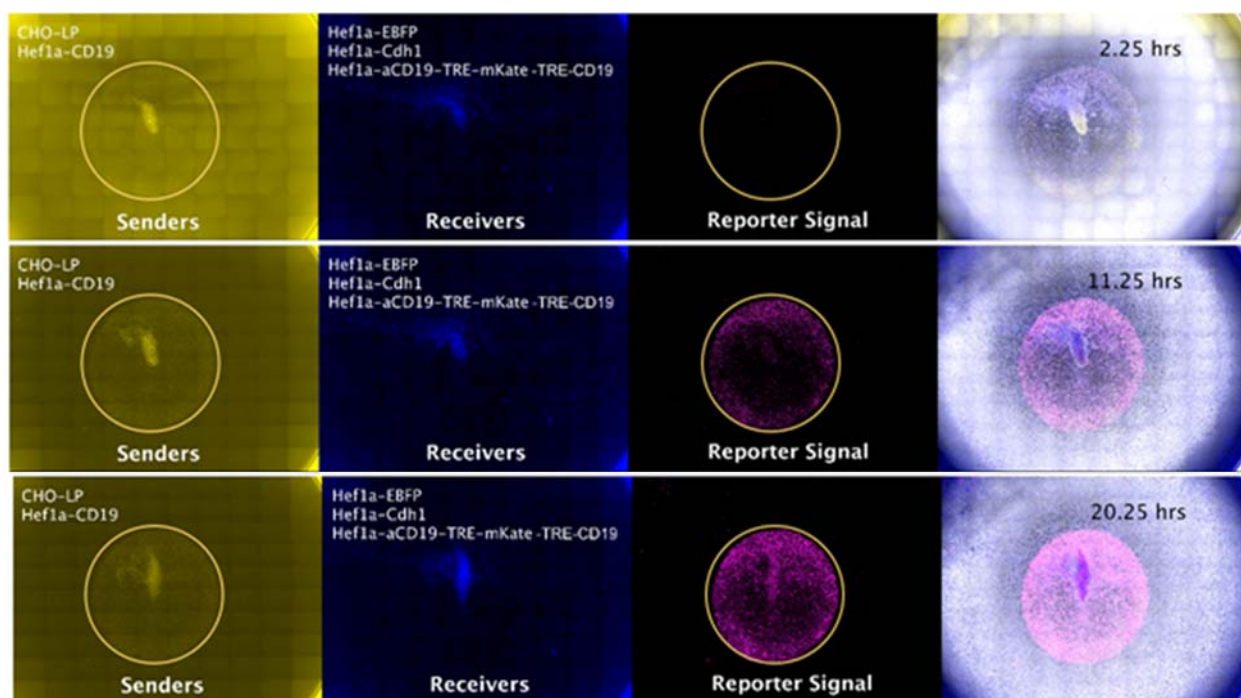


Figure 4. Co-culture of senders and receivers in patterns for time-lapse microscopy. CHOK1-LP sender cells expressing CD19 are seeded in the inner ring (depicted by yellow), while the receiver cells were seeded in an indiscriminate fashion inside the well. Within 20 hours post-co-culture the entire population of receiver cells reported mKate expression.

We recently explored patterning the synNotch CHO cells in a 3D collagen matrix in order to assess propagation. One such scheme is presented in **Fig. 5**. The sender CHO cells seeded in the central discoidal patterns engulf the receiver cells patterned in a cross shape. Within 12 hrs, the mKate reporter signal turns on and propagates along the region where no sender cell is present. At the red square mark, where the receiver cells are disjointed from the rest of the population, the mKate expression is not present at 12 hrs, highlighting the likelihood of signal propagation. Further controls, and time-lapse images are currently being explored. We are further

investigating the extent of the reporter signal leakiness. If considerable, we will devise alternative circuit design strategies to reduce this background.

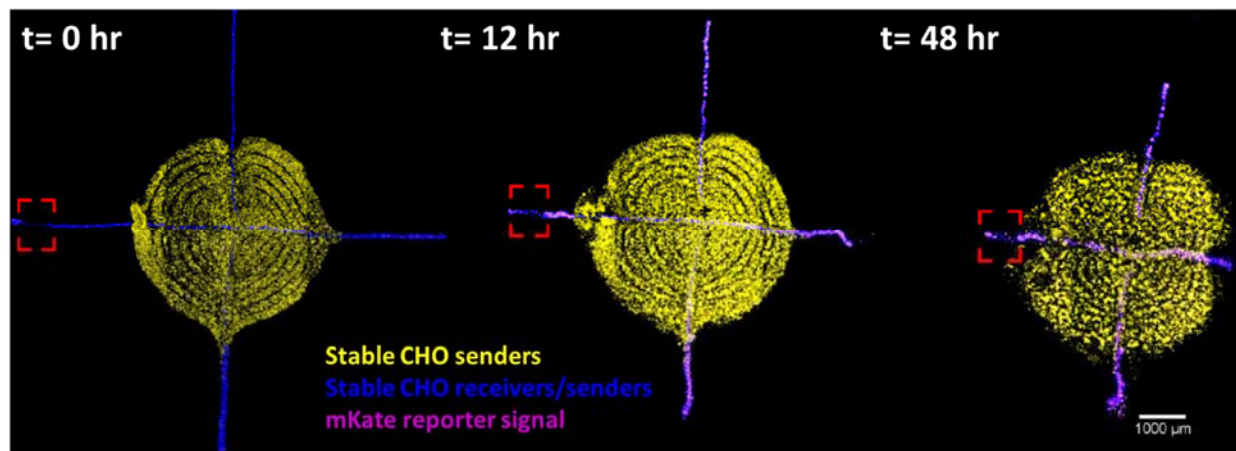


Figure 5. Co-culture of senders and receivers in patterns for propagation assessment via imaging. CHOK1CHOK1-LP sender cells (yellow) expressing CD19 are seeded in the central discs, while the receiver cells (blue) were engulfed within the senders in a cross shape. Within 12 hours post-co-culture the mKate reporter expression is observed beyond where the receiver/original sender contact site. The red square marks where the receiver population is seeded at a gap from neighboring cells.

Circuit design and optimization of the phloretin systems: In the end we were able to show greater than 10-fold induction in HEK293FT cells; however, our latest preliminary results in CHO cells suggest the system may behave quite different in CHOK1 cells. There was less overall activation in CHO cells, likely due to differences in signaling efficiency.

Computational model: Technically, our simulations described disk-like agents moving with constant magnitude of velocity along the direction determined by the external forces. In particular, assuming the interaction potential between the cells due to adhesion and steric repulsion, random forces, and the external force due to gravity, we were able to calculate forces on all cells at each simulation step. These forces were then used to determine the changes in the directions of cell movements. In particular, we assumed that at any given time, each cell moves along the direction of the net force acting on it. We used our model to simulate cell sorting in two-component cell aggregates. Assuming the above dynamics, we started with a well-mixed initial configuration and observed cell sorting under different conditions. In particular, we varied the adhesion strengths between the two cell types, total cell count, and the composition of the aggregate, i.e. fraction of type-1 vs. type-2 cells and quantified the structure of the cell aggregate by measuring the number and sizes of clusters of a given cell type.

Cell-sorting based shapes and image analysis: By computing statistics over all clusters and replicates, we found systematic relationships between both mean area and mean velocity and between time and mean velocity. We hypothesize that this may indicate cluster formation can be viewed in terms of random walks driven perturbation of clusters by unbound cells, which thus reduces as the clusters grow in size.

5. IMPORTANT FINDINGS AND CONCLUSIONS

Oscillator: Negative feedback circuits, resembling the circadian rhythm in the human body, have small windows to behave as robust oscillators or damped oscillators. Yet experimentally searching for those windows for each circuit is nontrivial. Sophisticated methods should be applied to meet the complexity.

3D synNotch Sender-Receiver: We compared our 3D culture FACs values to their corresponding 2D culture FACs values and found improvement in HEK293FT synNotch signaling via 3D culture of senders and receiver as depicted in the figure below.

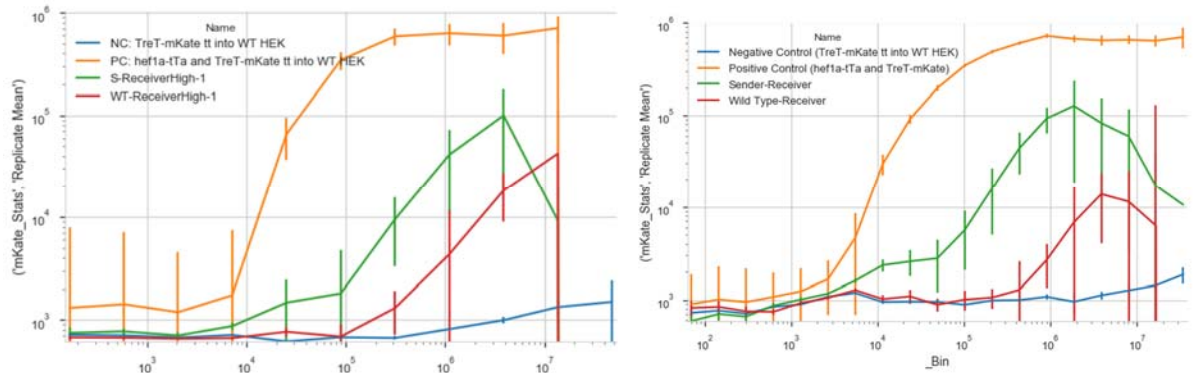


Figure: The left panel is the 2D synNotch sender-receiver experiment. The right panel is the 3D synNotch sender-receiver experiment.

Circuit design and generation of stable synNotch cell lines in HEK293FT cells: As depicted in the figure below we have planned to test this with one of Cadherin derived shapes for further symmetry breaking.

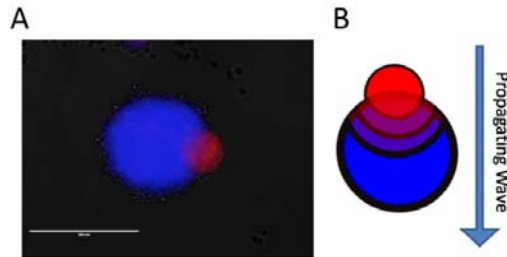


Figure: Panel A Illustrates the polarity we can achieve by mixing Cadherin 6 and Cadherin 1 with just enough cells to create only two separate spheres. The blue sphere is E Cadherin (CDH1) expressing, and the red sphere is K-Cadherin (CDH6) expressing. Panel B illustrates the propagating wave we plan to build from this polarity.

Cadherin based circuits for CHOK1 cells were also designed however initial tests suggested stable integration allow was sufficient to increase CHOK1 synNotch performance.

Circuit design and optimization of the phloretin systems: Phloretin is the first completely orthogonal mammalian system capable of both generating and responding to in own freely diffusible small molecule. Due to the quorum sensing-like nature of this system, development of this system should open up a whole field of pattern generating circuits currently available in bacteria making them usable in mammalian systems.

3D synNotch Sender-Receiver in CHO: Considerations of circuit leakiness, sender/receiver seeding population and patterning are the key experimental factors that need to be fine-tuned for synNotch performance consideration. We have successfully engineered both the sender and receiver circuits in CHOK1 cells and have arrived at monoclonal populations of each. Preliminary results highlight the likelihood that we have further engineered signal propagation which we are now actively exploring and characterizing.

Computational model: We found that the adhesion strengths alone give a wide range of cell configurations in two-components cellular mixtures. In particular, we observed that the final cell configurations, i.e. after the sorting period, form either completely sorted states (a single cluster of strongly-adherent cells engulfed by weakly-adherent cells), completely dispersed states (strongly-adherent cells are dispersed and individually separated by weakly-adherent cells), partly sorted transient states (many clusters of strongly-adherent states surrounded by weakly-adherent cells), or trapped states where sorting cannot occur due to strong adhesion and thus limited cell movements.

Next, by varying the overall composition of the aggregate, we were able to recapitulate an abrupt transition from the multi-cluster regime, which was observed experimentally at low fractions of strongly-adherent cells, to a single-cluster regime, observed at high fractions. This confirmed the validity of our model, which not only captures different configurations of two-component aggregates based on differential adhesion, but can also predict how these configurations change upon varying the composition. Furthermore, our model predicted, that the structure of these aggregates can be manipulated by varying the total cell count. In particular, our simulations showed that the aggregates in the multi-cluster regime increase the number of clusters with the total cell count approximately linearly. This prediction was tested and confirmed by experiments and further supported by simple dimensional-analysis-based scaling arguments.

Shape compiler: With the compiler, we have incorporated a modular differentiation circuit, so that a differentiated region can further differentiate again. This works by a competitive recombinase-based switch that differentiates a region into two cell strains, one of which expresses an adhesion protein while the other does not. We have then further modified this switch template to be able to be triggered by the output of another switch, which will allow specification of structures with an arbitrary number of cell types.

Cell-sorting based shapes and image analysis:

We analyzed self-organizing cadherin-based shape formation through a variety of lenses. We completed analysis of the size of the features to show that the theory of percolation was a valuable framework to view pattern formation. Additionally, we performed deeper analysis of the statistics of tracked cell clusters over time. We found that area goes through a transition of first compacting, then growing, presumably as the cells are dividing over time. Velocity showed a clear correlation with cluster size. Both of these have strong trends associated with the percentage of high-adhesion cells as well.

6. IMPLICATIONS FOR FUTURE RESEARCH

Oscillator: We are currently working on using “one-pot” transfection and single cell tracking to generate matrices based on large cell populations, where copy number ratios of activators and repressors are nearly continuously changed, and the dynamics are tracked. By doing so, we hope to gain great insights into negative feedback circuits in mammalian cells and potentially find oscillation windows.

3D synNotch Sender-Receiver: As the 3D coculture improved system performance we went on to test the system performance in CHOK1 cells. Future research should take into careful consideration the difference in efficiency and behavior between 2D and 3D systems.

Circuit design and optimization of the phloretin systems: As the project was coming to a close we decided to put future effort into optimization of induced pluripotent stem cells, iPSCs, instead of CHOK1 cells. Future research should focus on identifying specific causes for differences between the efficiencies and signaling strengths of different cell lines.

3D synNotch Sender-Receiver in CHO: Assessing how the synNotch performance changes as a function of cadherin expression was explored, however detailed characterization and comparison between Cdh1+ and Cdh1- CHO cells remains. We are also envisioning wiring the reporter signal to a physiological output. This allows us to engineer biological functions such as propagating matrix deformation or cell differentiation in desired patterns mimicking a biological context.

Computational model: The most important result of our research was to show that the structure of two-component cell aggregates can be manipulated in a controlled and predicted way by varying physical parameters such as total cell count and the composition of the aggregate. This result is important, because it is often easier to experimentally control these parameters rather than target particular values of adhesion strengths, which depend on molecule-scale details. In the future, we would like to extend our approach to multi-component tissues to achieve more complex structures and shapes of cell aggregates and possibly generalize our approach to three dimensions. Finally, it would be interesting to use similar approach using a more detailed representation of the tissue, where shapes of individual cells could be resolved and forces would be described at the subcellular level.

Shape compiler: Future research in self-assembling shape formation can use our shape compiler to design and simulate the construction of living shapes. The compiler could be expanded in many ways, including by adding other morphogenetic features like directional cell movement and conditional proliferation. With these features, more varieties of shapes could be designed and generated, and the principles underlying living shape formation could be examined.

Cell-sorting based shapes and image analysis: We have demonstrated that cell sorting is a powerful tool that will be useful in further research and generation of self-forming shapes. With the rules of self-assembly we have established, future research could build more complex shapes with more sophisticated temporal and spatial control of pattern. Our image analysis pipeline can be further used to analyze other self-forming aggregates, and would facilitate the numerical analysis of synthetic morphogenesis.