Biomolecular Breadboards for Prototyping and Debugging Synthetic Biocircuits

University of Minnesota

Richard Murray **Paul Rothemund** **Vincent Noireaux**
California Institute of Technology **U. Minnesota**

Clarmya Hayes Mikhail Hanewich-Hollatz Victoria Hsiao
Jongmin Kim Dan Siegal-Gaskins Vipul Singhal Zachary Sun
Anu Thubagere Zoltan Tuza Enoch Yeung

DARPA Living Foundries West Coast Regional Meeting
25 October 2012

Outline

I. Project overview
II. Technical results: TX-TL, biochemical wires, artificial cells
III. Next steps
IV. Summary of collaborations

Project Goals and Approach

Goal: >10X reduction in design cycle time

- Breadboard-based prototyping
- Parallel testing
- Predictive models

Cell-free (TX-TL)

- *E. coli*-based extract
- Linear DNA (PCR)
- Cheap: $0.03/ul

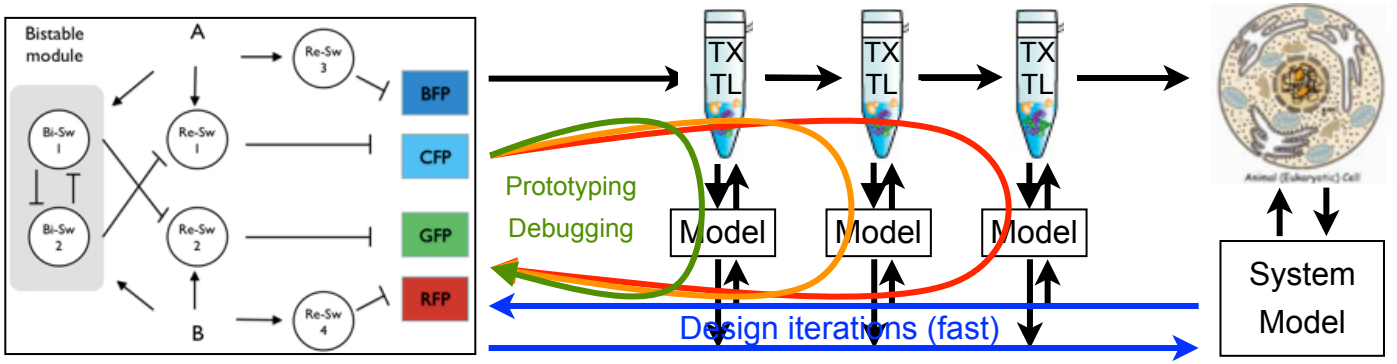
Biochemical wires

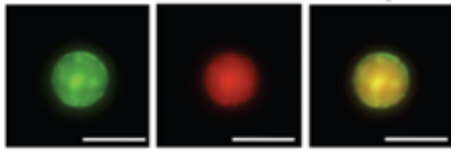
- Spatially localized reactions
- 10 um origami

Artificial cell

- Spatial constrained reactions
- 1-1000 fL volumes

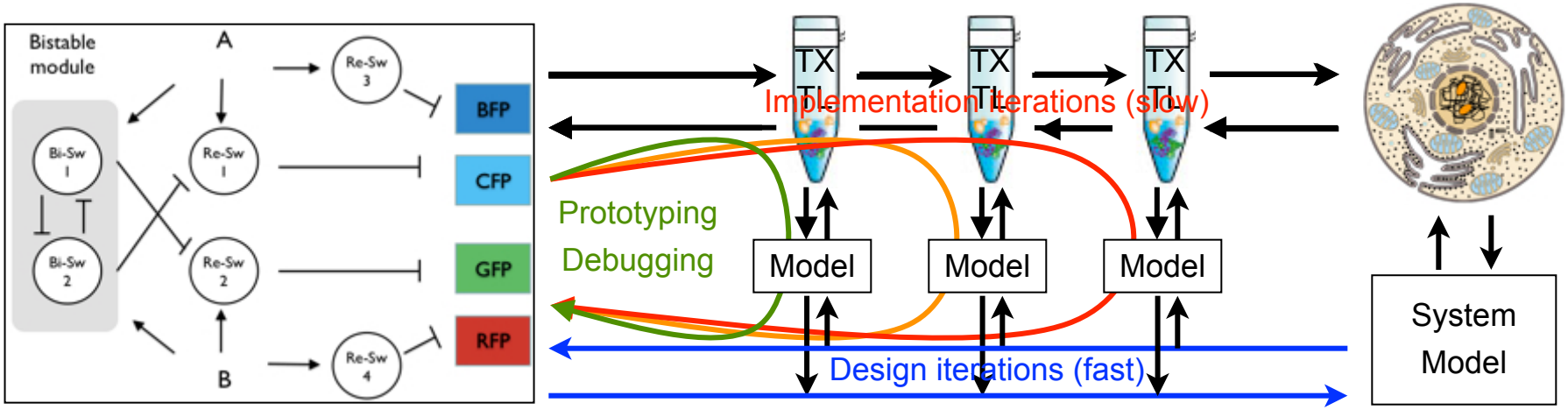
Program metric	Current	Phase I	Phase II
1. Time required to go from synthesized DNA sequences to measurement of circuit performance (on cell-free and origami breadboards)	1-2 wk	3 days	1 day
2. Time required to build a novel, modest complexity (6–8 unique pro- moter) circuit (existing design, novel components)	3-6 mo	1 mo	1 wk
3. Number of circuits that can be tested simultaneously, varying component concentration and/or cell-free toolkit parameters	5	25	100
4. Number of genes and regulatory parts characterized, modeled and available for use in cell-free circuits (and artificial cells)	2	5	10





http://www.openwetware.org/wiki/breadboards

Task 1.1: Cell-Free Circuits Breadboard



Key characteristics of the cell-free breadboard (Noireaux et al)

- Inexpensive and fast: ~$0.03/ul for reactions; typical reactions run for 4-6 hours
- Easy to use: works with many plasmids or linear DNA (PCR products)
  - Can adjust concentration to explore copy number/expression strength quickly
- Flexible environment: adjust energy level, pH, temperature, degradation

Milestones/demo for Phase I http://www.openwetware.org/wiki/breadboards

✓ • Q1: Post protocol on web, along with controls + summary of costs

80% • Q2: Demonstrate breadboard on 2 circuits (eg, switch, IFFL), document iteration time

50% • Q3: Post complete protocols + variations (degradation, energy, ...) + validated models

10% • Q4: Demonstrate design of 6-8 promoter circuit with 3 day cycle time, 1 month total

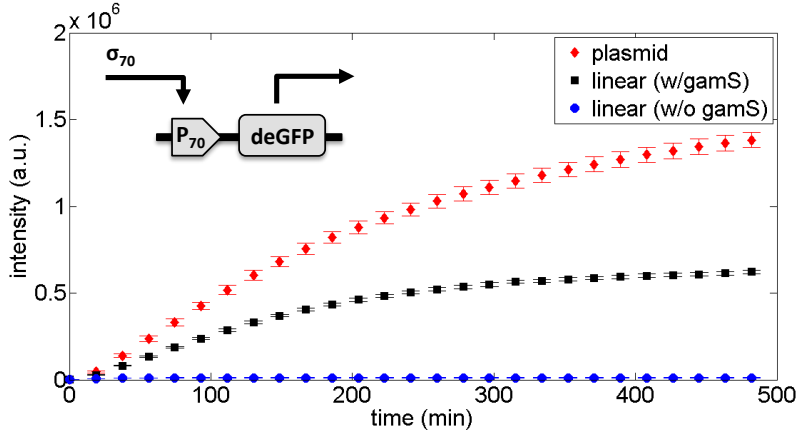
Phase II demo: 8-16 promoter circuit, 100 variations, 1 day cycle time, 1 week total

TX-TL Core Processes

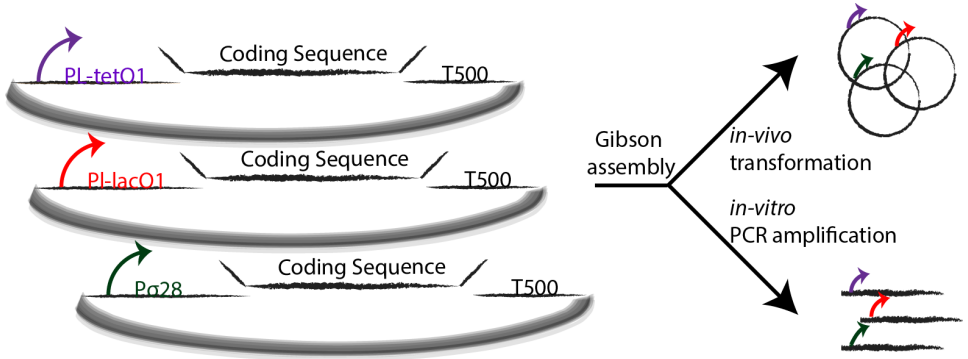
Zachary Sun, Vincent Noireaux

Rapid prototyping using linear DNA

- Use PCR products with GamS to get expression levels of ~60% of plasmid

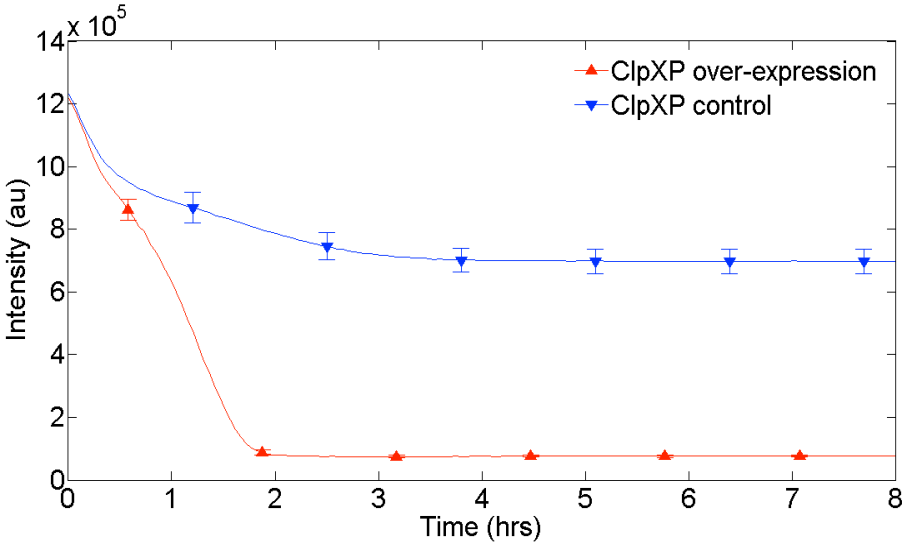


- Allows rapid assembly of constructs
  - PCR extension for simple circuits
  - IDT gBlocks + isothermal ass'y



Protein degradation

- Use clpXP machinery to degrade tagged proteins



Tested components

- RNA polymerases: E. coli*, T7
- Activators: sigma28*
- Repressors: tetR*, lacI*
- Reporters: deGFP*, MG, mSpinach
- DNA/RNA/protein deg: gamS*, clpXP*

* preliminary models also available

TX-TL Protocols, Documentation and Dissemination

Zachary Sun, Clarmyra Hayes, Vincent Noireaux

Online resources

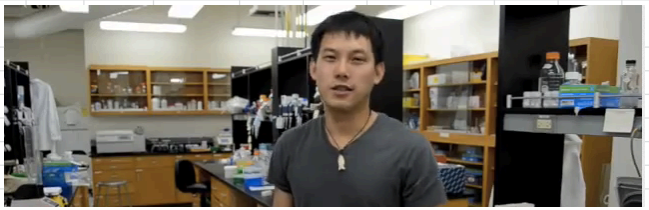
- Protocols + spreadsheet
- Test constructs
- Sample results
- Instructional videos
- Modeling library
- Cost analysis

41 Master Mix Preparation

42	Ingredient	Stock*	Final*	Master Mix (uL)
43	B2.X (buffer)	1	0.489	41.81
44	E2 (extract)	1	0.261	22.32
45	()			
46	()			
47	()			
48	()			
49			*Input as ratio	

50 DNA Prepa

51	ID
52	1
53	2
54	3
55	4
56	5
57	6
58	7
59	8



Component	Material price per µL of reaction	Labor price per µL of reaction	Total price per µL of reaction	Total price per 12 µL reaction
E. coli crude extract	0.45 cents	0.62 cents	1.07 cents	12.84 cents
Amino acids	0.21 cents	0.03 cents	0.24 cents	2.93 cents
3-PGA	0.39 cents	0.94 cents	1.33 cents	15.96 cents
Total price	1.05 cents	1.6 cents	**2.64 cents**	**31.7 cents**

Cycle time comparisons

- Standard cloning of genetic switch (from scratch), with 2 iterations: 10-14 days (est)
- Plasmid based breadboarding (from scratch), with 2 iterations: 6 days (measured)
- Linear DNA assembly, using PCR extension, with 2 iterations: 2 days (measured)

Circuit testing

- Testing out TXTL on multiple circuit technologies
- Stage 0: send us cells/plasmids
- Stage 1: we perform TXTL runs, send back data
- Stage 2: we send extract, you take the data
- Stage 3: we show you how to make extract
- Stage 4: you use TX-TL on your own

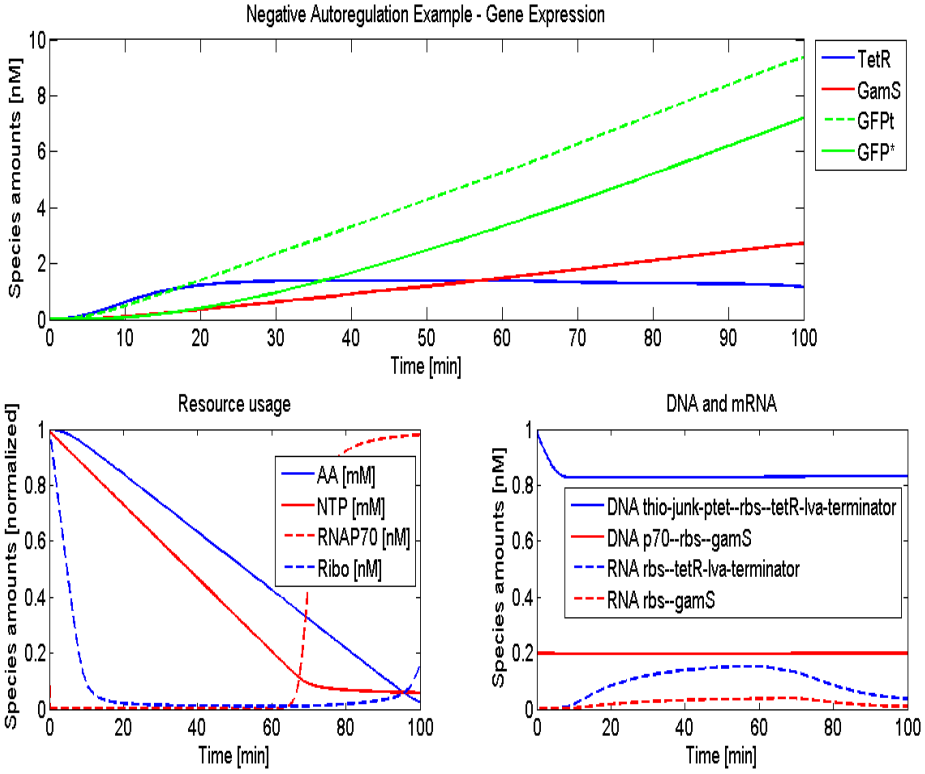
PI (+ contact)	Circuit/Technology	0 1 2 3 4
Lucks (CH)	RNA-sensing TFs	✓✓○ - -
Del Vecchio (EY)	Loading effects	✓ ○ - - -
Temme (VH)	Orthogonal RNAPs	✓ ○ - - -
Voigt (DSG)	4 input, 11 gene	✓ ○ - - -
[Tabor (JK)]	Green light sensor	✓ ○ - - -
Hutchison (ZS)	DNA replication	○ - - - -

TX-TL Modeling

Zoltan Tuza, Vipul Singhal, Dan Siegal-Gaskins

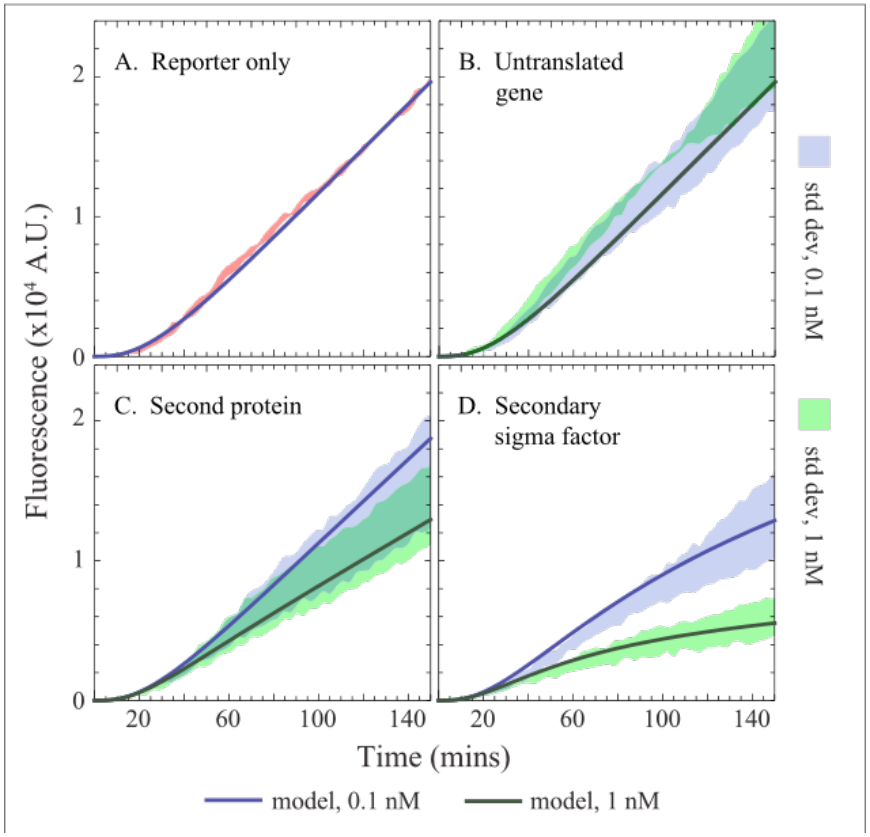
MATLAB toolbox (sf.net/projects/TXTL)

```
% Set up the standard TXTL tubes
tube1 = txtl_extract('e1');
tube2 = txtl_buffer('b1');

% Set up a tube that will contain our DNA
tube3 = txtl_newtube('circuit');
dna_tetR = txtl_dna(tube3, 'ptet', 'rbs', 'tetR', 100, 'linear');
dna_gamS = txtl_dna(tube3, 'p70', 'rbs', 'gamS', 10, 'plasmid');

% Mix the contents of the individual tubes and add some inducer
well_a1 = txtl_combine([tube1, tube2, tube3], [6, 2, 2]);
txtl_addspecies(well_a1, 'aTc', 0.1);

% Run a simulation
[t_ode, x_ode, names] = sbiosimulate(well_a1);
```



Resource utilization effects

- Model+TXTL shows effects of fixed number of RNAPs and ribosomes
- Additional sigma factor gene introduces significant 'crosstalk', reduces output
- Siegal-Gaskins, Noireaux & M, American Control Conf 2013 (submitted)

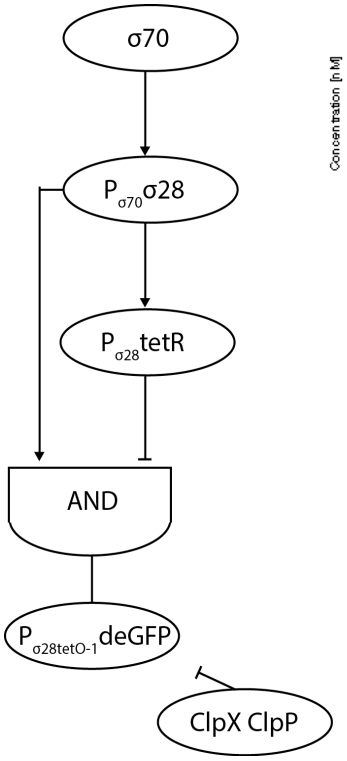


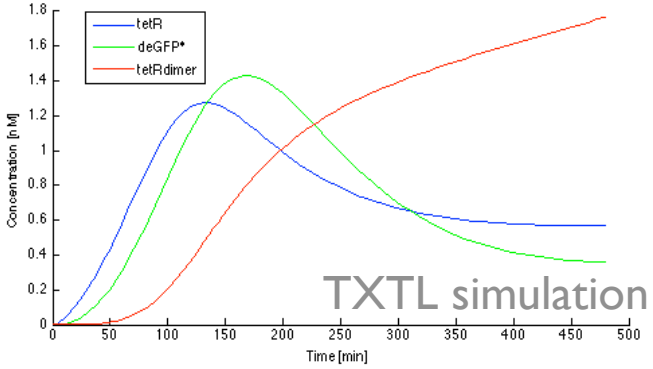
TX-TL Circuit Design

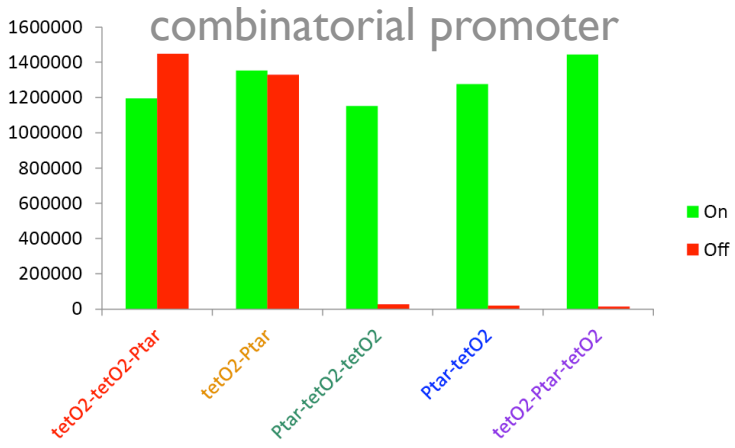
Zachary Sun, Zoltan Tuza, Dan Siegal-Gaskins

Incoherent Feedforward Circuit

- Designing feed forward loop to test protein degradation up regulation
- Simulation results using TX-TL toolbox
- Design of combinatorial promoter, tested in TX-TL assay

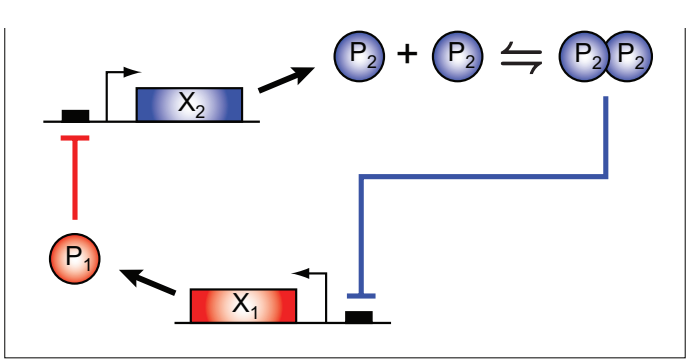


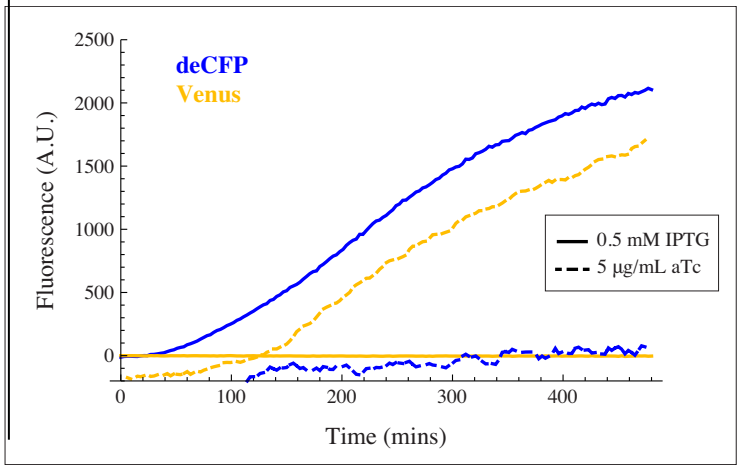




Genetic Switch

- 'Toggle' variant that uses a monomeric repressor is predicted to be bistable
- TetR-TetR fusion protein was built and tested, results suggest bistability
- Circuit implemented and working in TX-TL



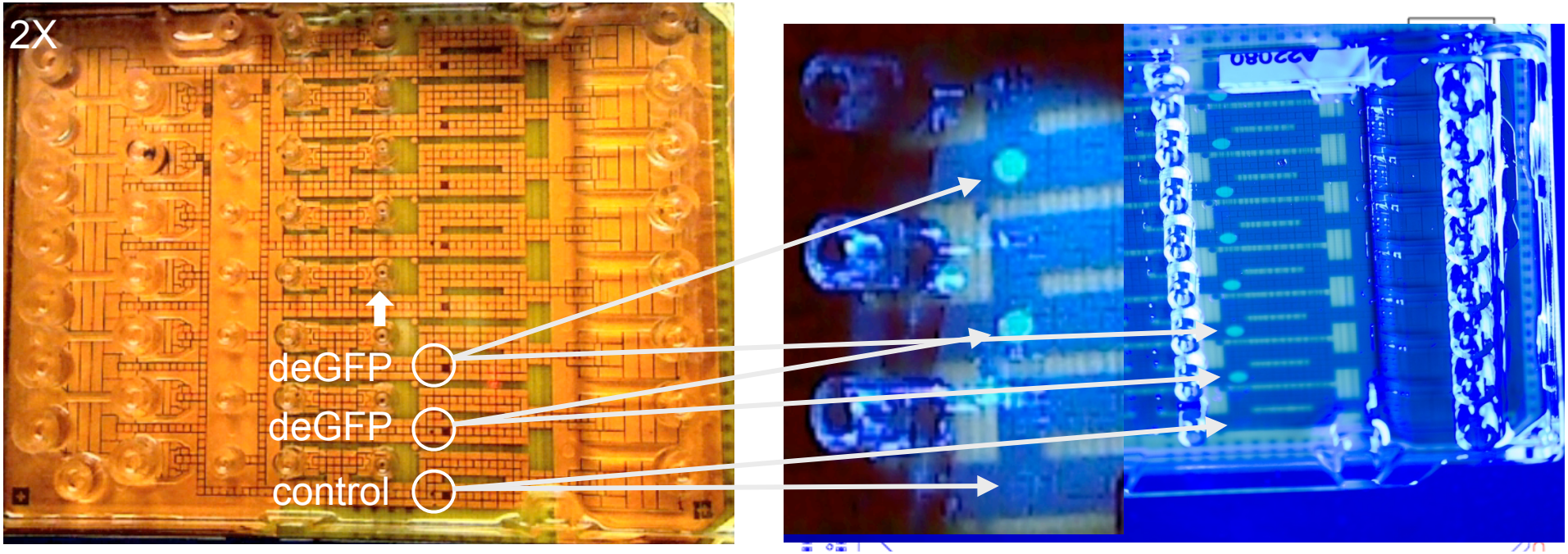


Droplet-Based TX-TL w/ Advanced Liquid Logic Analyzer

Enoch Yeung, Clarmyra Hayes, Jongmin Kim, Zach Sun + Lisa Bukovnic, Sri Punnamaraju, Nick Trotta

Merging cell-free breadboard and Advanced Liquid Logic droplet system:

- Enables rapid prototyping of circuit performance in small volume regime.
- Spatial manipulation of droplets allows for spatial modularity of reaction volumes:
  - merging droplets allows for infusion of new fuel molecules to extend the lifetime of a breadboard reaction indefinitely.
  - splitting a droplet of reaction mixture simulates division of artificial “cells” and RNA/ protein dilution



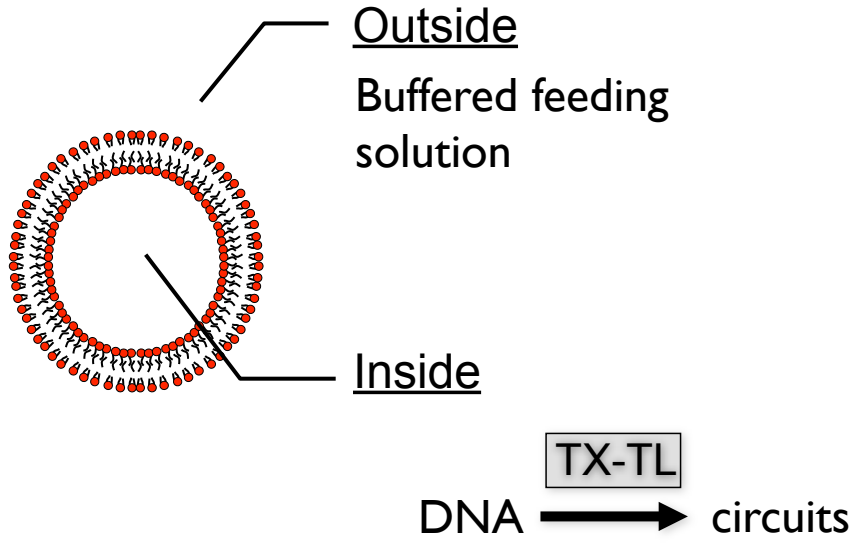
Task 1.2: Artificial Cells

Demonstrate the feasibility of a programmable synthetic phospholipid vesicle system with elementary synthetic gene circuits

- Create cell-sized (1-50 μm diameter) synthetic phospholipid vesicles containing TX-TL system and genetically encoded circuits
- External inducers (tetracycline, arabinose, ...) will diffuse through the membrane and activate the circuits or repress expression of fluorescent protein reporters

Approach

- Demonstrate stable synthetic liposomes capable of hosting transcriptional activation and repression units
- Demonstrate activation and repression units that can be turned on and off using inducers diffusing through the membrane (arabinose, lactose, tetracycline, …..)



Milestones/demo for Phase I

✓ • Q2: Investigators will demonstrate stable synthetic liposomes capable of hosting transcriptional activation and repression units

80% • Q4: Investigators will demonstrate activation and repression units that can be turned on and off using inducers diffusing through the membrane

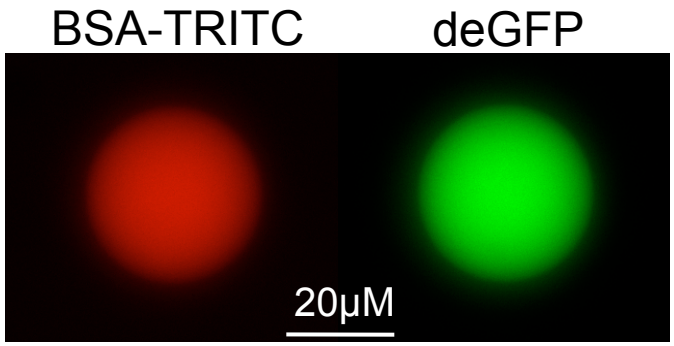
Inducible expression inside vesicles

Vincent Noireaux

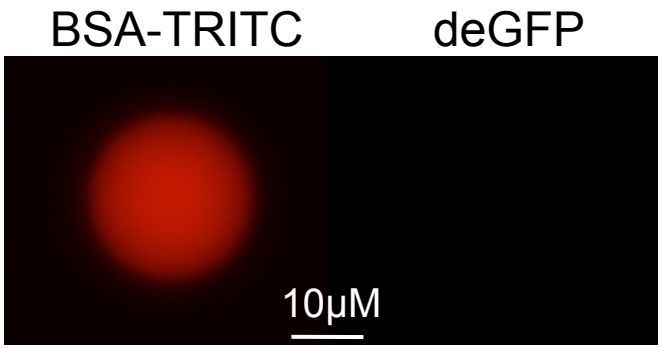
8 hr incubation, 40X

AraC

- pBAD promoter with AraC acting as repressor
- Arabinose inducer



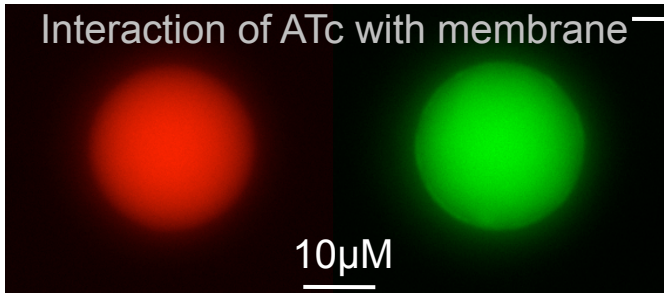
+ 10mM arabinose in feeding



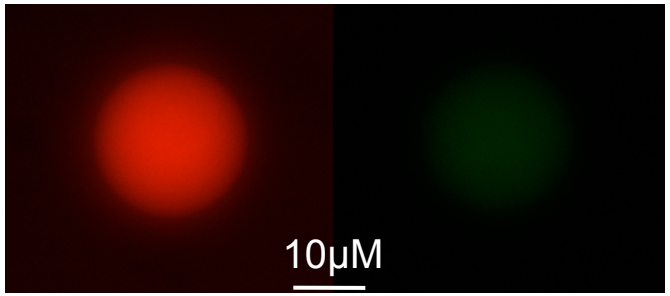
+ 0mM arabinose in feeding

TetR

- ptet promoter with TetR acting as repressor
- ATc inducer



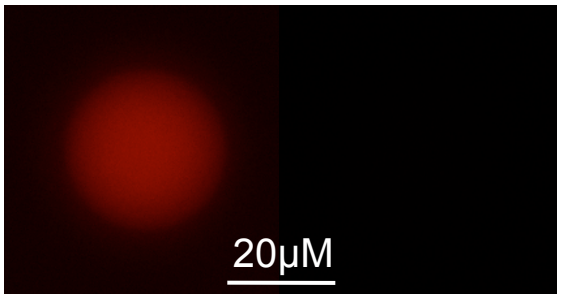
+ 20μM tetracycline in feeding



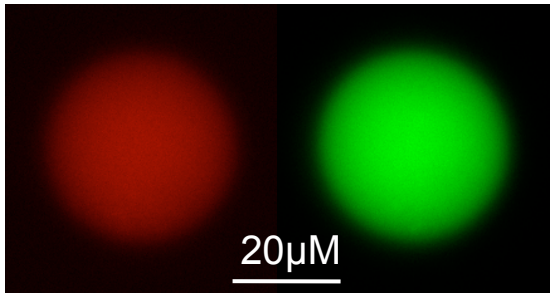
+ 0μM tetracycline in feeding

Melibiose

- pMelAB promoter with MelR acting as activator
- Melibiose inducer



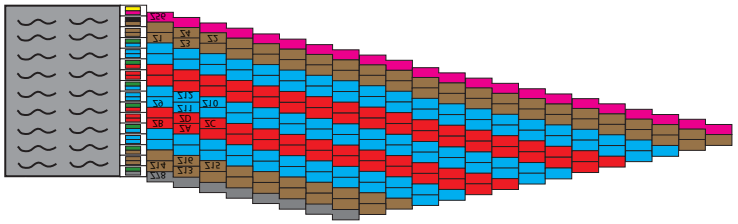
- 0 mM melibiose in feeding



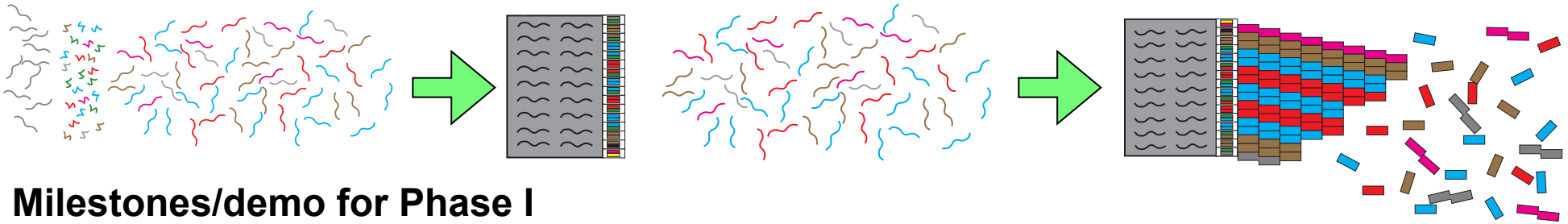
+ 10mM melibiose in feeding

Task 1.4: Biochemical Wires

Goal: demonstrate assembly of origami "wires" that spatially constrain reactions



- Create ~10 micron linear DNA structure with differentiated end that can serve as signal source
- Use genelets localized to the origami to generate RNA transcript signals.
- Use fluorescent reporters on ribbon-wires to measure passive signals.
- Compare to active signal propagation mediated by bistable genelets along the length of the wire.



Milestones/demo for Phase I

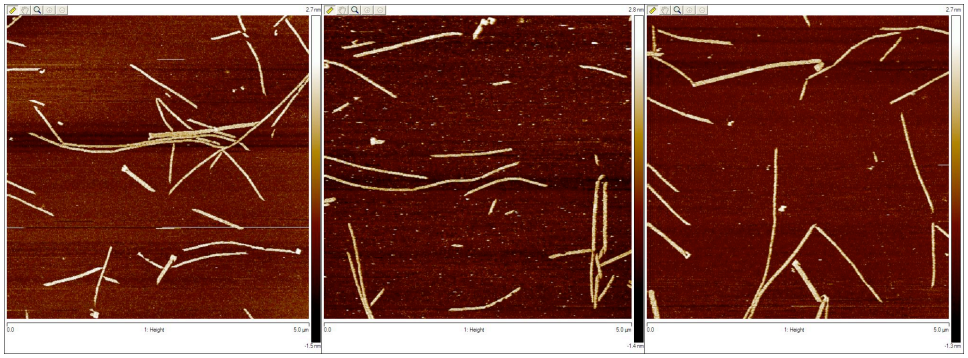
50%
- (Q2) Demonstrate high-yield assembly of fluorescent origami/ribbon wires; optimize light microscopy conditions to allow simultaneous visualization of origami and ribbon
- (Q3) Demonstrate operation of transcriptional elements (genelets) on DNA origami as signal source. Demonstrate diffusive propagation along DNA origami/ribbon wire
- (Q4) Demonstration of a directly activatible genelet system that will allow efficient (non-diffusive) signal propagation along an origami/ribbon wire.

Preliminary Results: Biochemical Wires

Paul Rothemund, Mikhail Hanewich-Hollatz

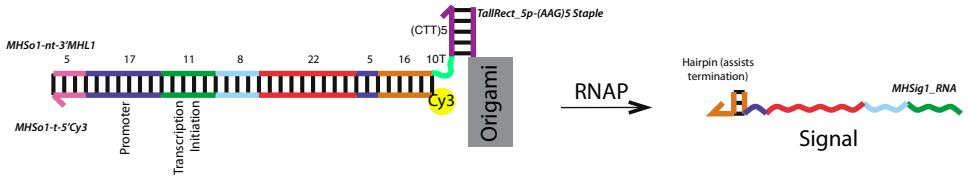
Growth of fluorescent origami-ribbons

Unlabeled **End-label** **Internal label**

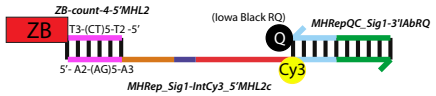


(5 micron AFM fields.)

Design of genelet / signal transcript.



Reporter:



MHSo1-nt-3'MHL1 (Sense): 5'- CACAC TAATACGACTCACTATA GGGAGAAGGAG AGGCGAAG ATTGAGGTAAGAAAGGTAAGGA TAATA CTGACAAAGTCAGAAA TTTTTTTTT CTTCTTCTTCTTCTT -3'

MHSo1-t-5'Cy3 (Template): 5'- /5Cy3/ TTTCTGACTTTGTCAG TATTA TCCTTACCTTTCTTACCTCAAT CTTCGCCT CTCCTTCTCCC TATAGTGAGTCGTATTA GTGTG -3'

MHSig1_DNA (DNA Signal): 5'- GGGAGAAGGAG AGGCGAAG ATTGAGGTAAGAAAGGTAAGGA TAATA CTGACAAAGTCAGAAA -3'

ZB-count-4-5'MHL2 (Modified Tile Sticky End): 5'- TTTCTCTCTCTCTTT ATGACGGATACGGTCAACCACGTCAT -3'

MHRep_Sig1-IntCy3_5'MHL2c (Fluorescent Reporter): 5'- AAAGAGAGAGAGAAA TTTCTGACTTTGTCAG TATTA TCCTTACCTTTCTTACCTCAAT /iCy3/ CTTCGCCT CTCCTTCTCCC -3'

MHRepQC_Sig1-3'IAbRQ (Quencher): 5'- GGGAGAAGGAG AGGCGAAG /3IAbRQSp/ -3'

Other progress:

- Designed dye-quencher reporter for measuring signal propagation
- Designed and tested two linkers for attaching reporters and genelets to wire

Problems:

- First linker system resulted in no wire growth from origami
- Second linker system resulted in poor wire growth from origami and a large amount of undesired DNA crystals

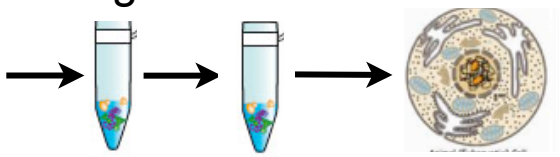
Solutions:

- Determine how linkers affect wire melting temperature relative to that of unlabeled wires. Then…
- Optimize annealing protocol -or-
- Switch to tubes that are less sensitive to modification with linkers

Upcoming Technical Work and Goals

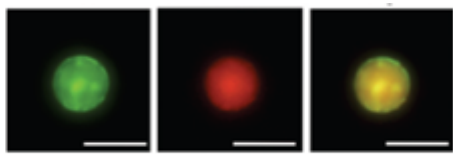
Cell-free breadboard

- Linear DNA assembly (build on work of others)
- Implement/test 6 circuits
- Document design cycle times (vs std cloning)
- Extract preparation video (→ JoVE?)
- Predictive, modular models for switch, IFFL, neg fbk



Artificial Cells

- Kinetics of expression inside vesicles
- Statistics of expression and induction (% of vesicles induced)
- Expression (and induction) as a function of vesicle size



Biochemical Wires

- Optimize assembly of origami wires w/ linkers
- Test signal carrying ability by hybridizing and dehybridizing fluorescent reporters
- Demo transcription of signal from genelets on microscope slides.

Open source information

- TX-TL protocols, data, tools: http://www.openwetware.org/wiki/breadboards
- TX-TL modeling library: http://www.sourceforge.net/projects/txtl
- TX-TL announcements mailing list: http://groups.google.com/d/forum/txtl-announce

Collaborations and Needs

TX-TL circuit testing

- Cornell (Lucks)
- Pivot Bio (Temme)
- MIT (Voigt)
- MIT (Del Vecchio)
- Hutchison (JCVI)
- Hoping to make all TXTL data available on internal LF website

Laboratory automation

- Stanford/Advanced Liquid Logic
- Visiting ALL on 8 Nov to work through details of TXTL based protocols
- Hoping to make use of Stanford protocols as they become available

Living Foundries web site

- Boyden, Lucks, Murray
- Draft site set up
- Would like to use for posting presentations (east/west coast LF meetings) + internal data
- Looking for volunteers to help test & maintain

What we need from others

- Help in trying out the protocols and identifying things that work and don't work
  - Protocols available on web: http://www.openwetware.org/wiki/breadboards
  - Workshops in Phase II, but happy to work with individuals at any time
- Larger collection of *in vitro* reporters (bulk + droplets); faster response times
- RNA scaffolds/origami for trying out biochemical wires in cells
- Sign up for web site: http://sites.google.com/site/livingfoundries
- TXTL announcements mailing list: http://groups.google.com/d/forum/txtl-announce