Biomolecular Breadboards for Prototyping and Debugging Synthetic Biocircuits

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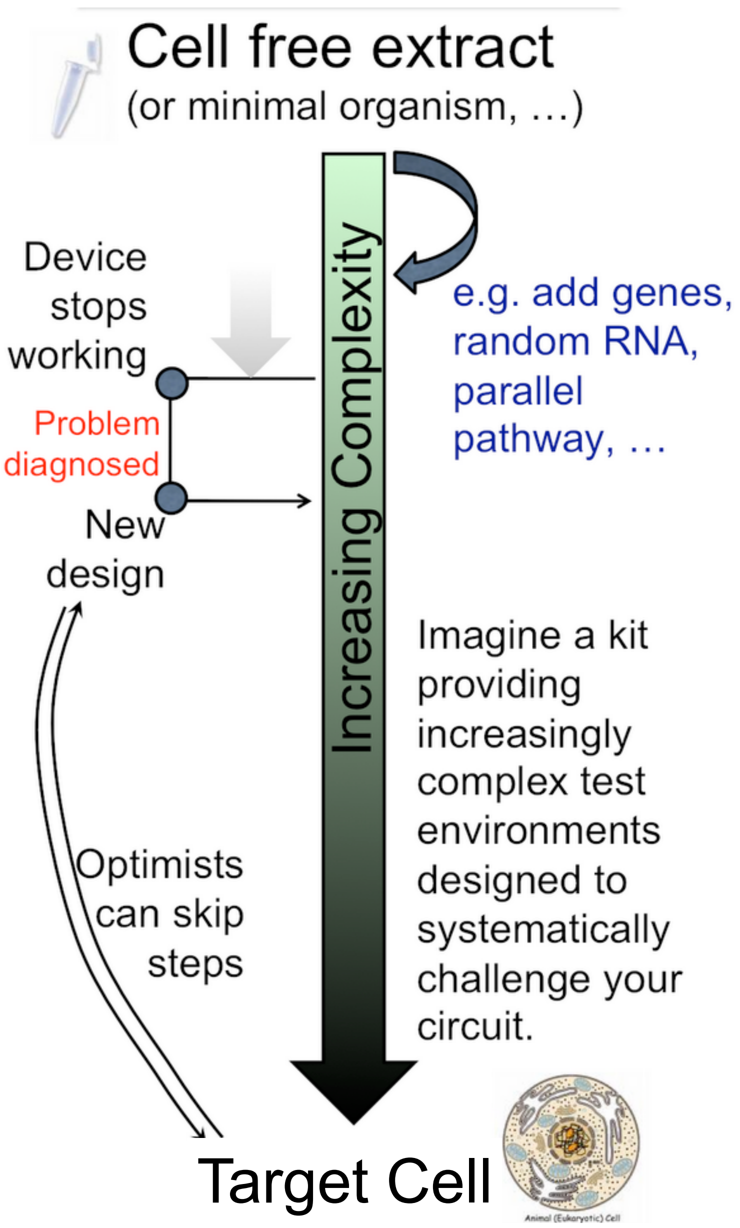
Biomolecular Breadboarding (“Wind Tunnel” [Klavins])



Cell free extract
(or minimal organism, …)

Device stops working

Problem diagnosed

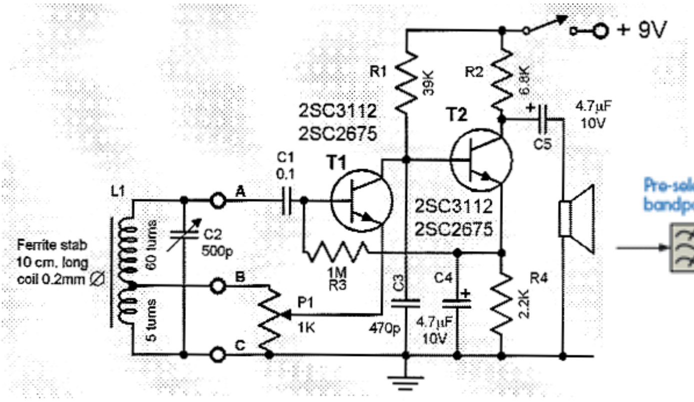
New design

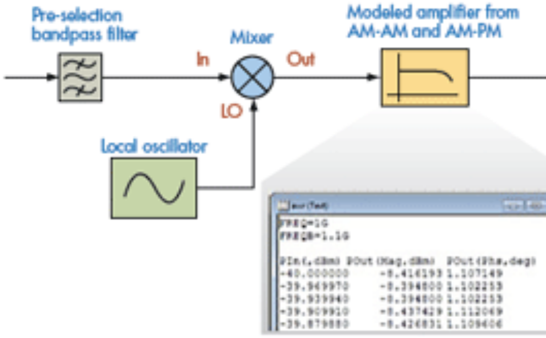
e.g. add genes, random RNA, parallel pathway, …

Optimists can skip steps

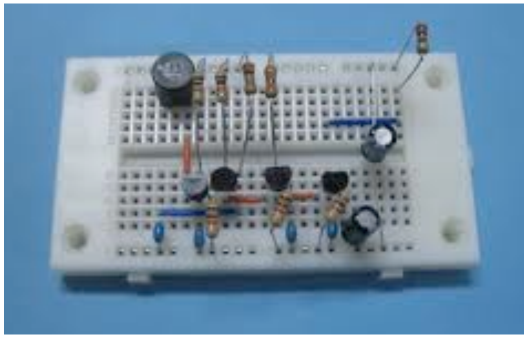
Imagine a kit providing increasingly complex test environments designed to systematically challenge your circuit.

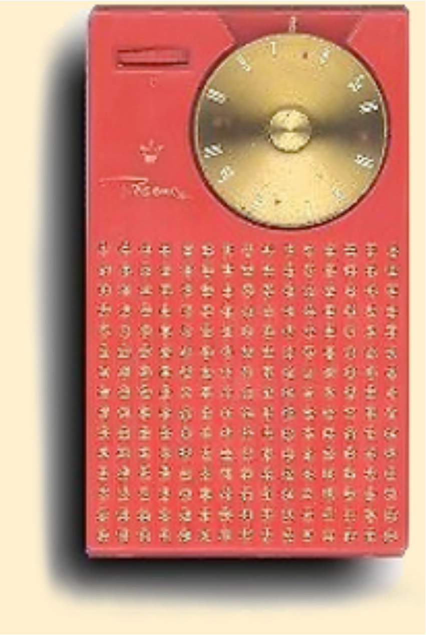
Target Cell





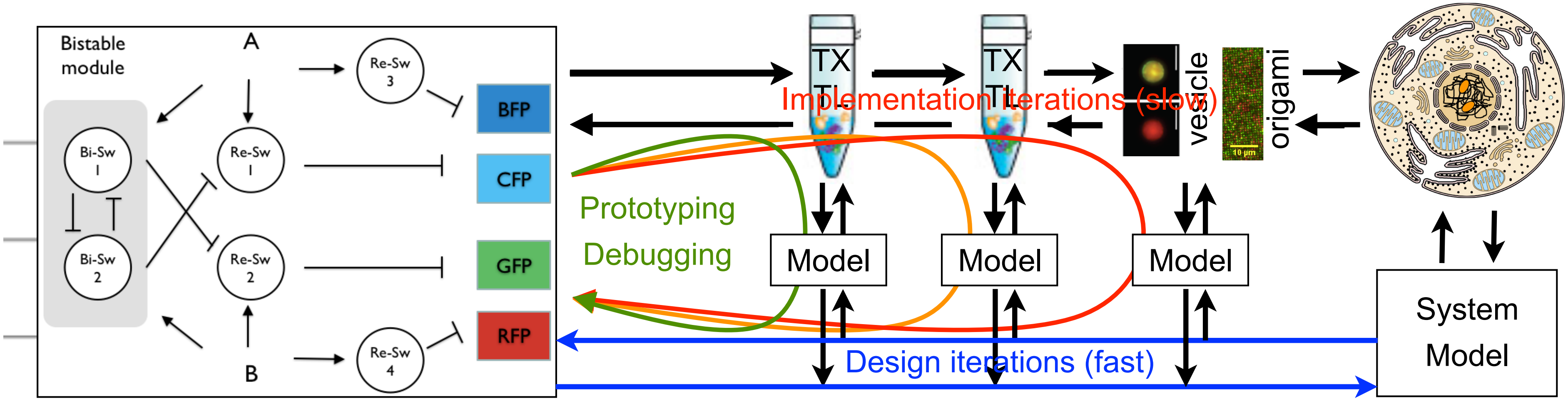
```
FREQ=1G
FREQB=1.1G

PIn(,dBm) POut(Mag,dBm)  POut(Phs,deg)
-40.000000     -8.416193 1.107149
-39.969970     -8.394800 1.102253
-39.939940     -8.394800 1.102253
-39.909910     -8.437429 1.112069
-39.879880     -8.426831 1.109606
```





Sun et al, JoVE 2013
Sun et al, ACS Syn Bio, 2013

Cell-Free Biomolecular Breadboards



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

- Inexpensive and fast: ~$0.03/ul ⇒ $0.30/ expt; 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
- Many mechanisms tested in TX-TL:

PI (+ contact)	Circuit/Technology	1 2 3
Lucks (CH)	RNA-sensing TFs	✓✓✓
Del Vecchio (EY)	Loading effects	✓✓✓
Temme (VH)	Orthogonal RNAPs	✓? -
Voigt (DSG)	4 input, 11 gene	✓x -
Tabor (JK)	Green light sensor	✓?○
Endy (VH)	DNA memory	✓○ -
Del Vecchio (SG)	Phospho-insulator	✓✓✓
Kortemme (EdIS)	Molecular sensors	✓○ -
Jewett (YW)	Butanediol pathway	✓✓○

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

Breadboards Project Goals and Objectives

Develop, demonstrate, document, and disseminate (two) new "biomolecular breadboards" that provide engineers with 10-100X improvement in time required to conceive, design and implement working biomolecular circuits

Program metric	Current	Phase I	Phase II
1. Time required from synthesized DNA sequences to measurement of circuit performance (on cell-free breadboards)	1-2 wk	3 days ✓	1 day ✓
2. Time required to build a novel, modest complexity (6-8 unique promoter) circuit - existing design, novel components (**in vitro**/**in vivo**)	3-6 mo	1 mo ✓	1 wk **1-2w**/**2-4m**
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 ✓	20 ✓
5. Number of circuit combinations that can be screened per day, varying component concentration and/or genetic elements	5	N/A	10^6 ✓

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

- Z. Z. Sun *et al*, Protocols for Implementing an *Escherichia Coli* Based TX-TL Cell-Free Expression System for Synthetic Biology. JoVE, 2013
- Z. Z. Sun *et al*, Linear DNA for rapid prototyping of synthetic biological circuits in an *Escherichia coli* based TX-TL cell-free system. *ACS Syn Bio*, 2013
- D. Siegal-Gaskins *et al.*, Resource usage and gene circuit performance characterization in a cell-free 'breadboard', *ACS Synthetic Biology*, 2014
- M. Takahashi *et al*, Rapidly characterizing the fast dynamics of RNA genetic circuitry with cell-free transcription−translation (TX-TL) Systems, *ACS Syn Bio,* 2014 [2013 CSHL course]
- M. Takahashi, C. Hayes *et al,* Characterizing and prototyping genetic networks with cell-free transcription-translation reactions, *Methods,* 2015 [s]
- H. Niederholtmeyer, Z. Z. Sun *et al,* A cell-free framework for biological systems engineering, 2015 [s]

Clare Hayes, Shaobin Guo

Sample TX-TL Based Design Process

Stage 0: modeling with TX-TL modeling toolbox

- Desired function + specs → set of possible designs

Stage 1: prototyping with linear DNA (0.5-1 day/cycle)

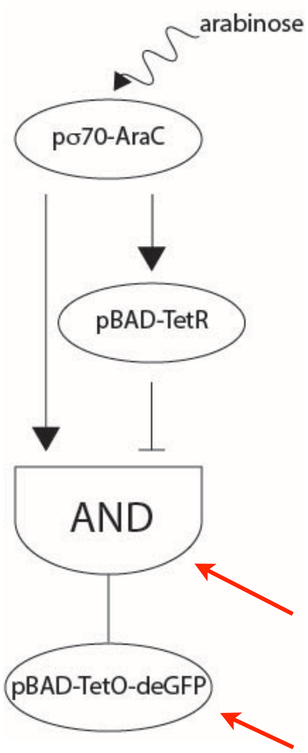
- Components from std library or PCR extension (no cloning)
- Compare w/ models; insure we can model what we see
- Downselect 4-8 designs to test in plasmids

Stage 2: prototyping with plasmid DNA (2 days/cycle)

- Clone into plasmid(s), using std sequences/protocols
- Verify operation in TX-TL, incl copy number variability
- Match results to models and linear DNA

Stage 3: validate in cells (2-3 days/test)

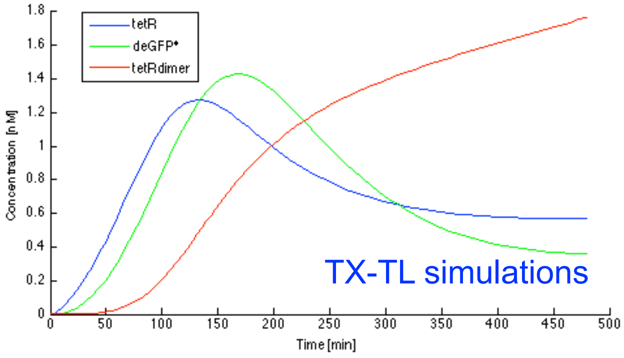
- Test top constructs from plasmid-based TX-TL assay



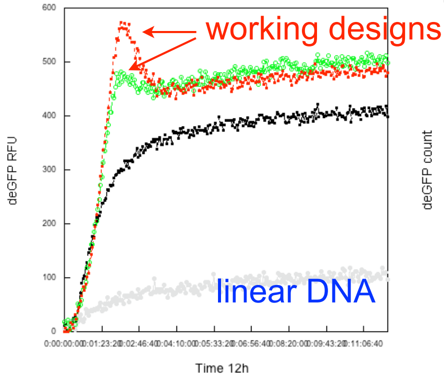
Novel combinatorial promoter (Hayes)

FFL output: pulse

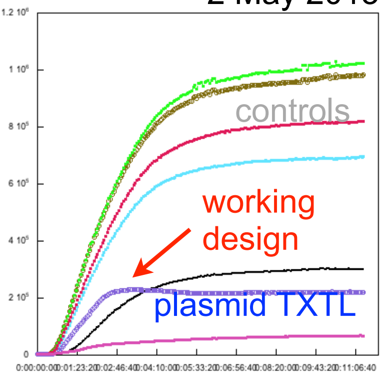
Project start: 15 Apr 2013



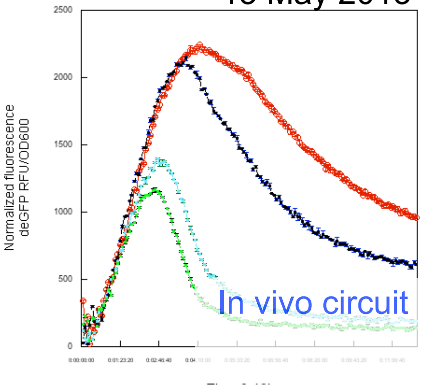
28 Apr 2013



2 May 2013



13 May 2013



Shin and Noireaux, *ACS Syn Bio*, 2012
Sun et al, JoVE 2013

Noireaux Cell-Free Expression Toolbox (TX-TL)

Basic Protocol Steps (extract prep)

- BL21 Rosetta2 cells grown at 37°C in 2xYT medium up to OD600 = 1.5
- Cells broken with a bead-beater (Biospecs) using 0.1 mm glass beads
- Clarified by centrifugation at 30000 g for 25 minutes [remove beads/membrane]
- Pre-incubated 80 minutes at 37°C followed by a centrifugation at 30000 g for 10 minutes [remove DNA/RNA?]
- Dialyze against S30 buffer B for 3 hours [removes small molecules]

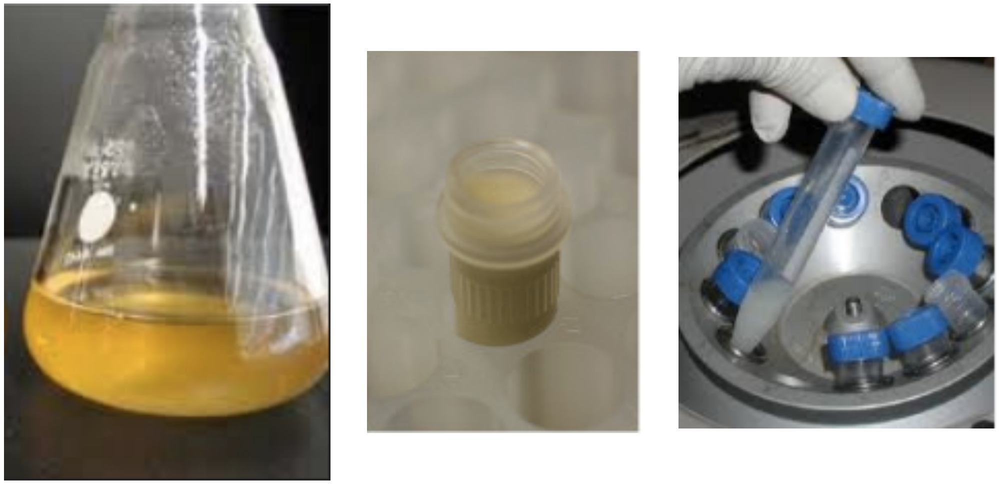
Tube 1: buffer

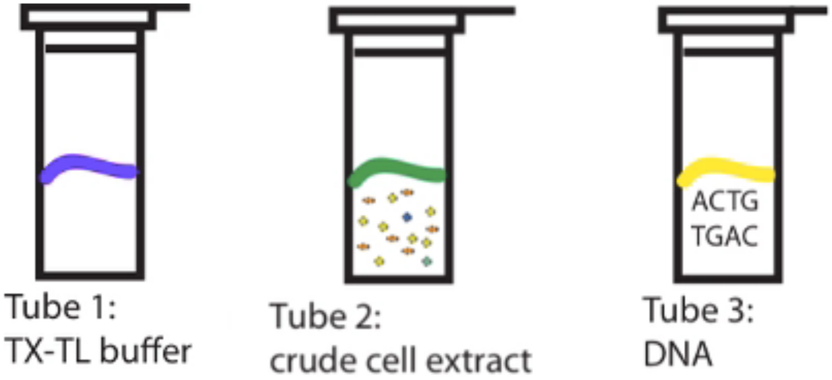
- Amino acids + energy solution (NTPs) + MG/K (calibrate for max expr)

Tube 2: extract

- 27-30 mg/ml of proteins (~1/30 E. coli)
- Stable at least 1 year at -80°C

Tube 3: DNA encoding circuit





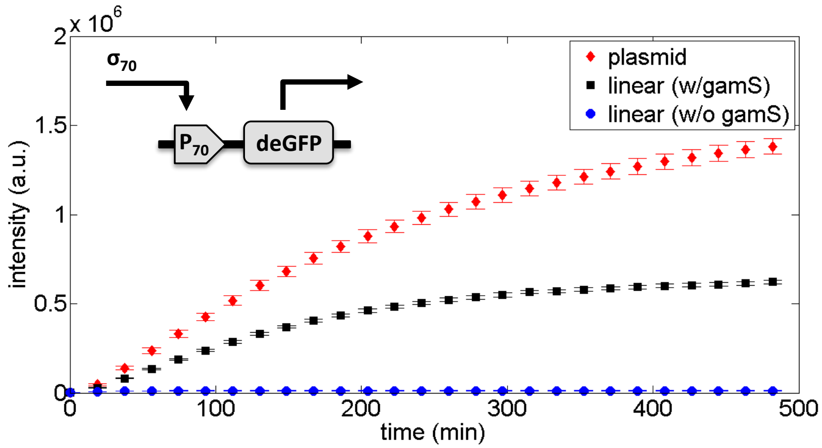
Sun, Hayes et al, *ACS Syn Bio*, 2013

TX-TL Core Processes

Zachary Sun, Vincent Noireaux

Rapid prototyping using linear DNA

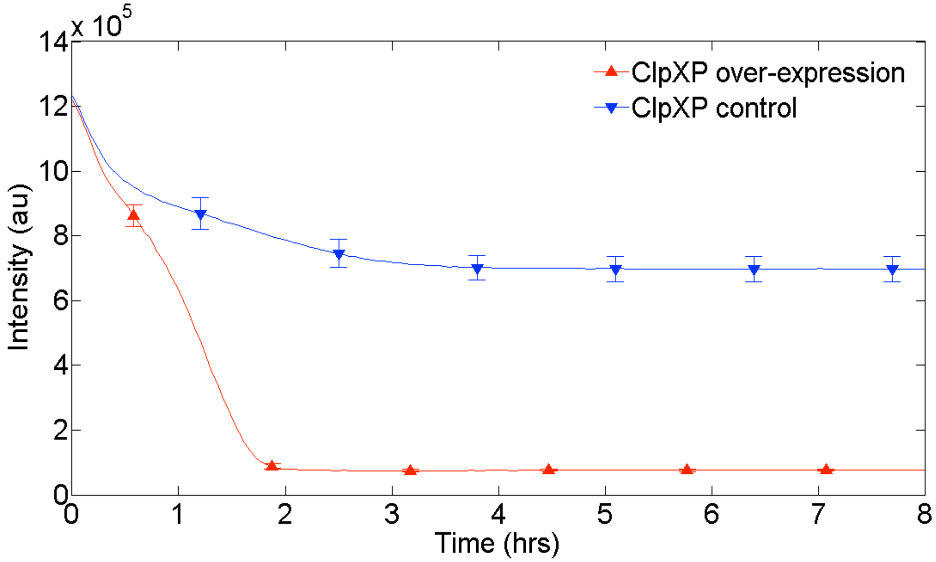
- Use PCR products with GamS to get expression levels of ~60% of plasmid

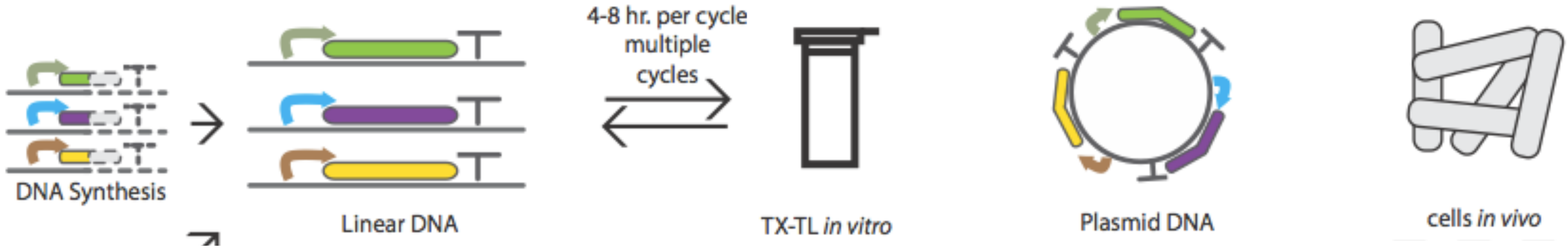


- Allows rapid assembly of constructs
  - PCR extension for simple circuits
  - Golden gate ass'y of components

Protein degradation

- Use clpXP machinery to degrade tagged proteins





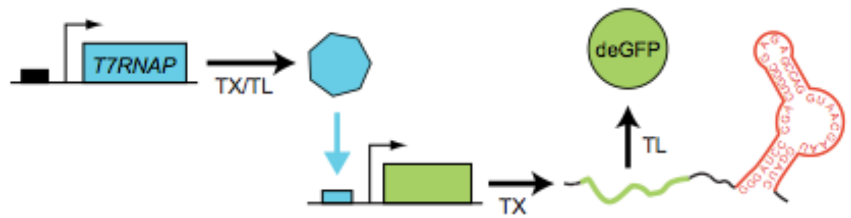
Siegal-Gaskins, Kim, Tuza, Noireaux, M, ACS Syn Bio, 2014

Resource Limits

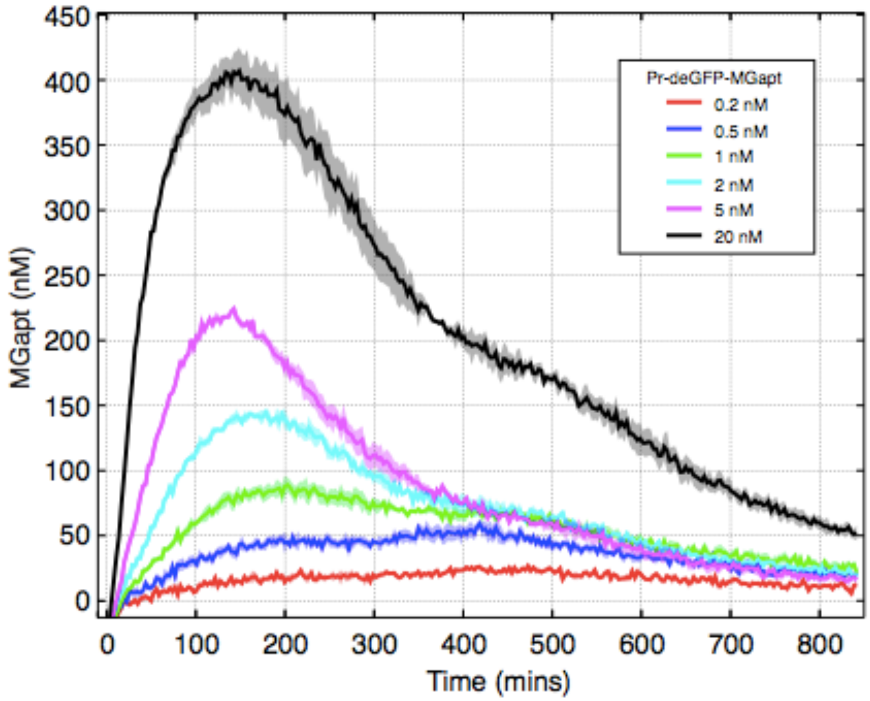
Limited capacity affects performance

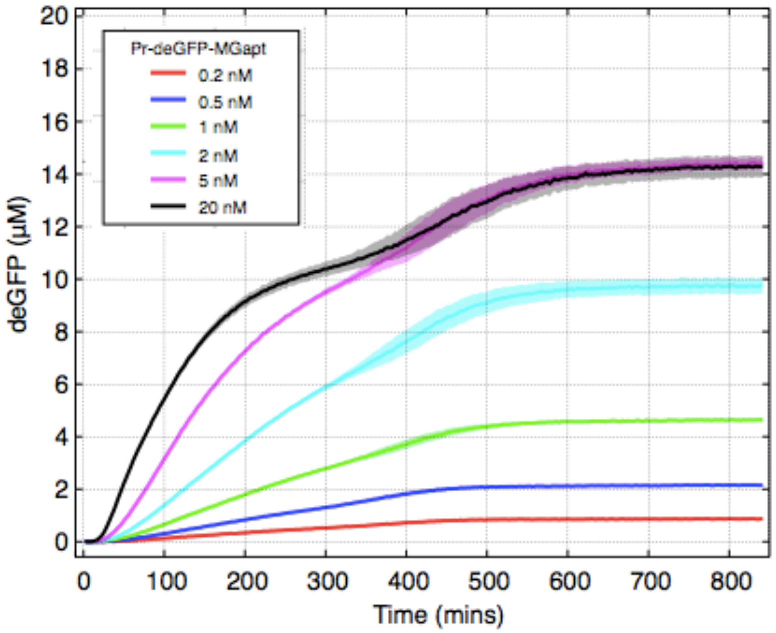
- Saturate transcriptional and/or translational machinery

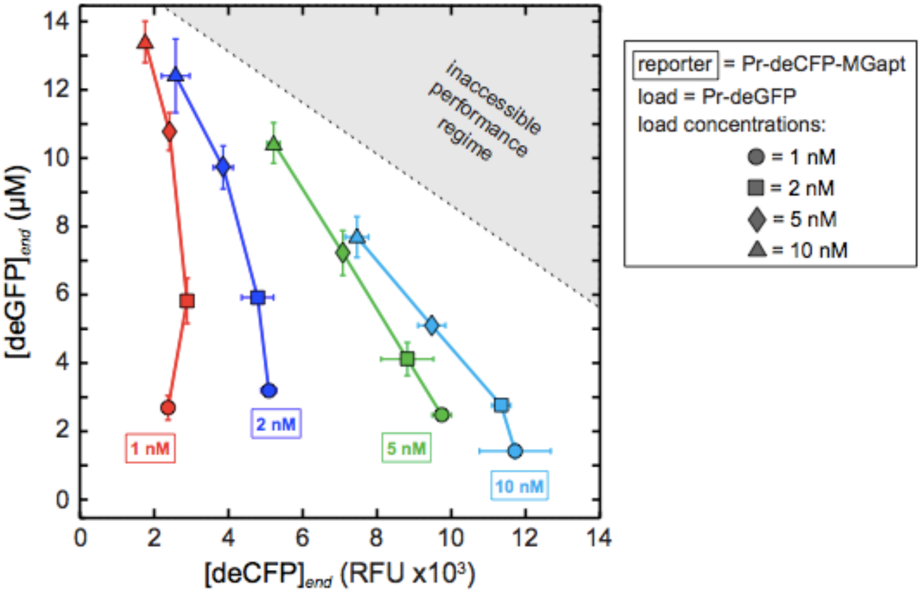
A



B





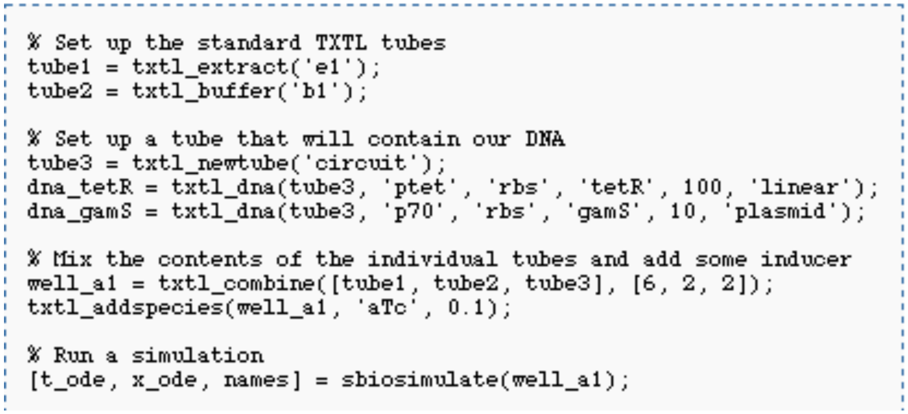


Tuza, Singhal, Kim and M, CDC 2013

TX-TL Modeling

Zoltan Tuza, Vipul Singhal, Dan Siegal-Gaskins

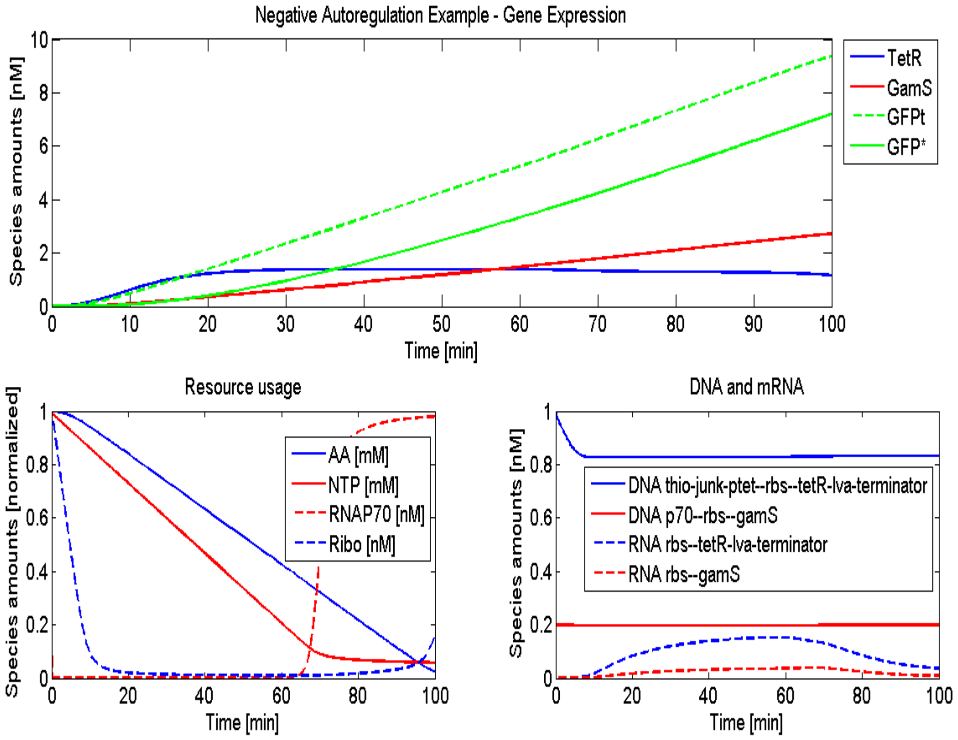
MATLAB toolbox (sf.net/projects/TXTL)

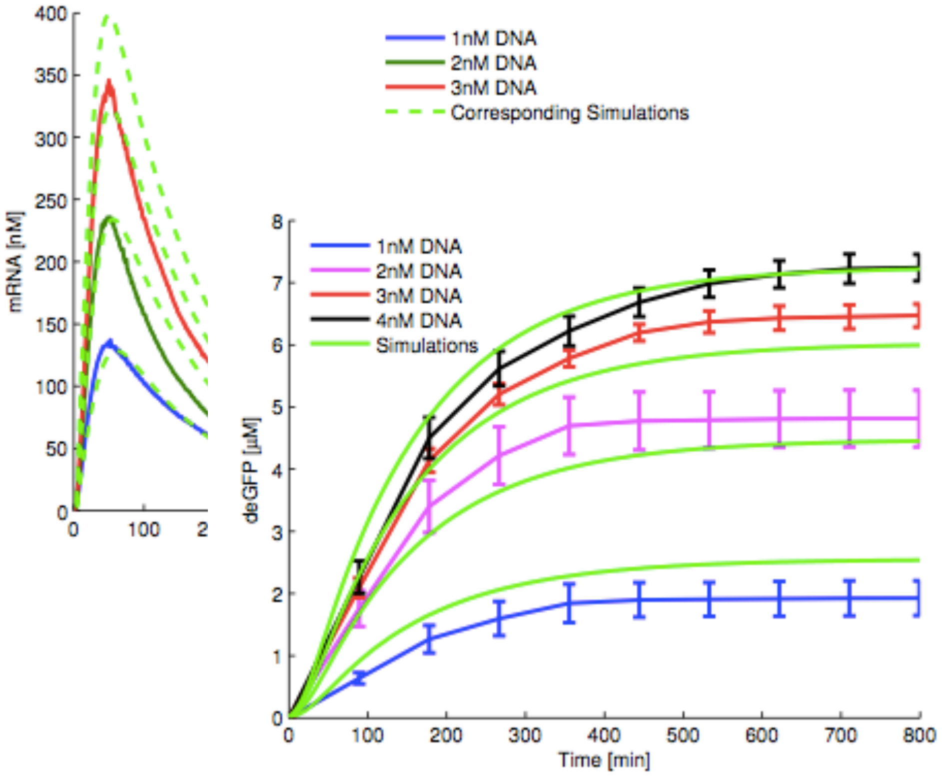


```
% Set up the standard TXTL tubes
tube1 = txtl_extract('e1');
tube2 = txtl_buffer('b1');

% 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
- Calibrated models that match experimental results

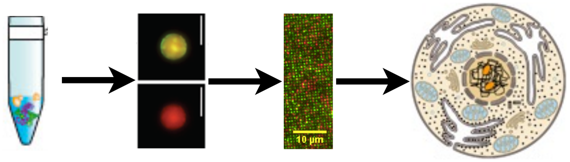




Phase I Accomplishments (21 May 2013 - 20 May 2014)

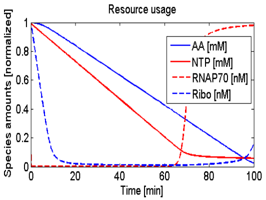
TX-TL cell-free toolbox

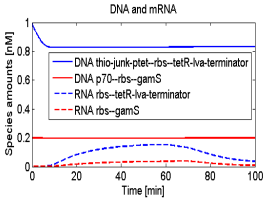
- $0.03/ul, 1 day cycle time
- Linear DNA (w/ protect'n)
- Protein degradation (via YbaQ and ssrA tags)
- Detailed protocols (JoVE)
- Circuits: switch, IFFL, toxin-antitoxin + partners
- Design 3-6 gene circuit, *in vitro* → *in vivo* in 1 mo



TX-TL modeling toolbox

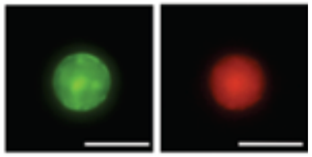
- MATLAB (Simbiology) based toolbox with 10 line circuit specs
- Validated models for gene expression, regulation, w/ resource lims
- Full source code and user documentation available on web

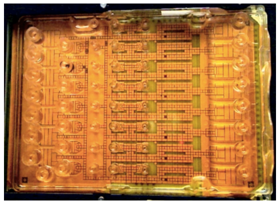




TX-TL vesicles & droplets

- Inducer-based expression in droplets
- Time course measurements of reporters in 0.3 µl droplets on ALL R110
- Hiccup: ALL acquired by Illumina, Oct 2013…

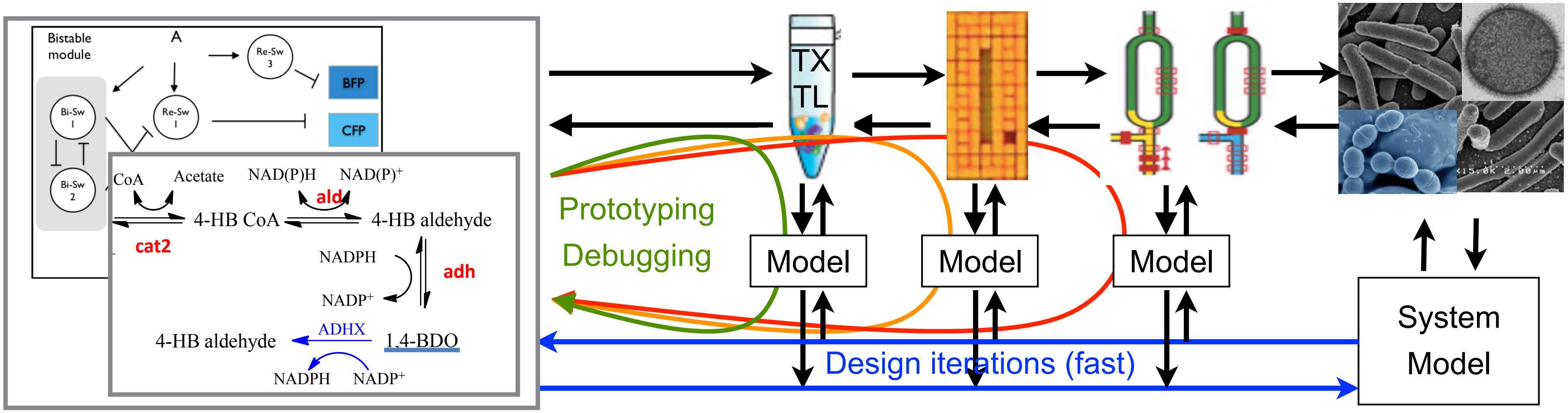




Publications: 3 journal, 6 conference/technical reports

- Z. Z. Sun *et al*, Protocols for Implementing an Escherichia Coli Based TX-TL Cell-Free Expression System for Synthetic Biology. JoVE, 2013
- Z. Z. Sun *et al*, Linear DNA for rapid prototyping of synthetic biological circuits in an Escherichia coli based TX-TL cell-free system. ACS Syn Bio, 2013
- D. Siegal-Gaskins *et al*, Resource usage and gene circuit performance characterization in a cell-free 'breadboard', ACS Synthetic Biology, 2014

Cell-Free Biomolecular Breadboards: Phase II



Focus areas for Phase II

- Improved circuit prototyping and debugging
- Microfluidics
    - Droplet-based microfluidics (ALL)
    - Ultra high throughput μfluidics (UCSF)
    - Continuous reaction loops (EPFL)
- Metabolic pathways: 2,3-BDO and 1,4-BDO
- Improved characterization and extract prep
    - *E. coli* strains and preparation methods
    - Organisms: *B. subtilis, L. Lactis* (ongoing)

Student/Postdoc	Circuit/Technology	MPC
Jongmin Kim	T7 RNAP aptamer	✓✓✓
M. Takahashi*	RNA-based SIMM	✓✓✓
Enoch Yeung	Event ordering	✓✓✓
Shaobin Guo*	Fold change detect	✓✓✓
Zach Sun / H. Niederholtmeyer	Novel repressilator	✓✓✓
	5-node repressilator	✓✓✓
Victoria Hsiao*	Freeze dried TX-TL	- ✓ -
Clare Hayes	4-input NOR gate	✓✓○
Yong Wu*	1,4 BDO pathway	○✓○

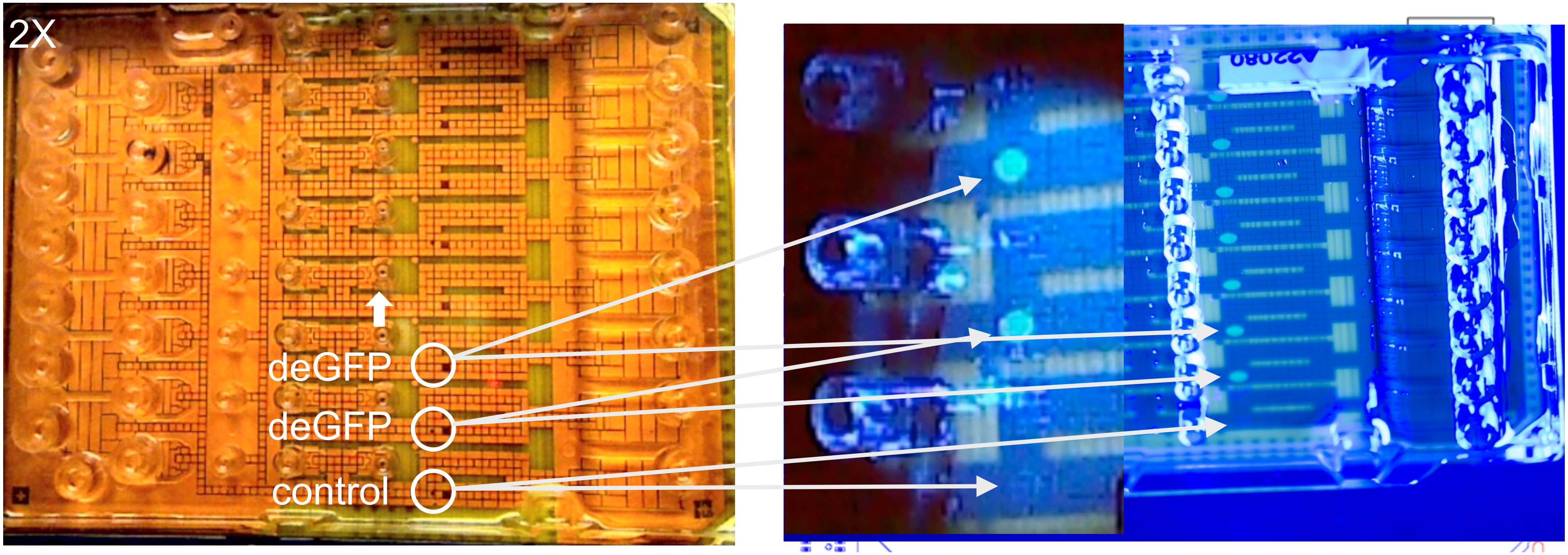
http://www.openwetware.org/wiki/breadboards

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

Enoch Yeung, Sean Sanchez (+ Lisa Bukovnic, Sri Punnamaraju, Nick Trotta @ ALL/Illumina)

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

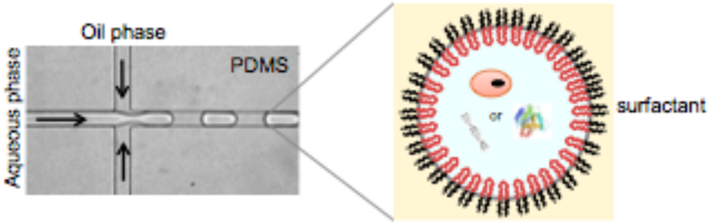
- Enables rapid prototyping of circuit performance in small volume regime.
- Spatial manipulation of droplets allows for spatial modularity of reaction volumes:
  - merging droplets allows for infusion of new fuel molecules to extend the lifetime of a breadboard reaction indefinitely.
  - splitting a droplet of reaction mixture simulates division of artificial "cells" and RNA/ protein dilution
- Status: all TX-TL operations can be performed in ALL, including chemical transform'n



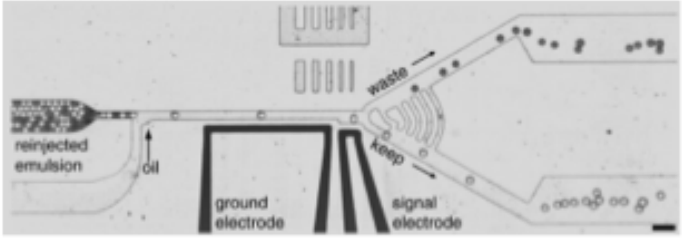
High Throughput Microfluidics (w/ A. Abate)

High throughput microfluidic platform

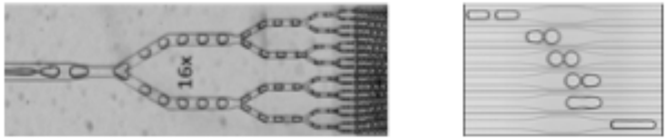
- Droplet generation



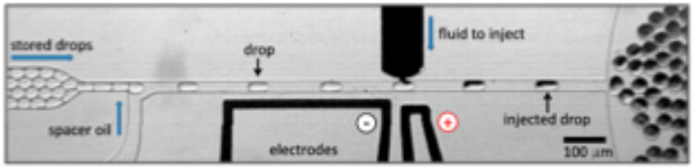
- Sorting



- Splitting



- Picoinjection

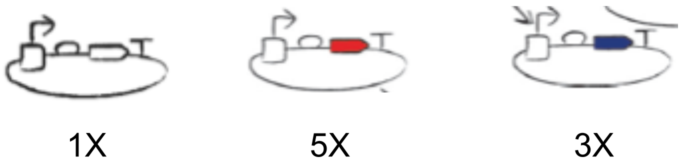


Goal: rapid design space exploration

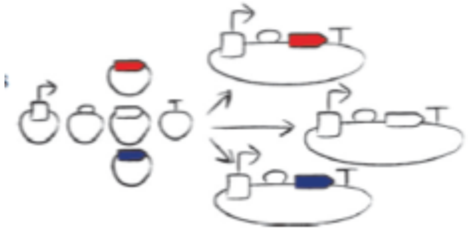
- Screen 10^6 circuit combination per day, varying component concentration and/or genetic elements
- Make use of data for mathematical modeling and circuit optimization
- Test on biosynthesis pathways

Exploration methods

- Component combinatorics



- TX-TL based genetic libraries



Challenges: measurement, combinatoric mixing, droplet ID, system ID, theory

Application: 1,4-BDO pathway exploration

1,4-BDO

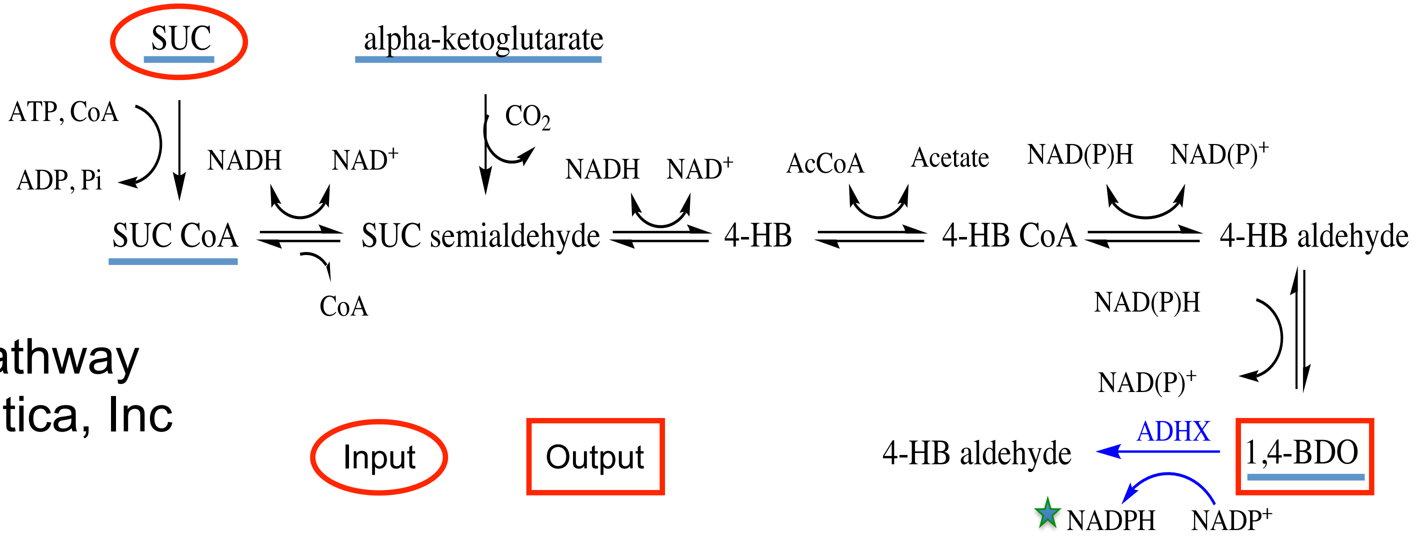
- High-value chemical used in plastics, elastic fibers, and solvents (~$33/L)
- *E. coli* biosynthesis pathway optimized by Genomatica, Inc (San Diego)

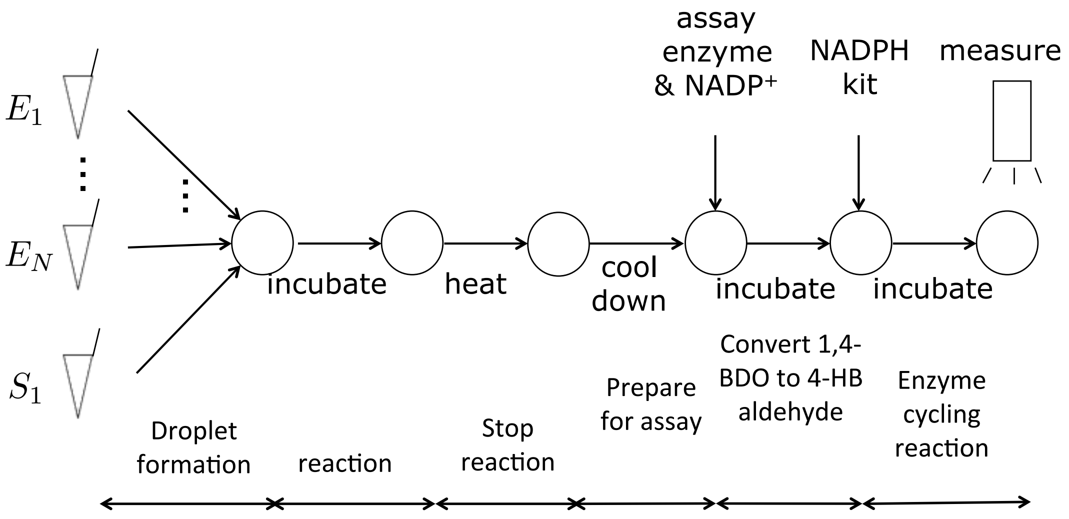
Design space exploration

- Use ultra-high throughput micro-fluidics with TX-TL to optimize pathway
- BDO sensing via engineered enzyme (from Genomatica)

Challenges

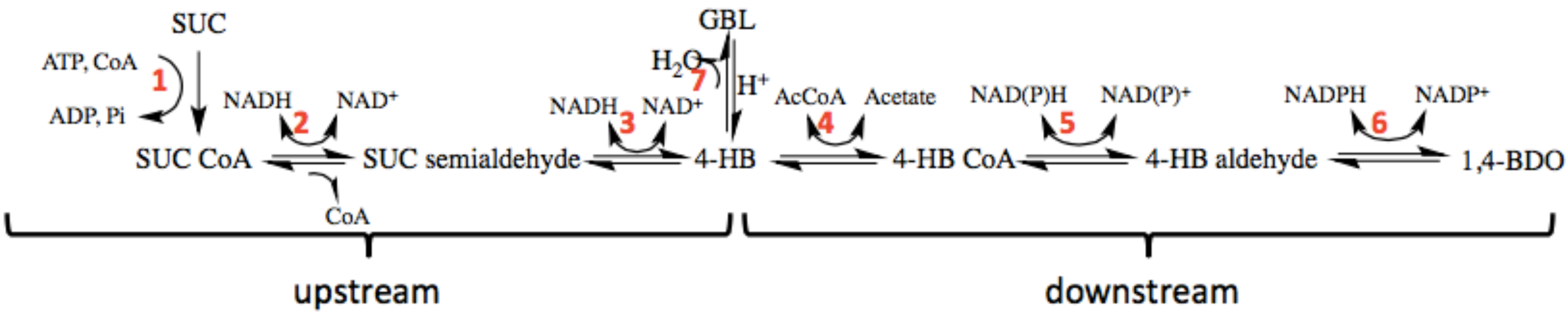
- Compatability of process steps with TX-TL and microfluidics
- Interaction of pathway (and co-factors) with native pathways





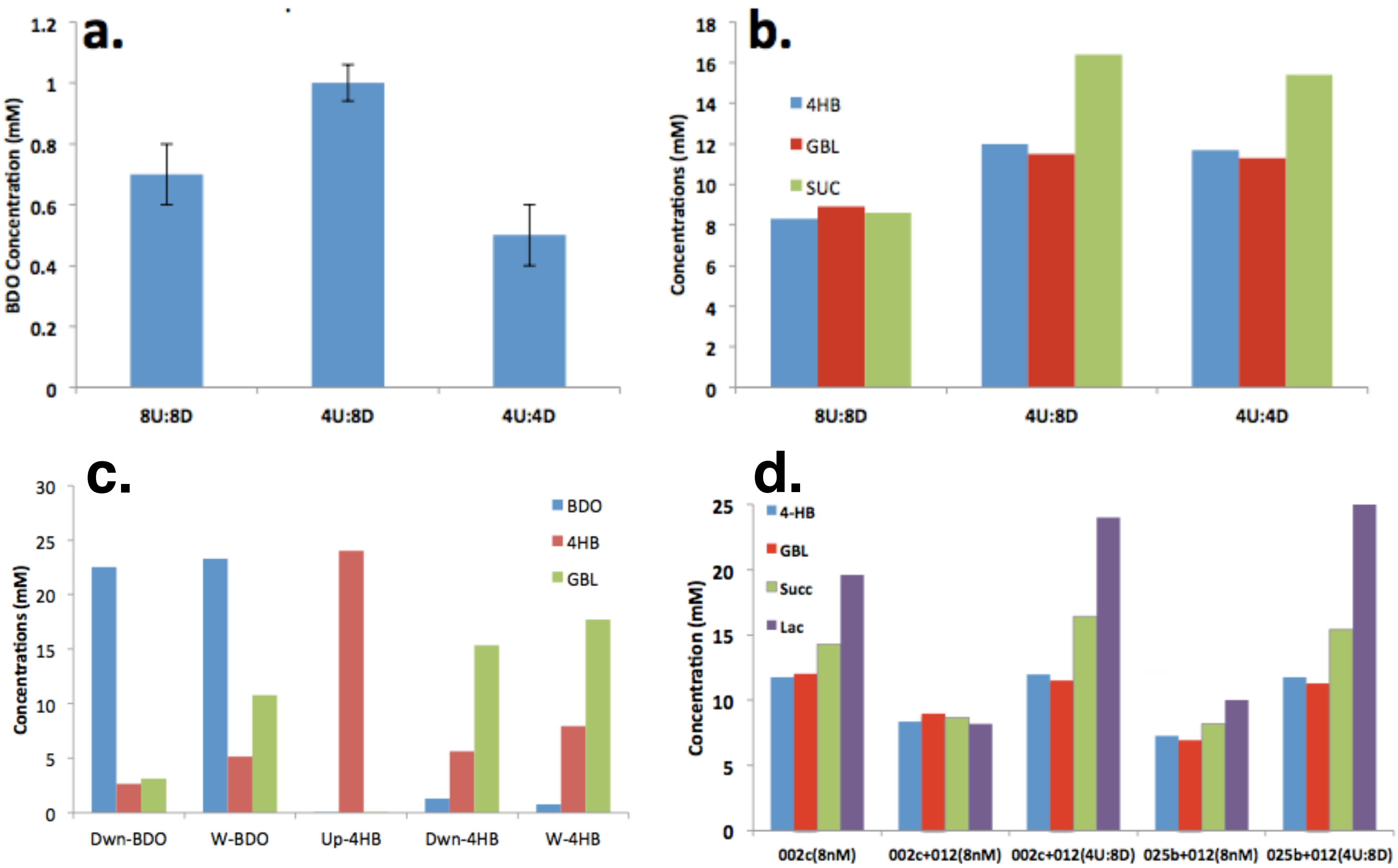
Wu et al, bioRxiv/SEED 2015 [a]

Prototyping 1,4-BDO Using TX-TL



(joint work with Genomatica, Inc)

- a, b: Vary upstream and downstream components to explore pathway fluxes
- c: Add intermediate metabolites with portions of pathway in place
- d: Explore mutated enzymes
- Not shown: vary redox potential

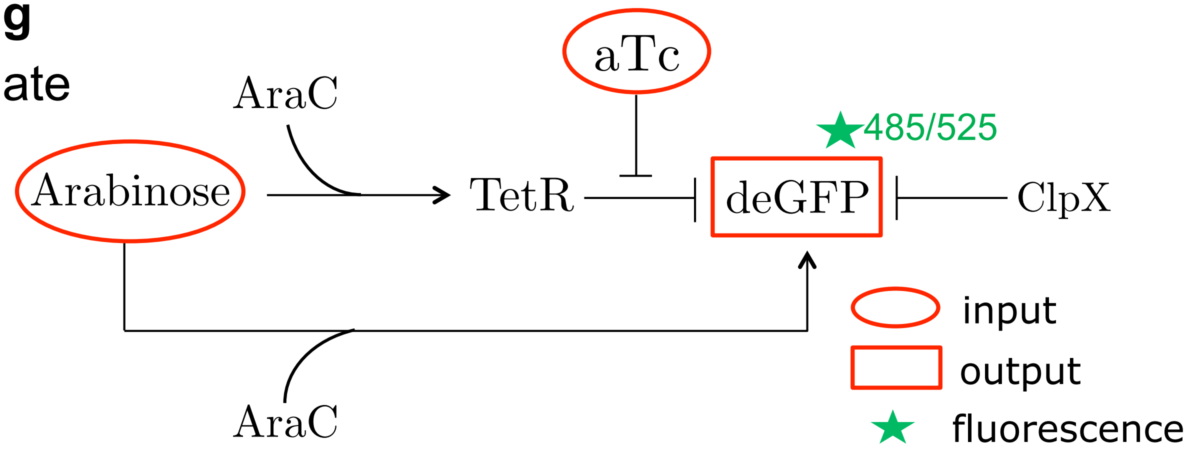


Y. Hori and M., ECC 2015 [a]

Modeling and System ID

Good progress in TX-TL modeling

- Testing IFFL as pathway surrogate
- Able to capture FFL performance across operation conditions with accuracy of ~10%



$$\dot{x} = -d_1 x + \bar{c}_1$$

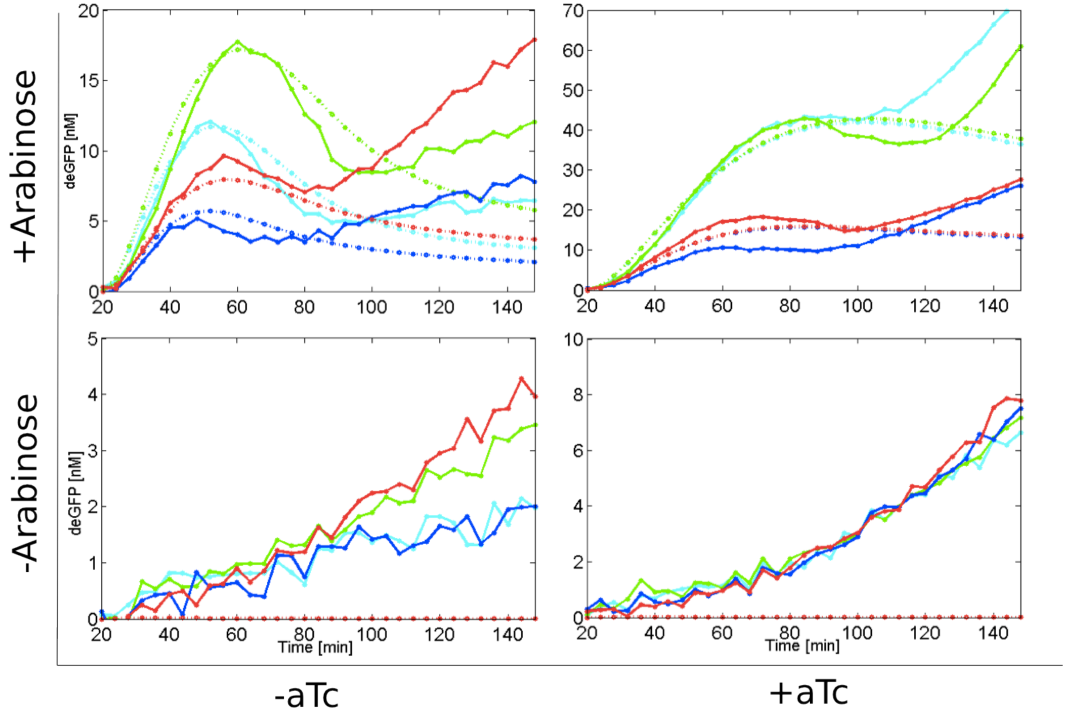
$$\dot{y} = -d_2 y + \bar{c}_2 \frac{x}{\bar{K}_1 + x} u_1 + \bar{b}$$

$$\dot{z} = -d_3 z - \bar{c}_\phi \frac{z}{K_\phi + z} \phi + \bar{c}_3 \frac{x}{(\bar{K}_5 + x) g(y, u_2)} u_1 + \bar{b}$$

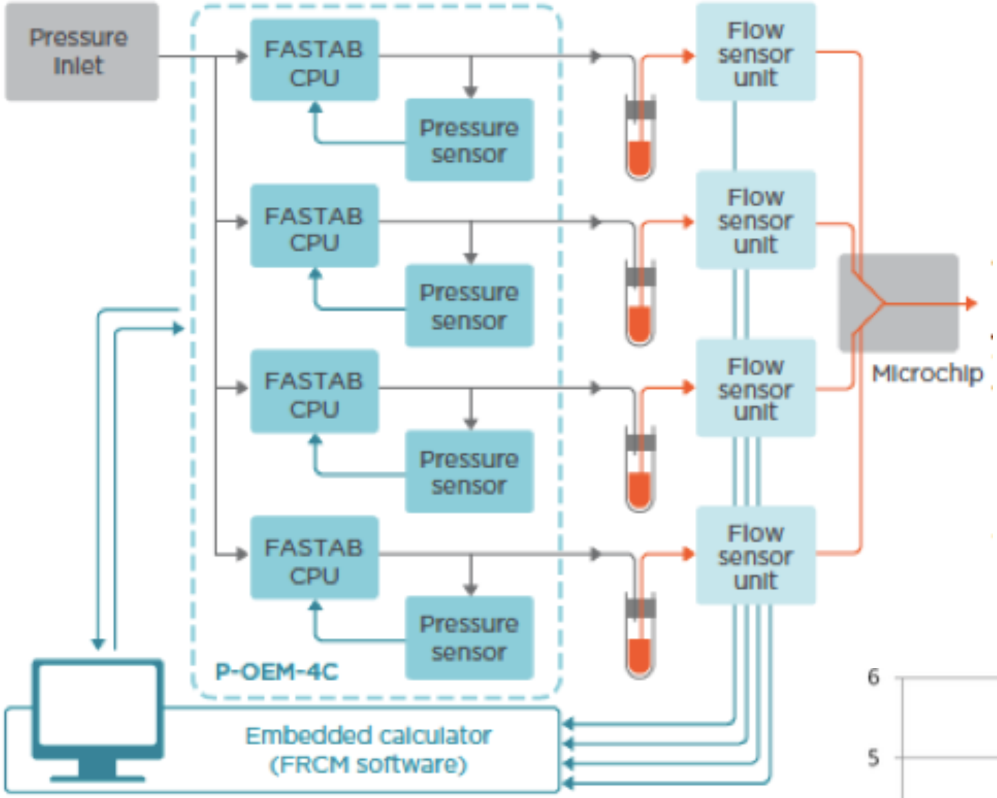
$$\dot{\phi} = -d_4 \phi + \bar{c}_4,$$

where $g(y, u_2) = \frac{-(\bar{K}_3 + u_2 - y) + \sqrt{(\bar{K}_3 + u_2 - y)^2 + 4y\bar{K}_3}}{2\bar{K}_7}$

u_1	Ratio of pBAD promoter induced by arabinose ($u_1 \in [0, 1]$)
u_2	aTc concentration [nM]
K_1	MM const. for AraC:pBAD-TetR binding [nM]
K_3	Dissoc. const. for aTc:TetR binding [nM]
K_5	Dissoc. const. for (AraC):(pBAD-tetO-deGFP) [nM]
K_7	Dissoc. const. for (TetR):(pBAD-TetO-deGFP) [nM]
d_i	degradation rates [min^{-1}]
$\bar{c}_i$	(Maximal) expression rate × DNA concentration [nM/min]
$\bar{b}_i$	leakiness × DNA concentration [nM/min]

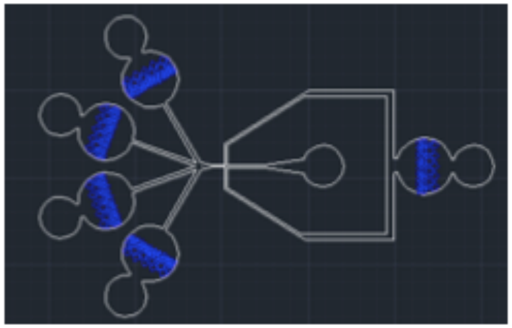


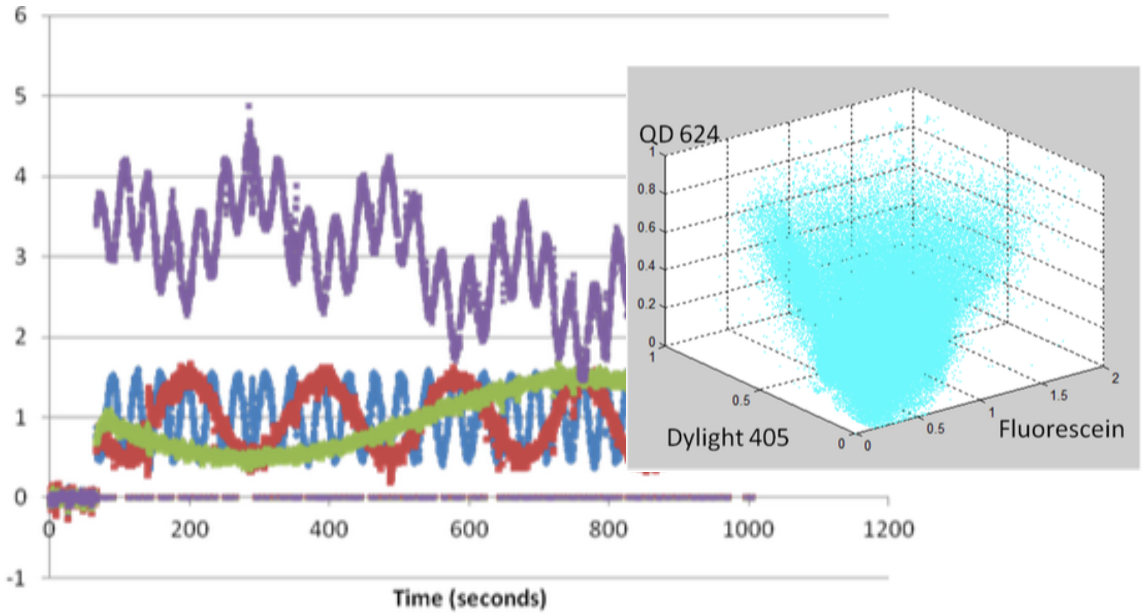
High Throughput Mixing (UCSF)



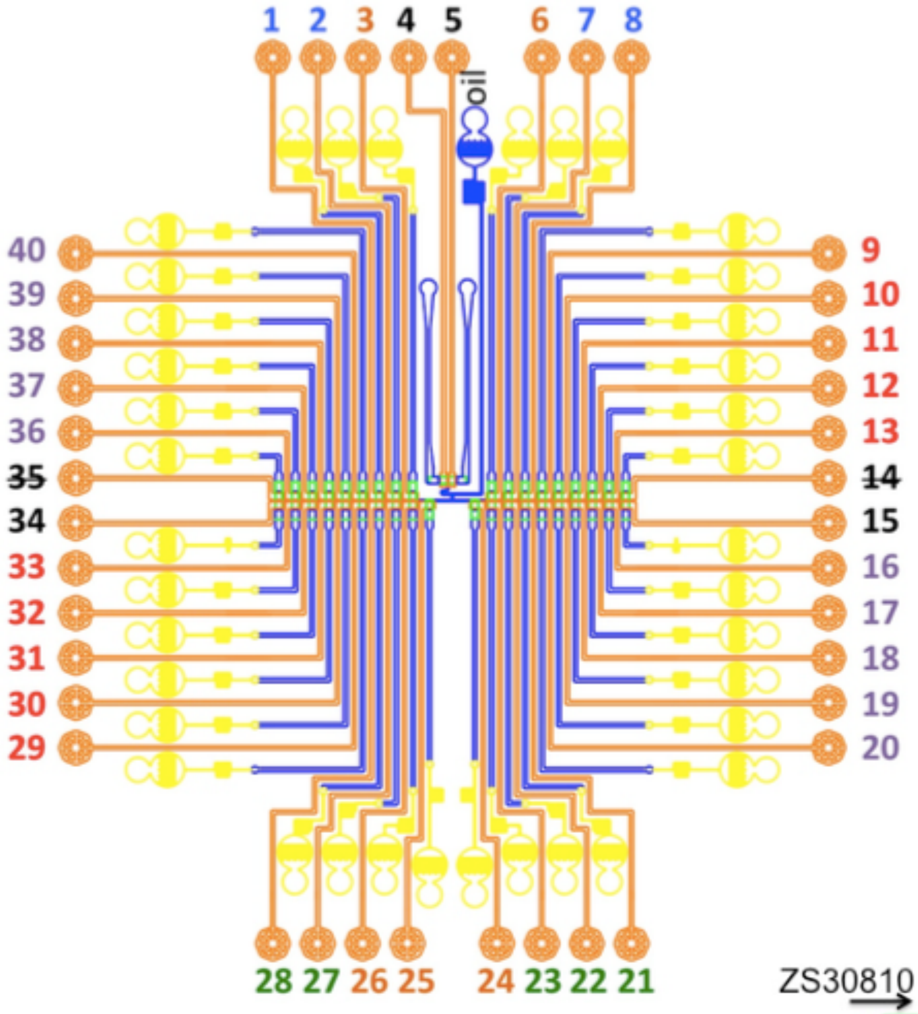
Built four-input multiplexer

- Capable of mixing 4 different inputs in combinatorial ratios
- Keep track of concentrations with dyes
- Tested on IFFL (surrogate) pathway
  - System ID and optimization
  - Direct design space exploration
- Demonstrated ability to test 10^4 combinations in < 1 day (using FFL)





High Throughput Assembly (UCSF)



Function	Valve
LIBRARY collect	4
WASTE Drops	5
ENZYME	15, 34

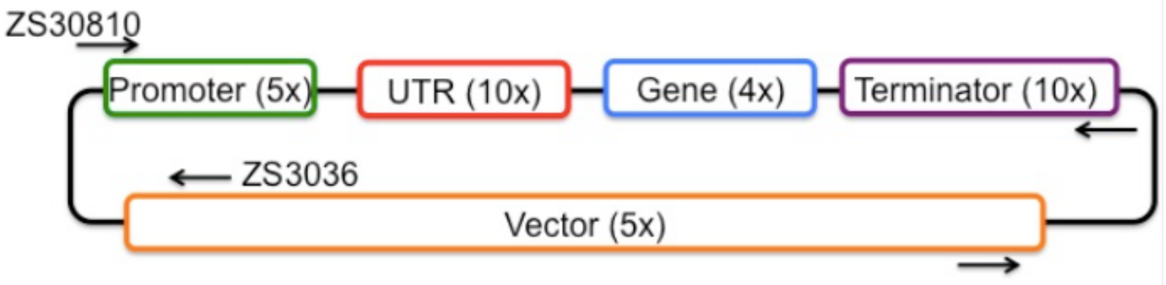
DNA	#	Valve
Prom	5	21,22,23,27,28
UTR	10	9-13 + 29-33
Gene	4	1,2,7,8
Term	10	16-20 + 36-40
Vector	5	3,6,24,25,26
reserve	*2*	*14 + 35*

Channel height

Blue (fluidic) :	50 um
Yellow (fluidic):	15 um
Green (valve):	35 um
Orange (pneumatic):	40 um
Dead volume:	25 nL
Operating pressure:	3 psi

GGA assembly

- Mix any combination of 40 parts
- Do Golden Gate Assembly and PCR in droplets
- Allows creation of structured DNA libraries
- Demonstrated 10k library < 3h, w/ 0.75 mL DNA (1M = 90 mL and 5d 19h)



Breadboards Project Milestone Summary

Phase I

- 🟢✓ 1.1: Develop, document and disseminate protocols for TX-TL based prototyping
- 🟢✓ 1.1: Demonstrate design of simple circuit (3–6 unique promoters) within 1 month
- 🟢✓ 1.3: Demonstrate complex circuits composed of 5–10 genes carried out in vesicles
- 🟢✓ 1.3: Engineer artificial cells with 1–2 different lipidic patterns

Phase II

- 🟢✓ 2.1: Run TX-TL workshop to train interested researchers (Aug 2013, Jun 2014)
- 🟢✓ 2.1: Use TX-TL design cycles to implement 4-8 promoter circuit in E. coli [delayed]
- 🟡✓ 2.1: Use TX-TL design cycles to implement 8-16 promoter circuit in 1 week [at risk]
  - 1 week cycle time for *in vivo* implementation is not feasible (expect 2-4 weeks)
  - Given delays in simpler circuits, may not make this milestone by target date
- 🟢✓ 2.6: Demonstrate all TX-TL protocols using Advanced Liquid Logic (ALL)
- 🟢✓ 2.6: Detect 5 properties in droplets using impedence/fluorescent spectroscopy
- 🟢✓ 2.6: Demonstrate ability to chemically transfer biological circuits into cells
- 🟢✓ 2.7: Screen 10^4 unique TX-TL combinations in <24 hours using <1 mL reagent
- 🟡✓ 2.7: Input/output dataset with $>10^6$ screened combinations of 6-8 components
- 🟡✓ 2.7: System ID for modeling, prediction, and optimization of pathway performance
- 🟡✓ 2.7: DNA assembly of 10^6 circuit variations in <48 hrs using <100 mL reagents

Niederholtmeyer and Sun et al., bioRxiv, 2015 [s]

Original Repressilator in Continuous TX-TL

Oscillation requires dilution

- Not possible to obtain enough degradation in TX-TL using just proteases

Use microfluidics to emulate

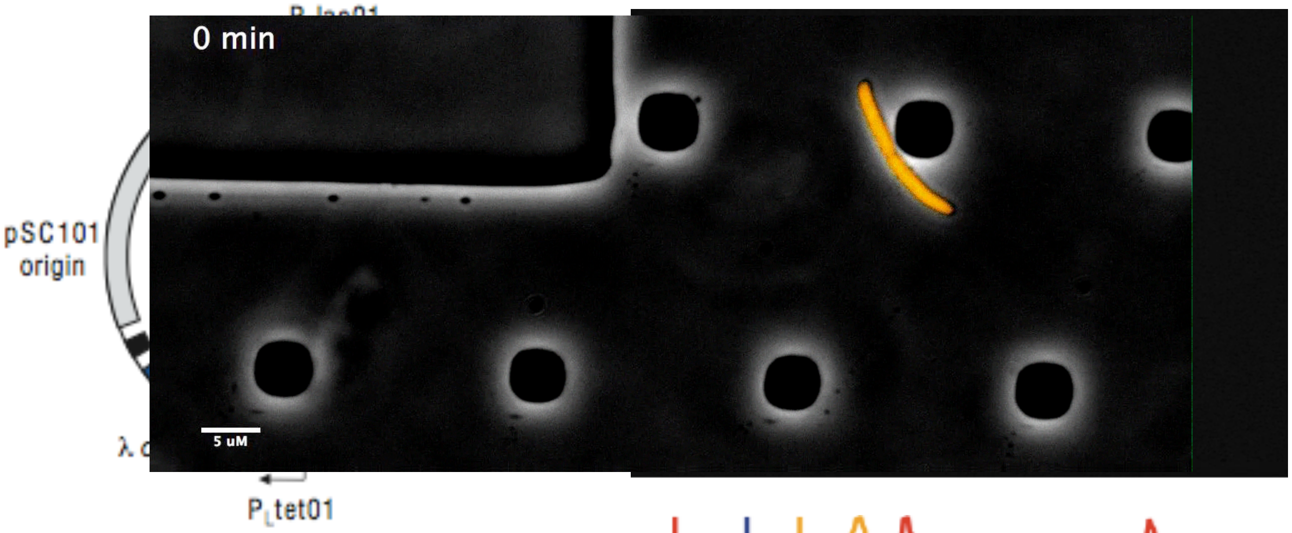
- Program dilution rate to explore amplitude, period
- Modified version with multiple reporters

Modeling matches *in vitro* experiments

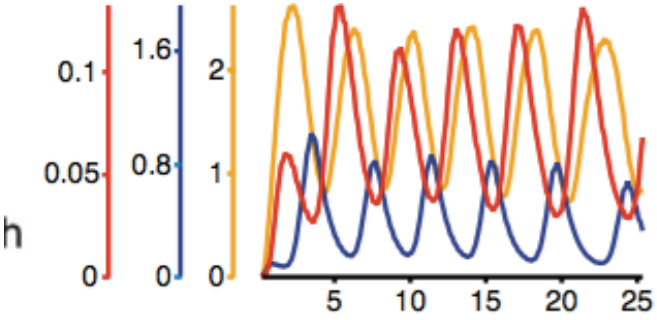
- ODE-based model that includes mRNA and protein dynamics

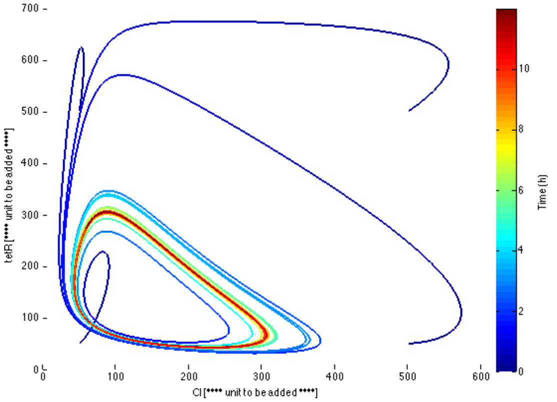
Very fast implementation

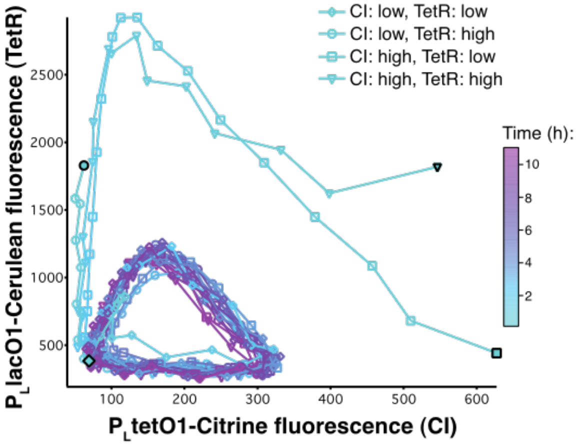
- Initial data obtained within days of sending DNA to EPFL
- Multi-color version *in vivo*



Vivo data: Elowitz and Liebler 1999 Nature
Vitro data: Collaboration with Niederholtmeyer and Maerkl, EPFL





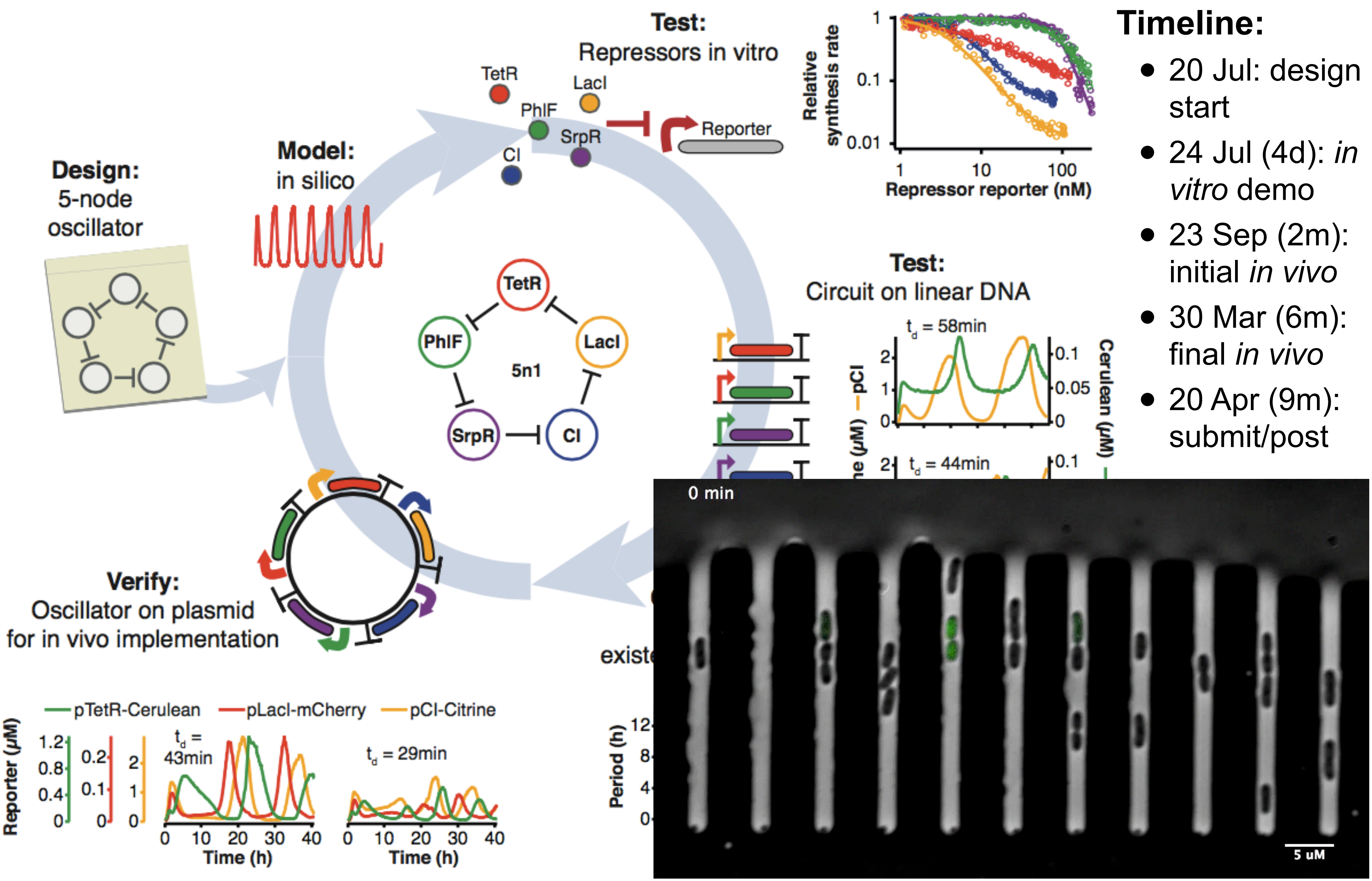


Niederholtmeyer and Sun et al., bioRxiv, 2015 [s]

5 Node Oscillator in TX-TL

Timeline:

- 20 Jul: design start
- 24 Jul (4d): *in vitro* demo
- 23 Sep (2m): initial *in vivo*
- 30 Mar (6m): final *in vivo*
- 20 Apr (9m): submit/post

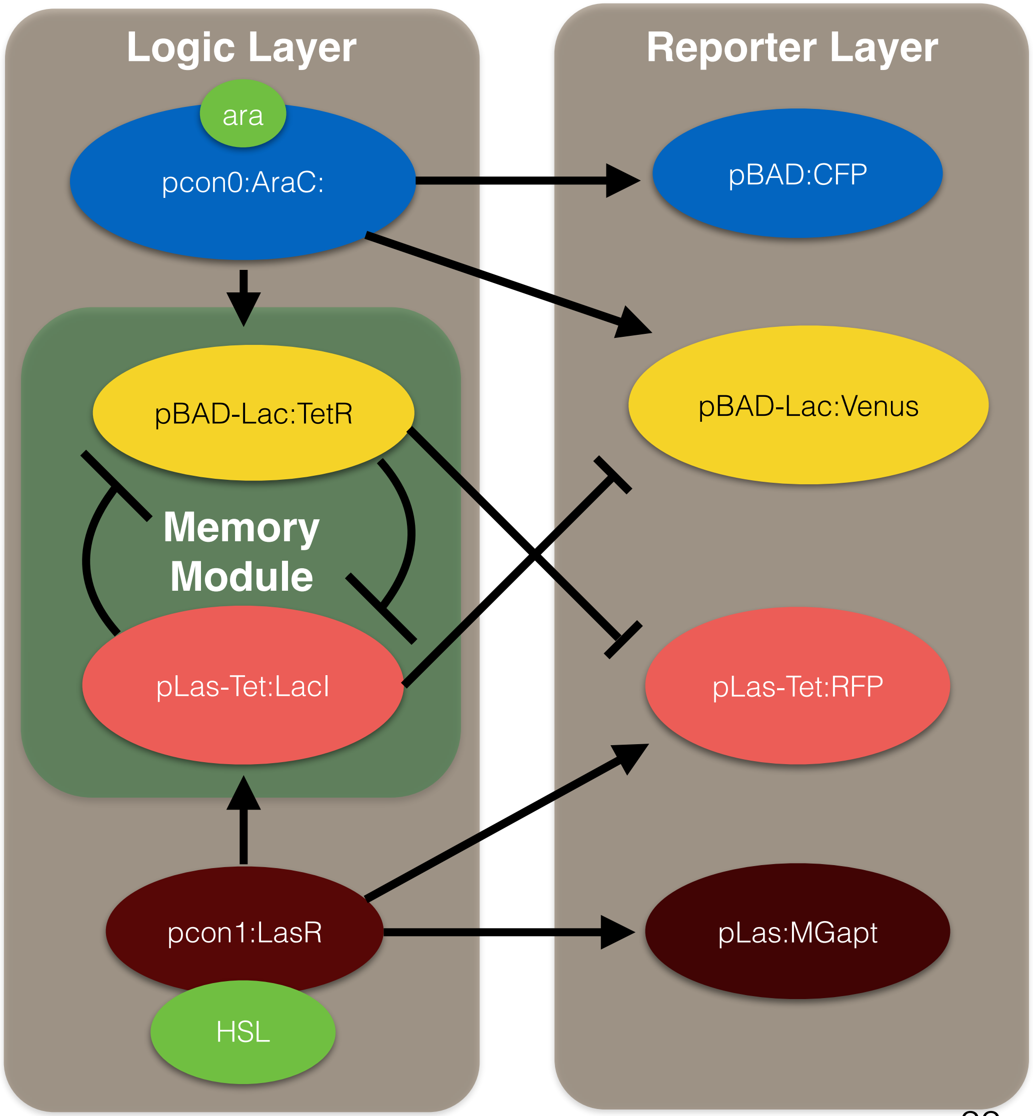


An Event Detector Biocircuit: DARPA Milestone 2.1.3

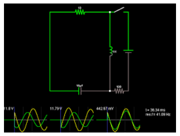
Milestone: Investigators will demonstrate the ability to get a modest complexity circuit (8–16 unique promoters, based on composing simpler circuits) working in E. coli, documenting the amount of time required for design iterations and debugging, as well as all protocols for breadboard environments and compensation mechanisms.

Enoch Yeung Kyle Martin
Department of Computing and Mathematical Sciences, Caltech

Event Detector Biocircuit



Biocircuit Components:
(0-3 days for curation)



Circuit Models & Simulations
(1-2 days)

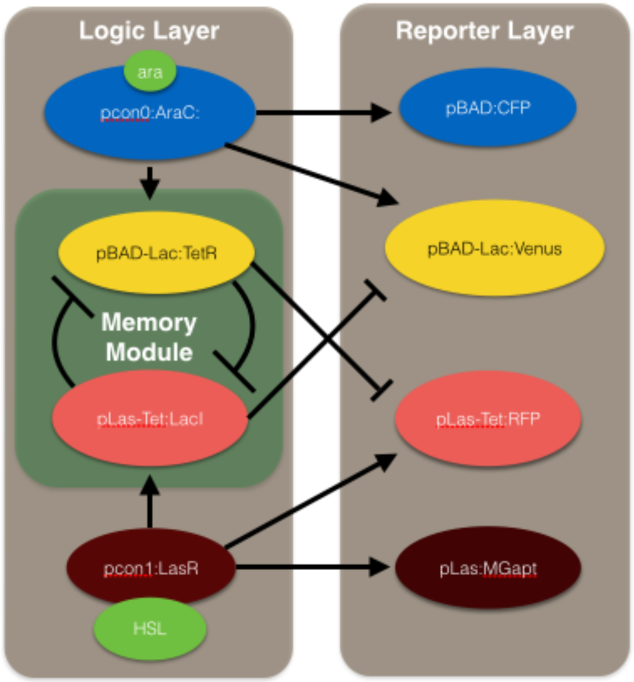
Breadboard Testing
(~2 weeks of
design-test cycling)

Functional Circuit Synthesis
(~2 months of cloning &
testing)

Field Applications
(in progress)

Biocircuit Modeling & Simulations Reveal a Functional Event Detector

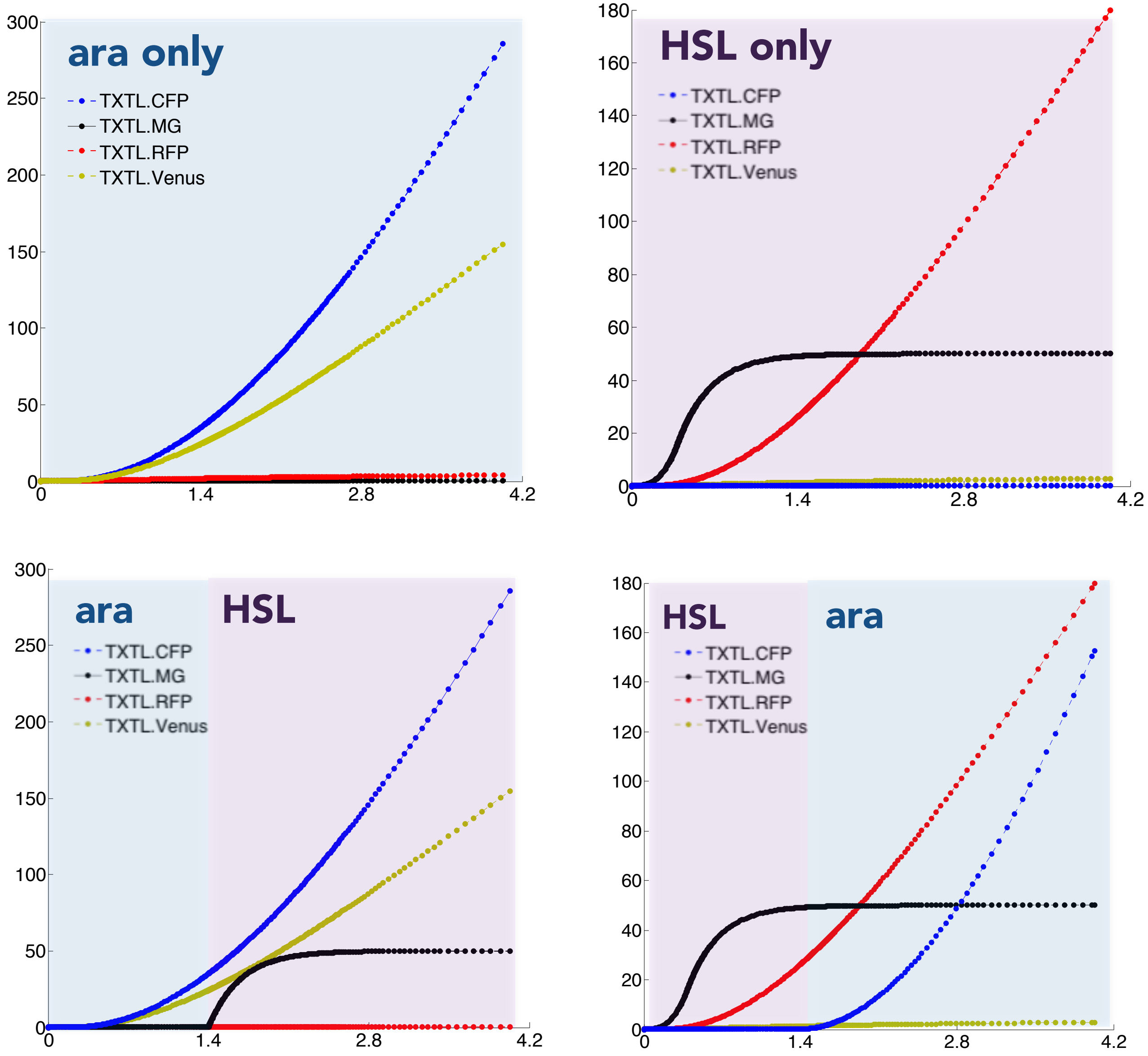
Event Detector Biocircuit



Expected (Steady-State) Outcomes

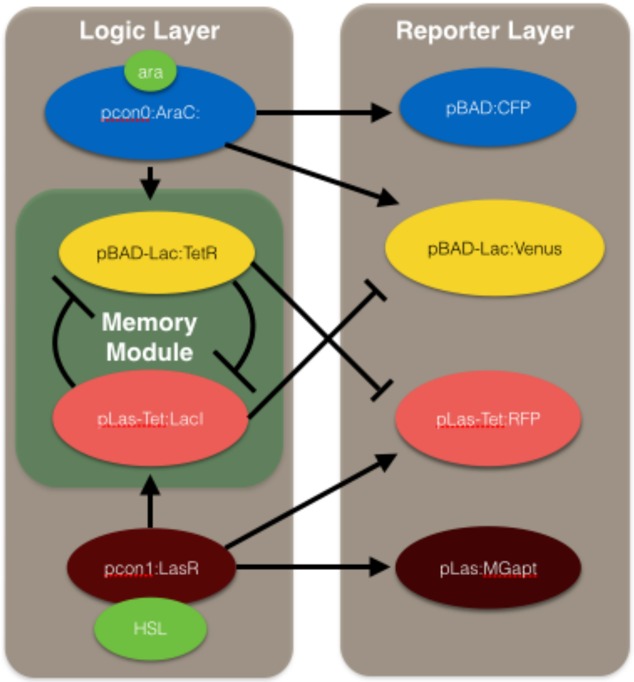
ara only	🔵	🟡		
HSL only			🔴	🟤
ara then HSL	🔵	🟡		🟤
HSL then ara	🔵		🔴	🟤

Simulation Results



Biomolecular Breadboard Experiments Confirm Model Predictions

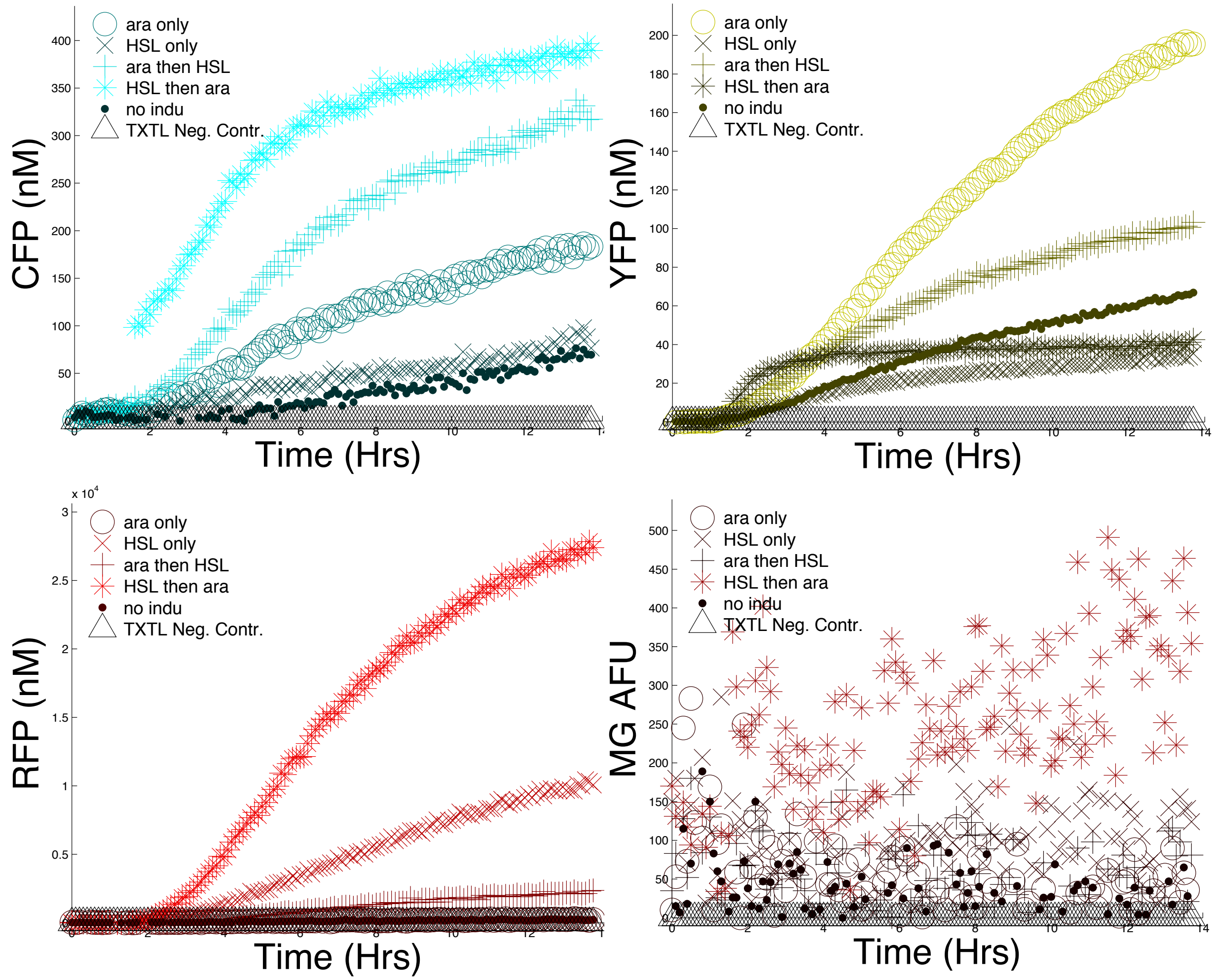
Event Detector Biocircuit



Expected (Steady-State) Outcomes

ara only	🔵	🟡		
HSL only			🔴	🟤
ara then HSL	🔵	🟡		🟤
HSL then ara	🔵		🔴	🟤

Biomolecular Breadboard (TXTL) Data

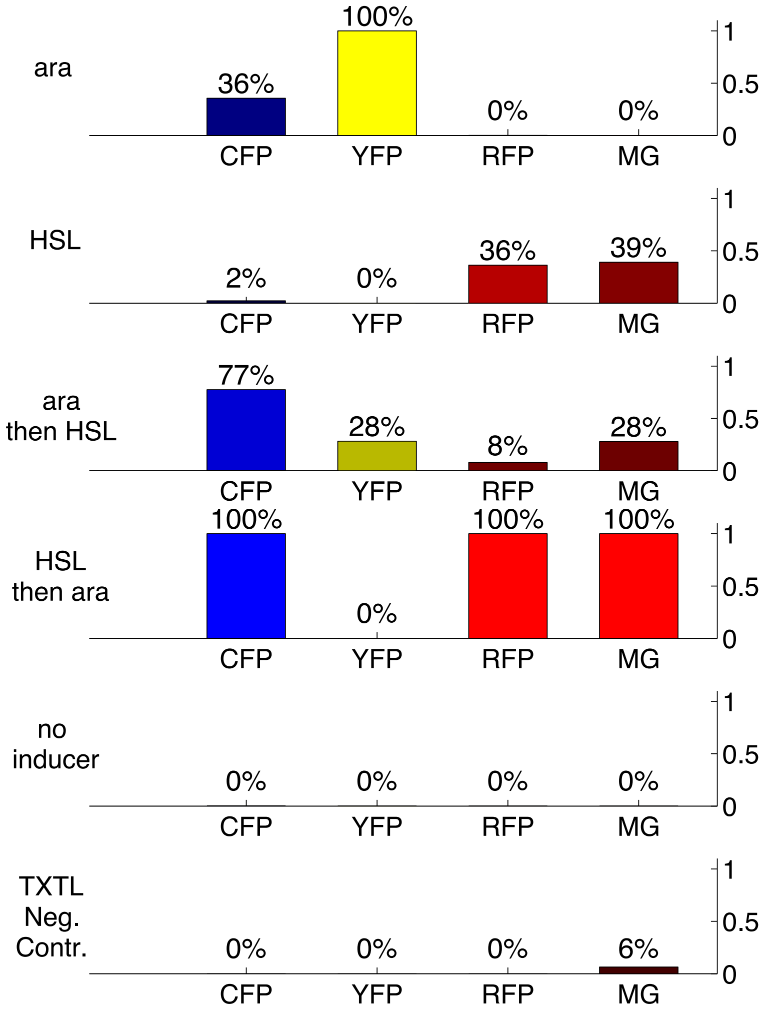


Biomolecular Breadboard Steady-State Outcomes Match Predictions

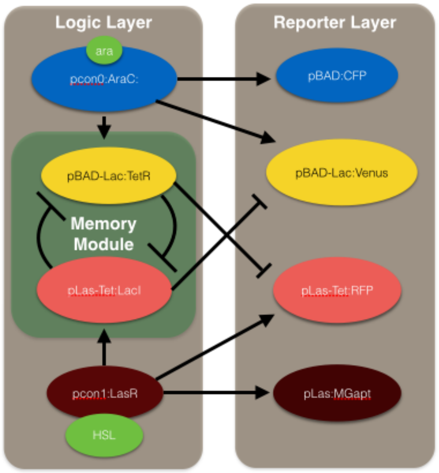
Expected (Steady-State) Outcomes

ara only	🔵 🟡
HSL only	🔴 🟤
ara then HSL	🔵 🟡 🟤
HSL then ara	🔵 🔴 🟤

Biomolecular Breadboard (TXTL) Steady-State Data



Event Detector Biocircuit



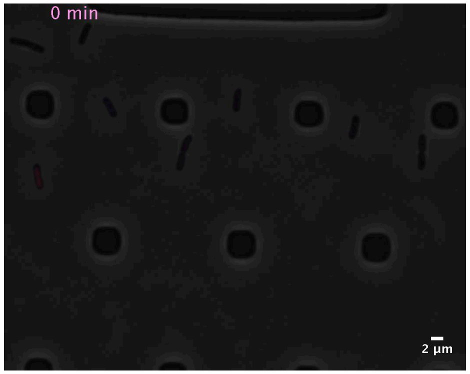
The event detector biocircuit functions *in vivo* in MG1655 *E. coli*

Expected (Steady-State) Outcomes

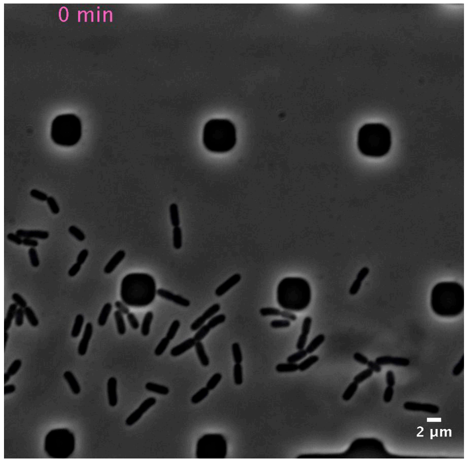
ara only	🔵 🟡
HSL only	🔴
ara then HSL	🔵 🟡
HSL then ara	🔵 🔴

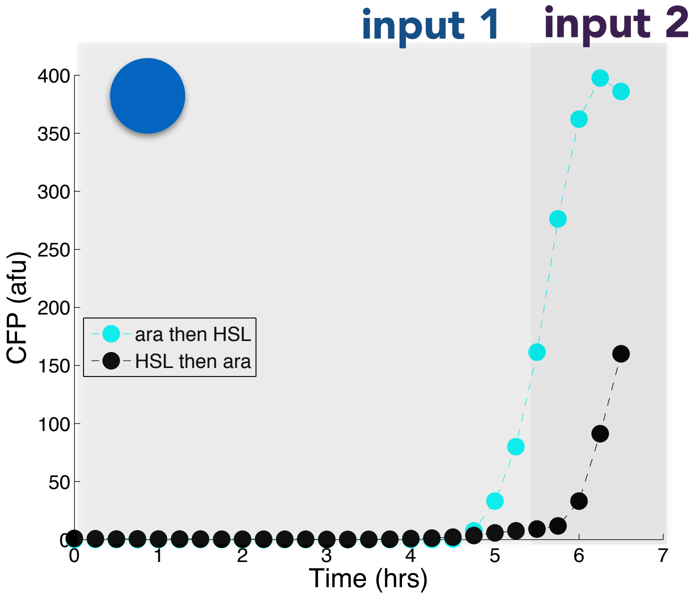
Preliminary Single Cell Microscopy Data

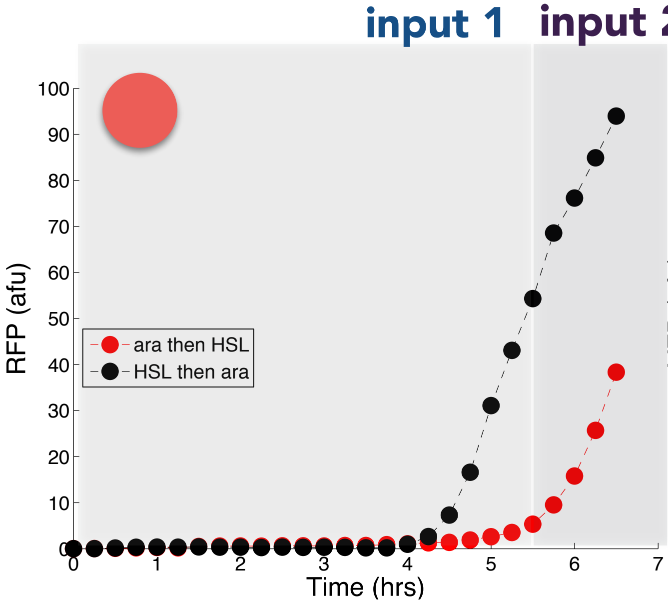
ara then HSL

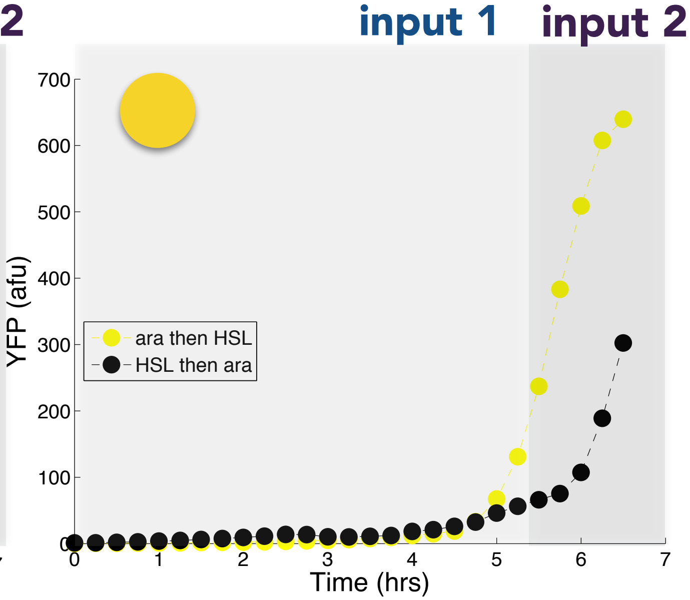


HSL then ara

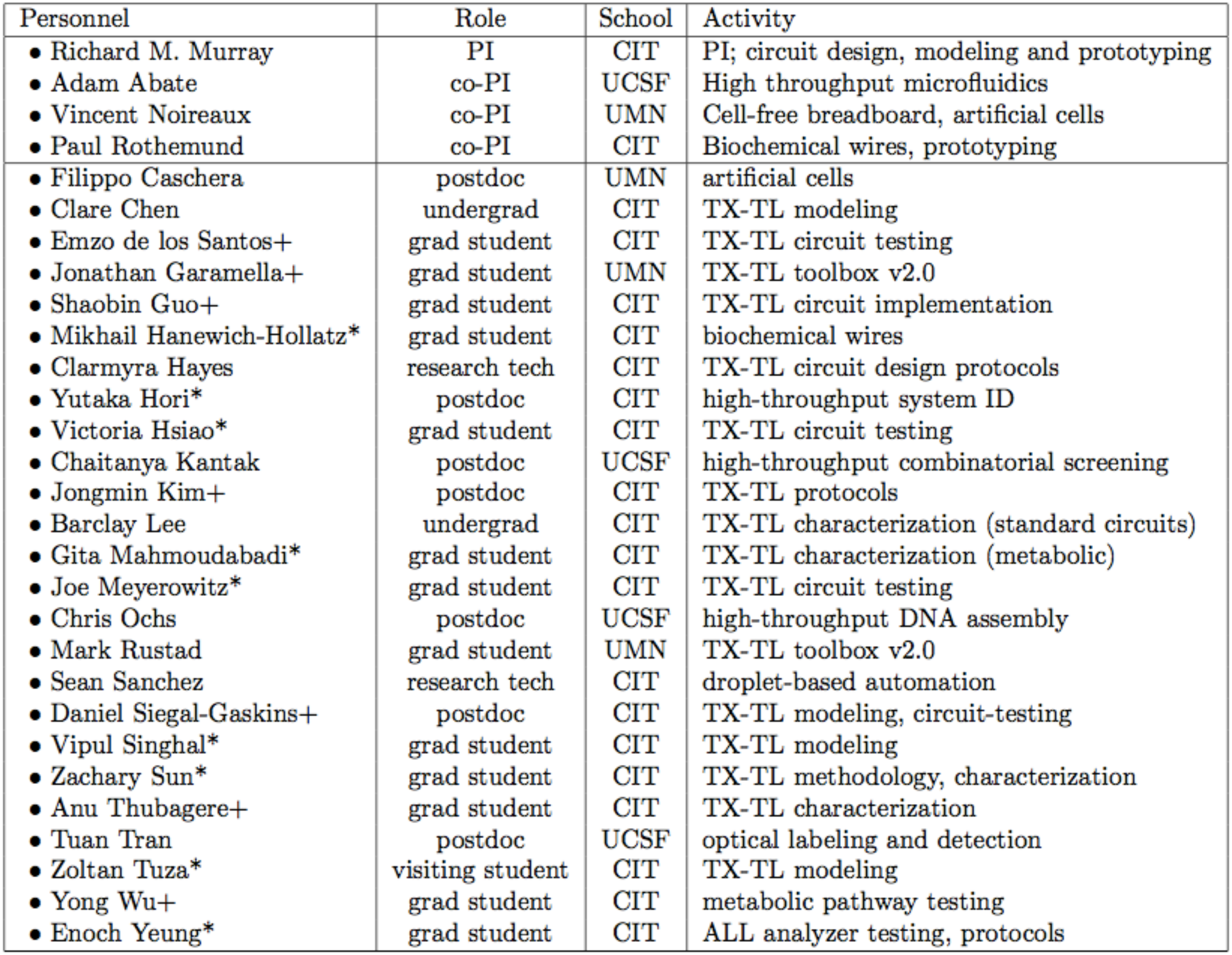








Breadboards Project Personnel



Personnel	Role	School	Activity
• Richard M. Murray	PI	CIT	PI; circuit design, modeling and prototyping
• Adam Abate	co-PI	UCSF	High throughput microfluidics
• Vincent Noireaux	co-PI	UMN	Cell-free breadboard, artificial cells
• Paul Rothemund	co-PI	CIT	Biochemical wires, prototyping
• Filippo Caschera	postdoc	UMN	artificial cells
• Clare Chen	undergrad	CIT	TX-TL modeling
• Emzo de los Santos+	grad student	CIT	TX-TL circuit testing
• Jonathan Garamella+	grad student	UMN	TX-TL toolbox v2.0
• Shaobin Guo+	grad student	CIT	TX-TL circuit implementation
• Mikhail Hanewich-Hollatz*	grad student	CIT	biochemical wires
• Clarmyra Hayes	research tech	CIT	TX-TL circuit design protocols
• Yutaka Hori*	postdoc	CIT	high-throughput system ID
• Victoria Hsiao*	grad student	CIT	TX-TL circuit testing
• Chaitanya Kantak	postdoc	UCSF	high-throughput combinatorial screening
• Jongmin Kim+	postdoc	CIT	TX-TL protocols
• Barclay Lee	undergrad	CIT	TX-TL characterization (standard circuits)
• Gita Mahmoudabadi*	grad student	CIT	TX-TL characterization (metabolic)
• Joe Meyerowitz*	grad student	CIT	TX-TL circuit testing
• Chris Ochs	postdoc	UCSF	high-throughput DNA assembly
• Mark Rustad	grad student	UMN	TX-TL toolbox v2.0
• Sean Sanchez	research tech	CIT	droplet-based automation
• Daniel Siegal-Gaskins+	postdoc	CIT	TX-TL modeling, circuit-testing
• Vipul Singhal*	grad student	CIT	TX-TL modeling
• Zachary Sun*	grad student	CIT	TX-TL methodology, characterization
• Anu Thubagere+	grad student	CIT	TX-TL characterization
• Tuan Tran	postdoc	UCSF	optical labeling and detection
• Zoltan Tuza*	visiting student	CIT	TX-TL modeling
• Yong Wu+	grad student	CIT	metabolic pathway testing
• Enoch Yeung*	grad student	CIT	ALL analyzer testing, protocols

* = fellowship
\+ = other project

Caltech: 21
UCSF: 4
UMN: 4

Journal papers
- 4 appeared
- 2 submitted
- ~4 in prep

Conf papers
- 7 appeared
- 3 accepted

Tech reports
- all q-bio, SEED submissions posted as bioRxiv preprints (on submission)
- 8 preprints

Breadboards Project Interactions

TX-TL short courses: 3 days, training + small project (data on day 1)

- Aug 2013 (17 participants): Amanda Loshbaugh (UCSF), Stephen Fried (Stanford), Ariel Hecht (Stanford/NIST), Lap Man Lee (U. Mich), Arnaz Khushroo Ranji (Northwestern), Cameron Myhrvold (Harvard), Hari Sivakumar (UCSB), Jaimie Stewart (UC Riverside), Phil Romero (UCSF), Liz Norred (U. Tenn/ONRL), Andrew Reimer (UC Riverside), Tuan Tran (UCSF), Scott Livingston (Caltech), Leopold Green (UC Riverside), Patrick Caveney (U. Tenn/ONRL), Brent Lutz (Covitect), Anna Paulson (Princeton)
- Jun 2014 (16 participants): Ania Baetica (Caltech), Anton Frisk (Lund), Deborah Fygenson (UCSB), Chaitanya Kantak (UCSF), Ashty Karim (Northwestern), Pulkit Malik (U. Marlyand), Chris Ochs (UCSF), Narajan Srinivas (Caltech), Jong Seto (UCSF), Brandon Wong (BU), David Younger (U Washington), Tiffany Zhou (Caltech) + 4 iGEM

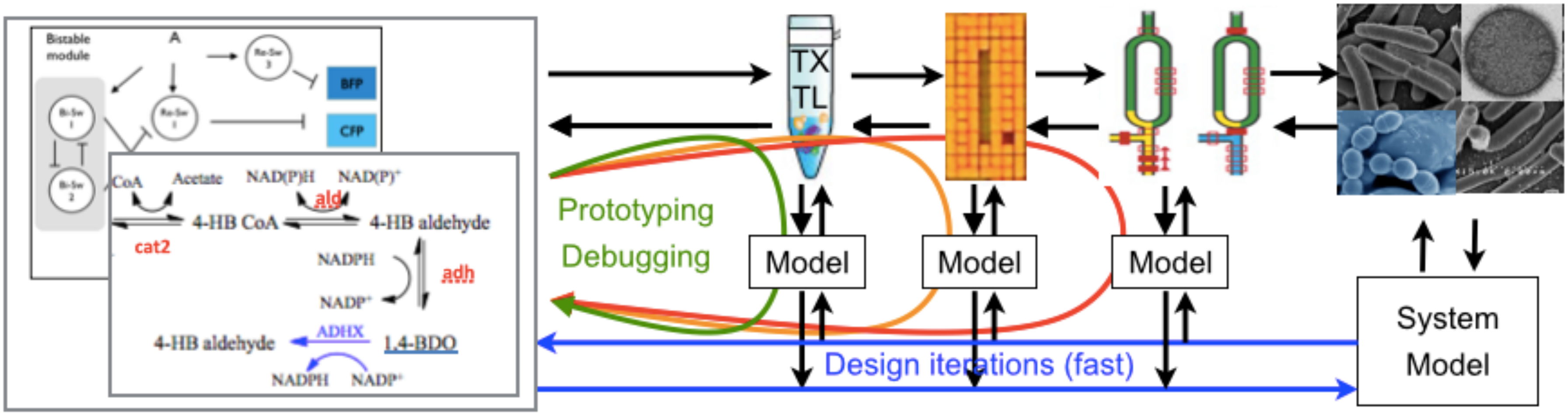
CSHL courses: two weeks, with TX-TL session (all) + TX-TL projects

- 2013 (6): RNA-based project (Lucks); published in *ACS Syn Bio,* 2014
- 2014 (16): RNA-based session + project, CRISPR, TX-TL characterization (*Methods*)

Labs using TX-TL:

- Developers: Caltech, Minnesota (Noireaux), Cornell (Lucks), Northwestern (Jewett)
- Users: Rice (Tabor), Princeton (Rabitz), Genomatica (Culler), Imperial (Ellis, Stan), ARL (Perkins, Warner), NIST (Munson, Hecht), EPFL (Maerkl), UCSF (Abate, Kortemme),
- Testing (they send circuits to us): MIT (Del Vecchio, Voigt), Harvard (Silver), U. Texas (Alper), Pivot Bio (Temme), Stanford (Endy), Rutgers (Sontag)

Next Steps, Transition Opportunities, Gaps



Ongoing activities

- Ongoing grants: NSF MPP (Winfree), ONR MURI (Voigt), AFOSR BRI (Del Vecchio), ARO ICB, HFSP (Maerkl)
- CSHL synthetic biology short course
- Caltech Grubstake - better extract
- DARPA 1KM (UCSF) - better μfluidics
- Metabolic pathways (w/ Genomatica)
- Next TX-TL bootcamp (June 2015)
- Synvitrobio, Inc (founded Mar 2015)
- Rotation + summer projects (many)

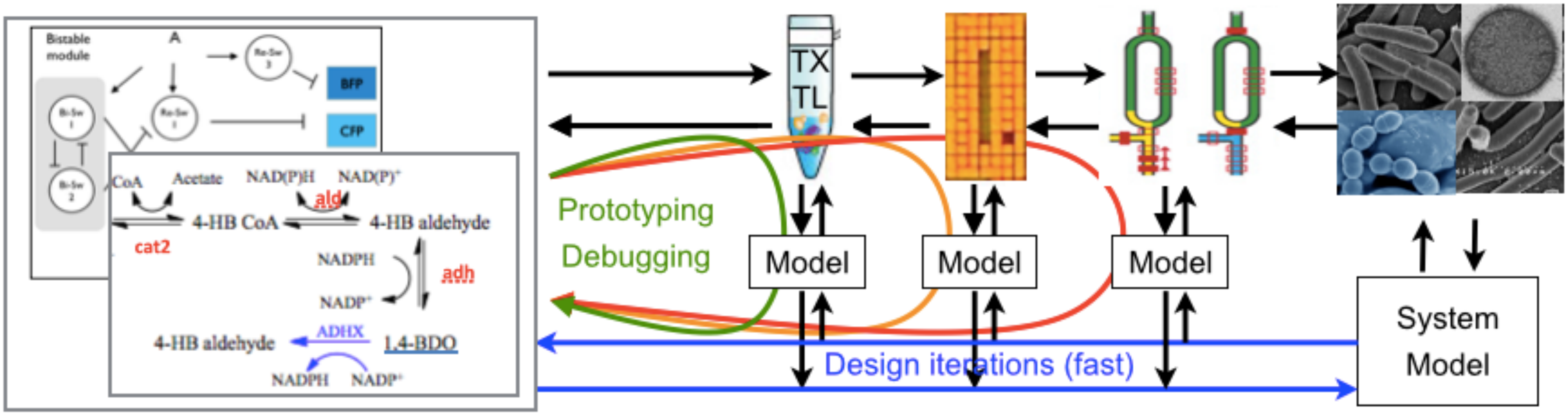
Additional opportunities

- TX-TL platform advances (Synvitrobio)
  - Better repeatability, lower cost
  - Educational kits (freeze-dried)
  - Extracts from other organisms
- Cell-free biosynthesis (Jewett)
- Paper-based TX-TL circuits (Collins)
- Biomolecular circuit theory (Fahroo?)

Gaps

- *Vitro→vivo*, resource lims, eukaryotes

Summary: Cell-Free Biomolecular Breadboards



Program metric	Current	Phase I	Phase II
1. Time required from synthesized DNA sequences to measurement of circuit performance (on cell-free breadboards)	1-2 wk	3 days ✓	1 day ✓
2. Time required to build a novel, modest complexity (6-8 unique promoter) circuit - existing design, novel components (**in vitro**/**in vivo**)	3-6 mo	1 mo ✓	1 wk **1-2w**/**2-4m**
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 ✓	20 ✓
5. Number of circuit combinations that can be screened per day, varying component concentration and/or genetic elements	5	N/A	10^6 ✓

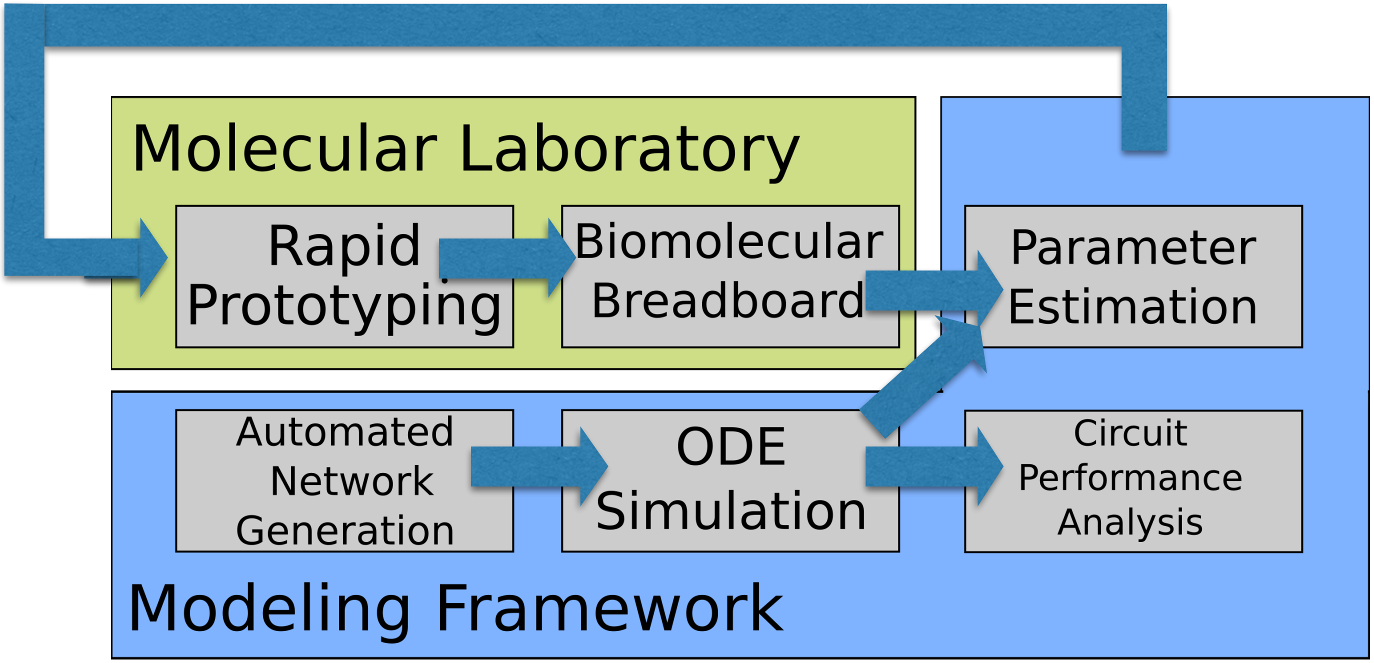
http://www.openwetware.org/wiki/breadboards

Backup

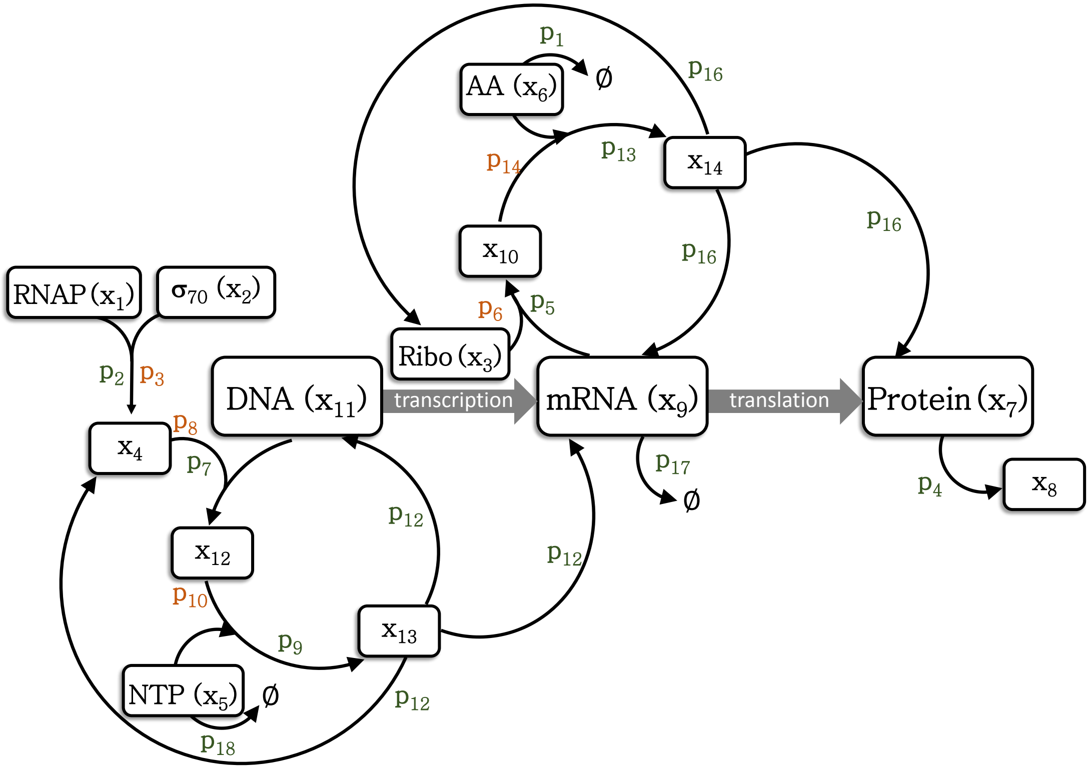
TX-TL Modeling Toolbox

Motivation

- Create a modeling framework to support rapid prototyping in a biomolecular breadboard.
- Modeling assumptions based on lab experiments
- Calibrate the model to experimental data
- Assist experimenters (Automatic exploration of the possible dynamics)
- Build a library of characterized parts for rapid in-silico prototyping of circuits



Modeling



Modeling assumptions

- Mass action kinetics
- Finite resources
- Coupling through resources
- TX: various stages of initiation lumped into a single step. Similarly for elongation and termination.

Our approach accounts for

- Resource utilization, Enzyme loading
- Sigma Factors

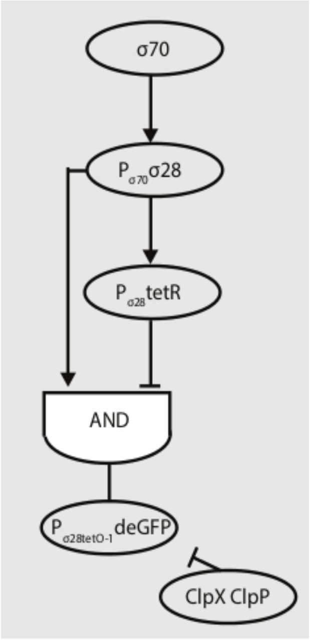
Not considered

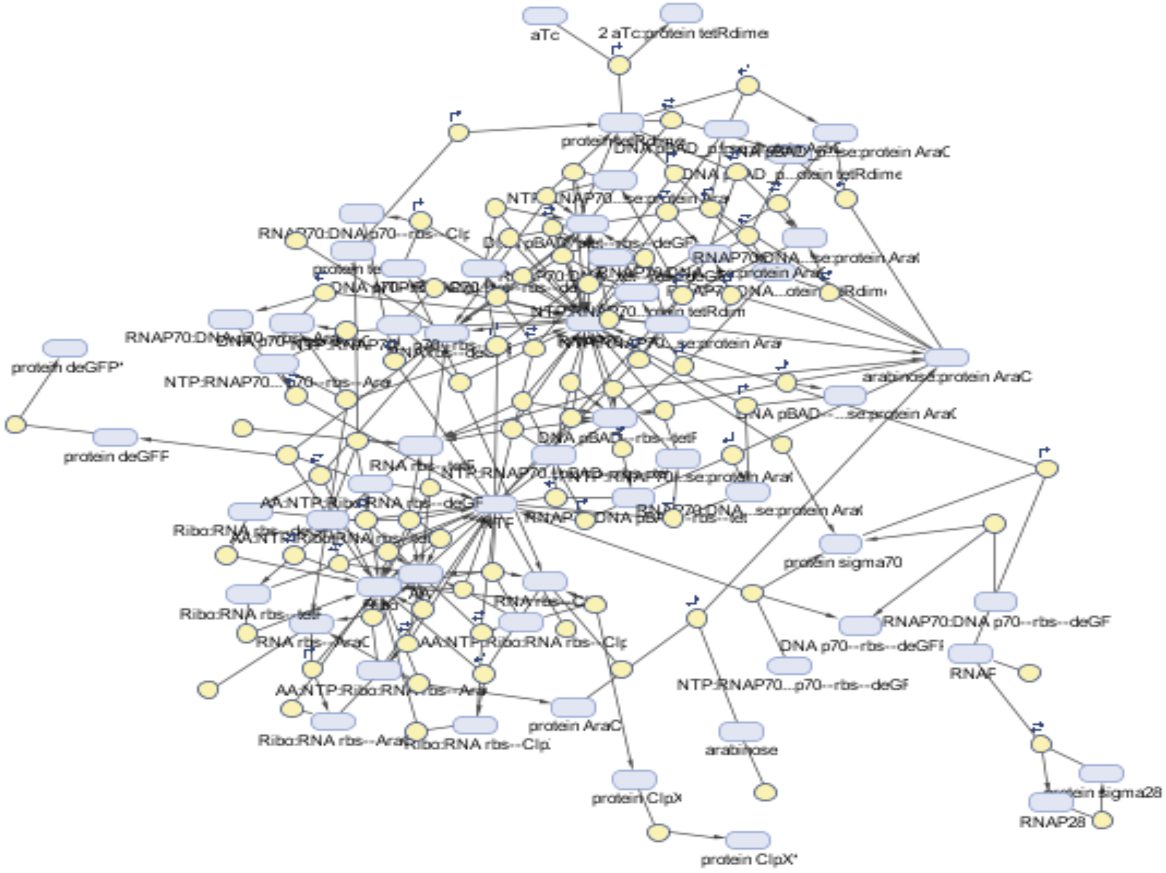
- Sequence information or spatial structure

Automatic Network Generation

Automatic generation of reaction network (simple gene expression vs. feed forward loop)

Making functional connections (based on the library of elements, e.g. Promoter: pLac + Protein: LacI → Repression)

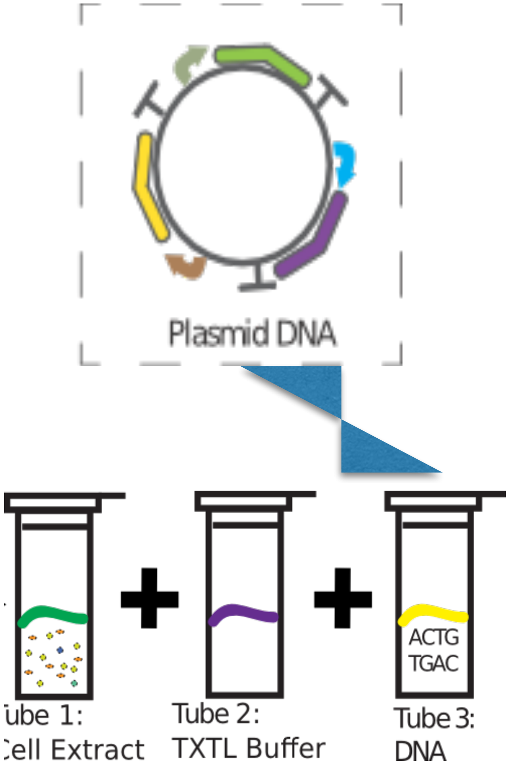


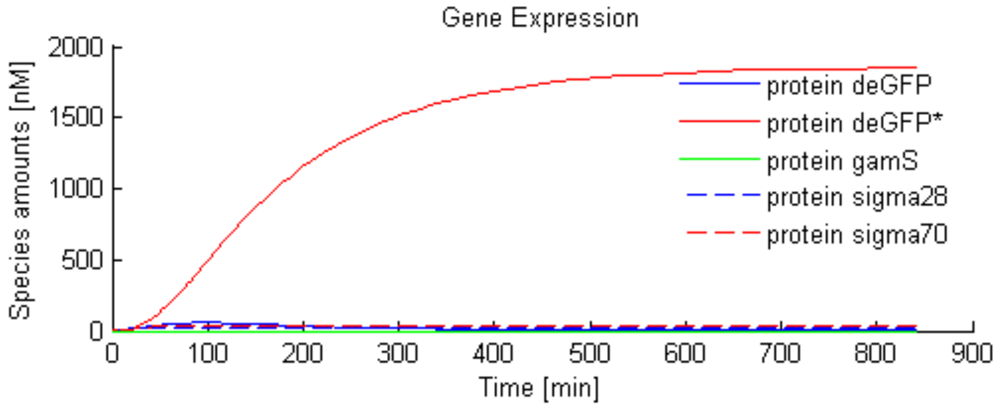


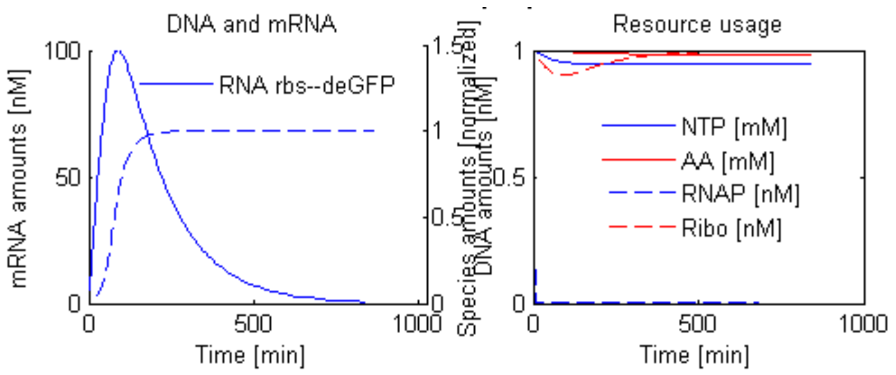
Few lines of code

Define basic components	tube1 = **txtl_extract**('E6');
	tube2 = **txtl_buffer**('E6');
	tube3 = **txtl_newtube**('circuit');

Define your circuit	**txtl_add_dna**(tube3, 'p70(50)', 'rbs(20)', 'deGFP(1000)', 4.0, 'plasmid');
Combine each components	Mobj = **txtl_combine**([tube1, tube2, tube3]);
Run the simulation	[t_ode,x_ode] = **txtl_runsim**(Mobj,simulationTime);
Plot the result	**txtl_plot**(t_ode,x_ode,Mobj);







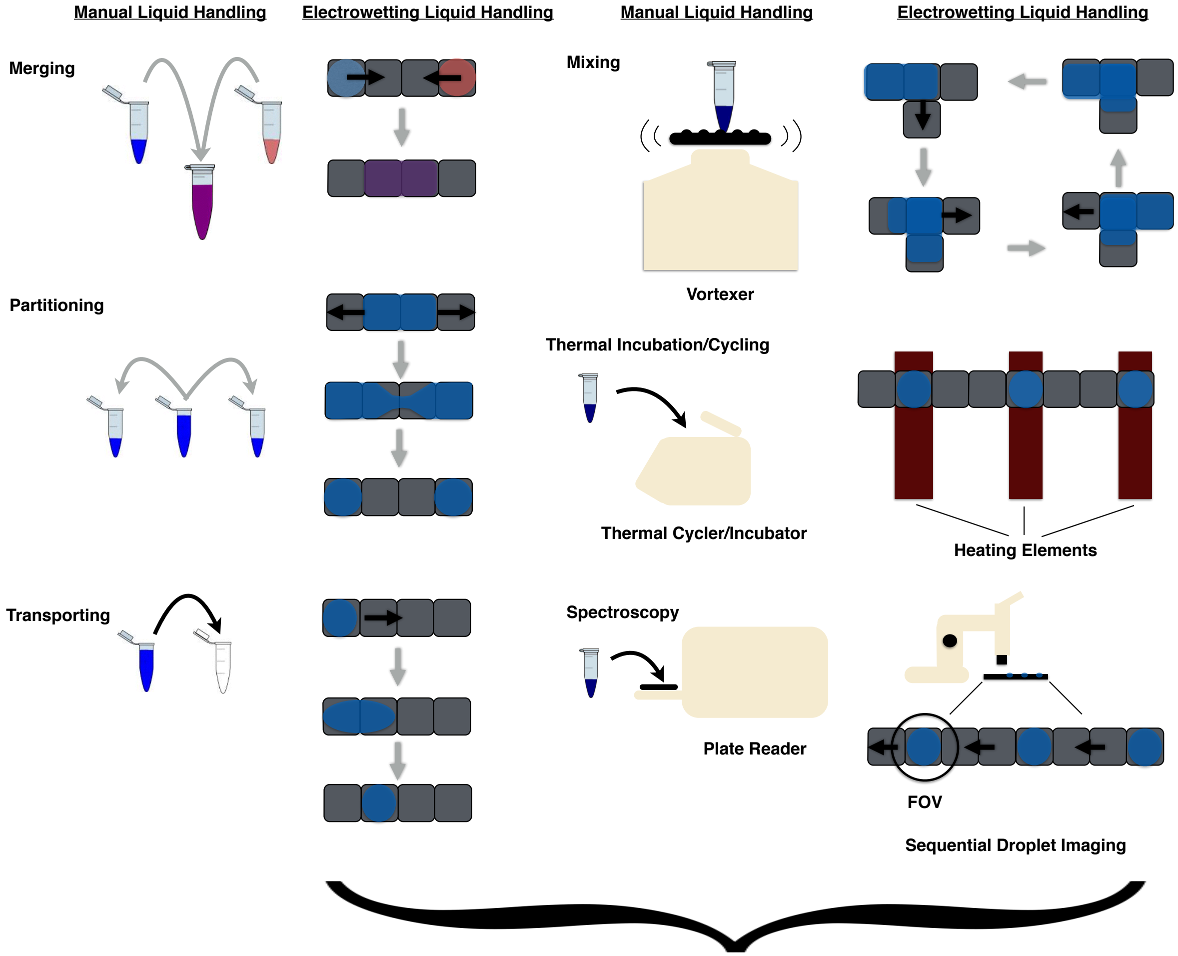
Automation Protocols in Electrowetting Microfluidics: DARPA Task 2.6

Statement of Work: Investigators shall develop, document and dessiminate a set of protocols and technologies enabling the use of digital microfluidics (droplets) to carry out TX-TL reactions for prototyping and debugging of biological circuits. Taken together, these new capabilities will enable real-time monitoring and cellular incorporation of synthetic gene *circuits.*

Enoch Yeung **Sean Sanchez**

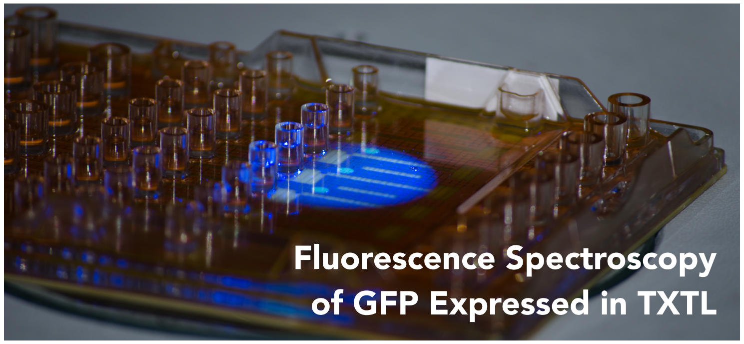
Engineering & Applied Science, Caltech

Basic microfluidic functions can be rapidly combined to program complex automation protocols



Programmable Protocols for Automating Biological Workflows

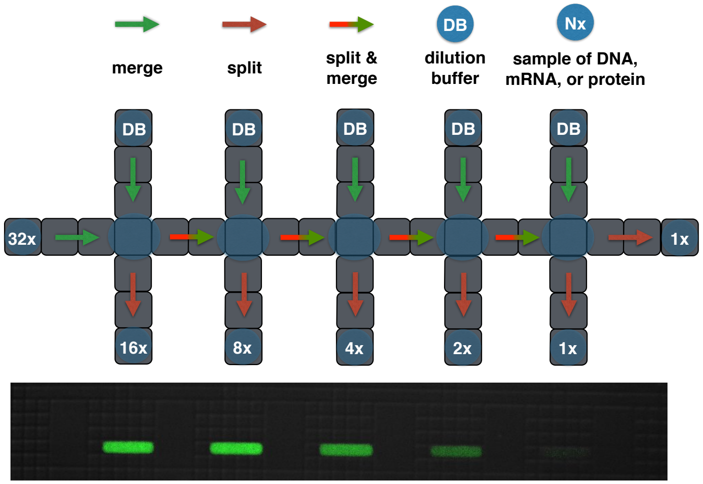
Microscopy Integrated with Microfluidics



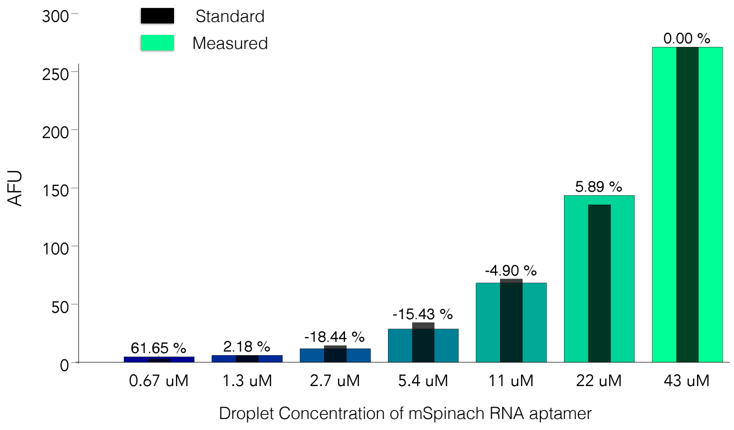
Fluorescence Spectroscopy of GFP Expressed in TXTL

EWOD microfluidics has compatible chemistry with protein, DNA, and TXTL

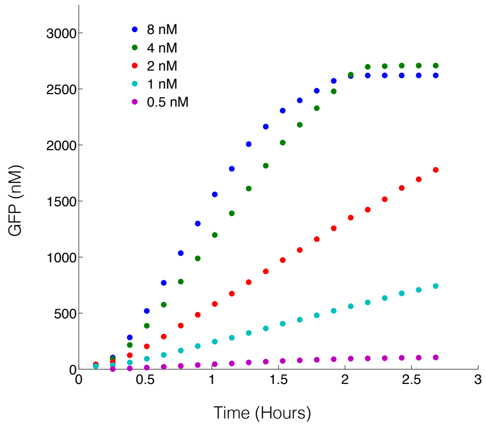
Basic Concept of Protocol

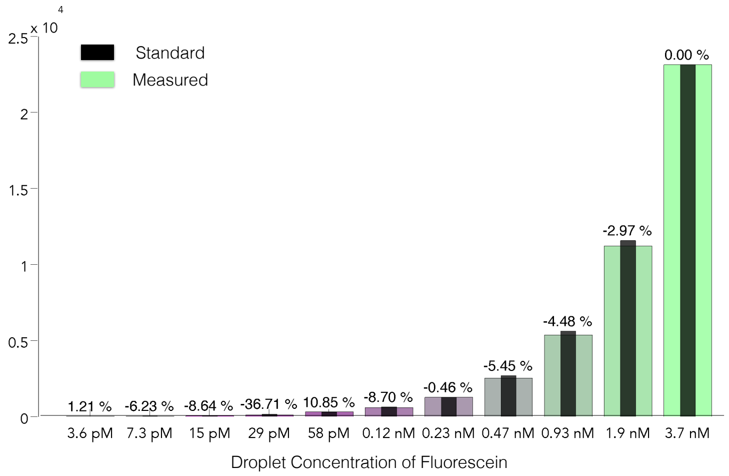


Serial Dilution of mSpinach RNA

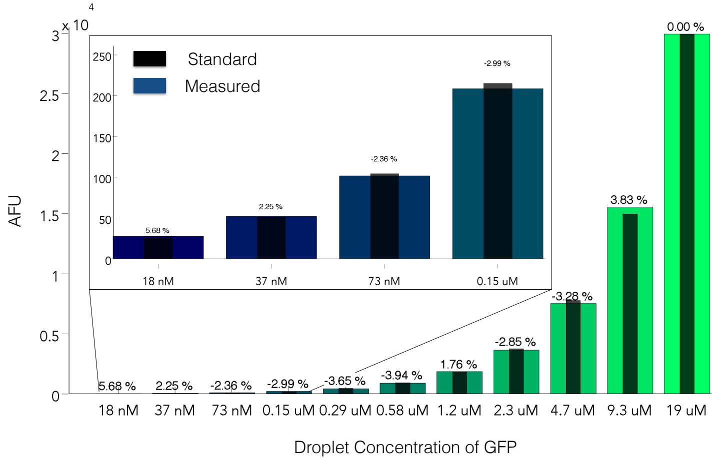


Serial dilution of plasmid DNA with TXTL master-mix

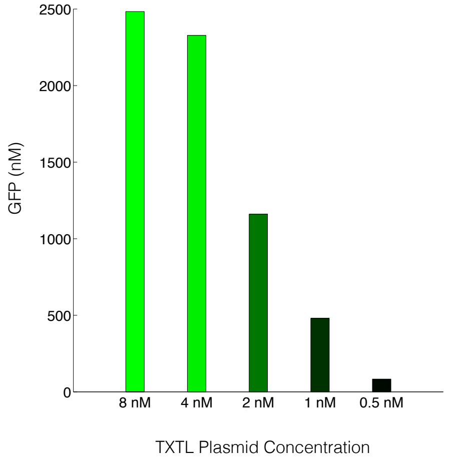




Serial Dilution of Fluorescein



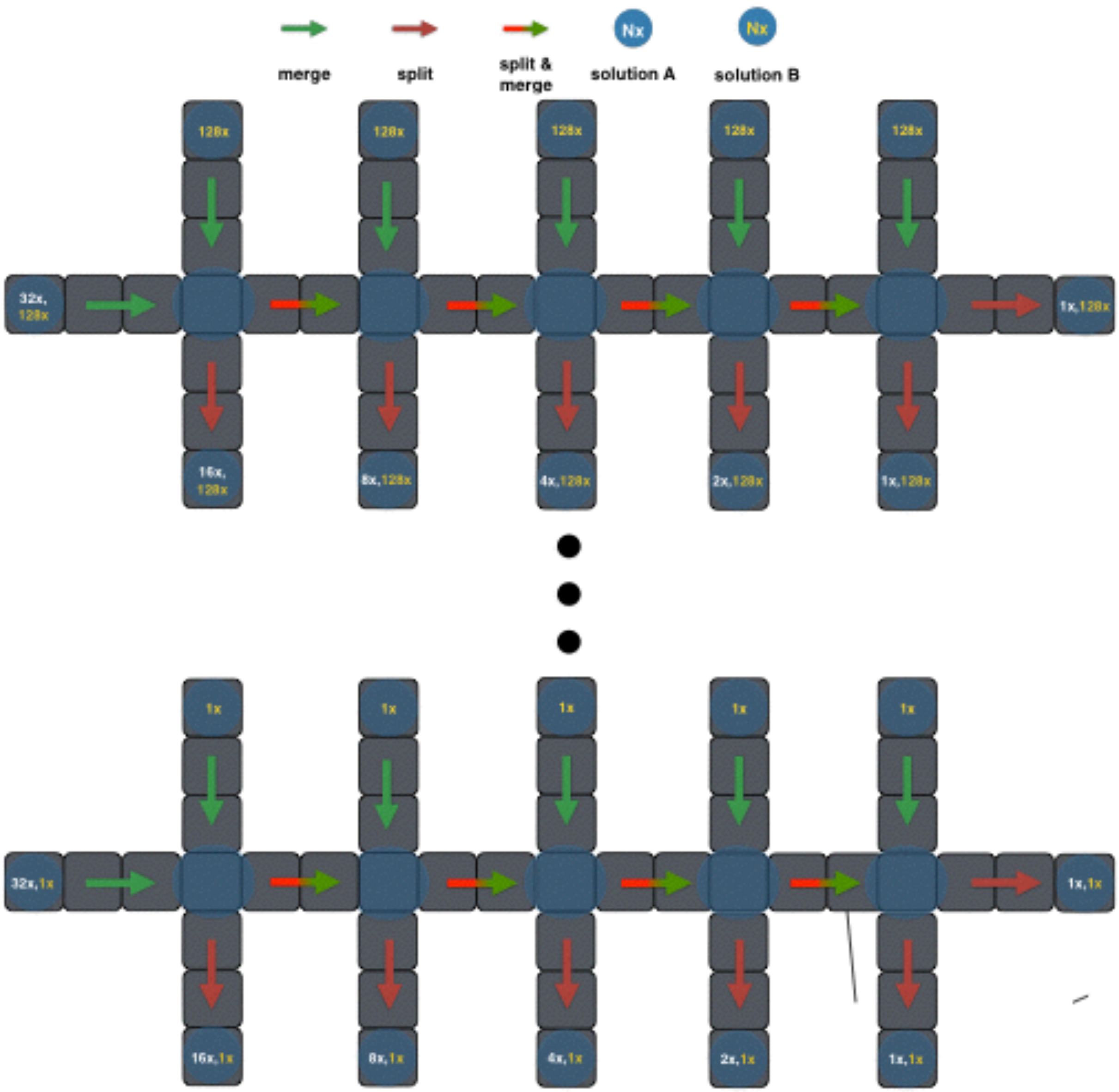
Serial Dilution of GFP



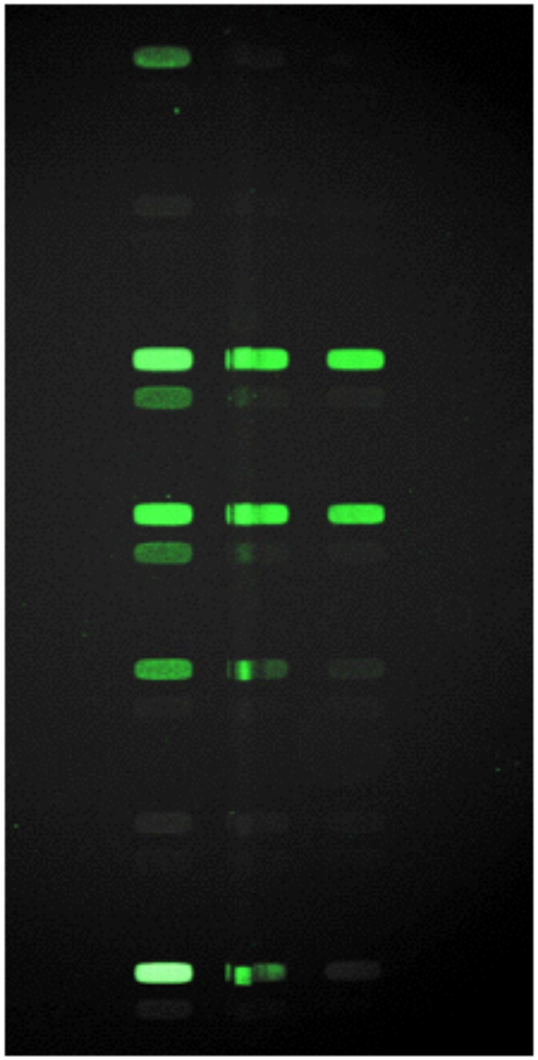
Steady-State Expression in TXTL

EWOD microfluidics can be programmed to perform the basic functions of TXTL sample prep and a plate reader.

EWOD microfluidics can be used to automate multi-variate titration assays

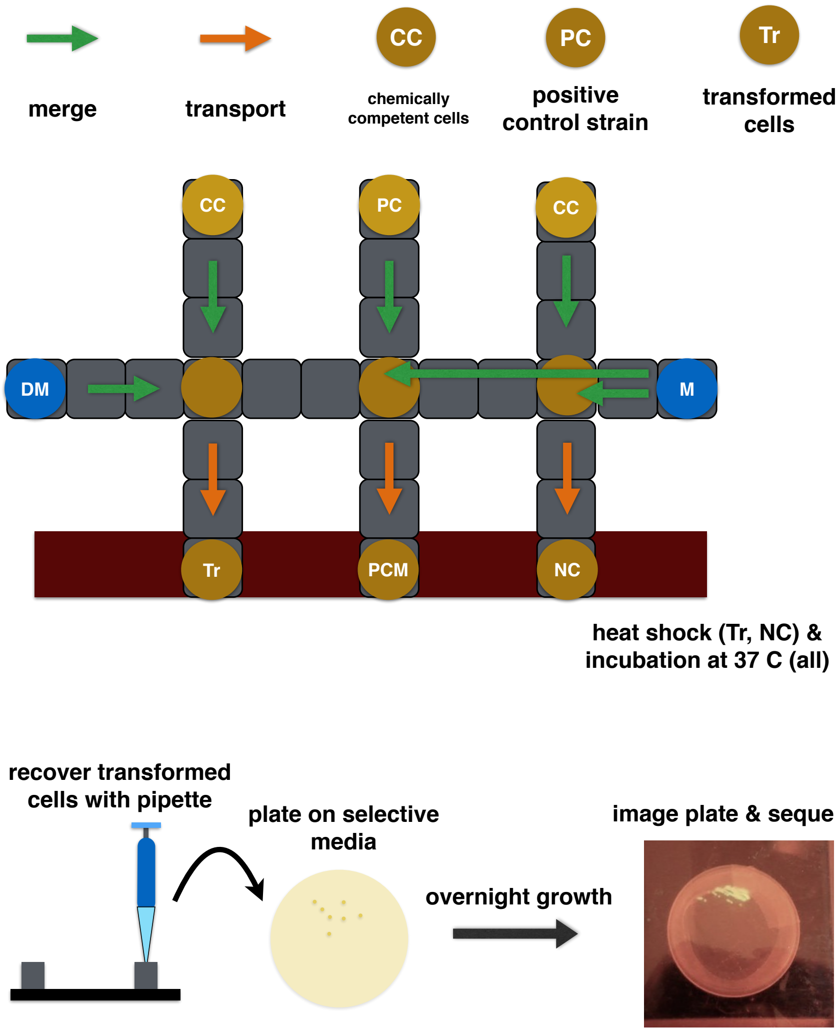


Steady-State Expression of 42 TXTL Reactions



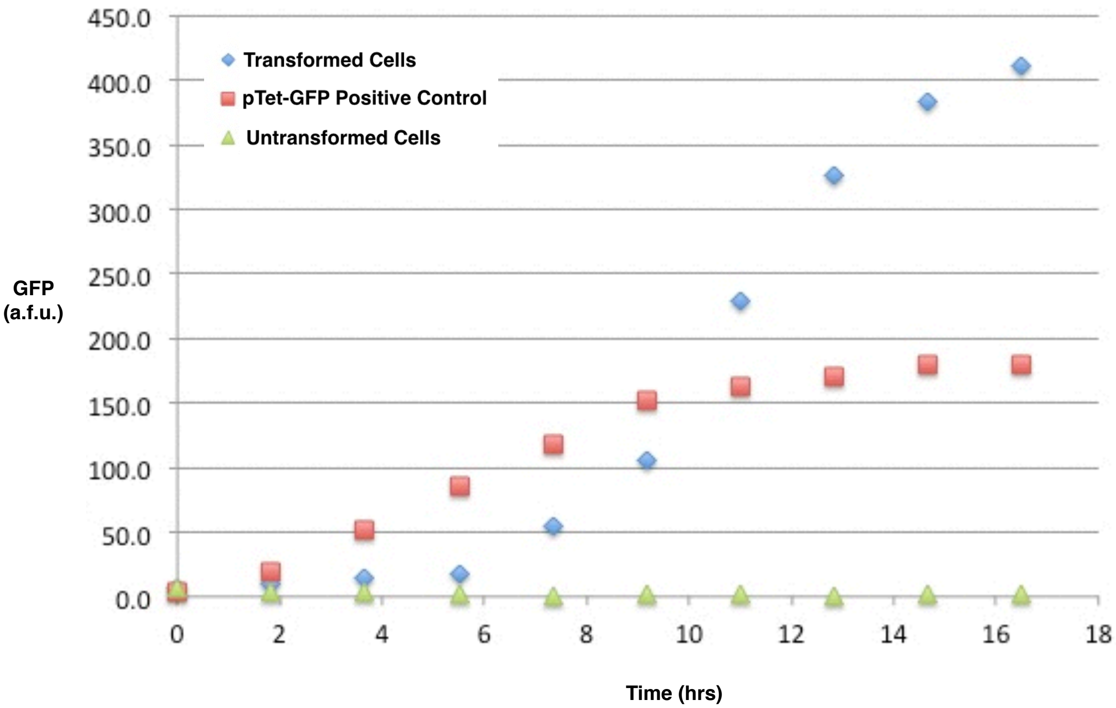
EWOD microfluidics can be programmed to automate extensive testing of the biocircuit design parameter space.

EWOD microfluidics can be used to automate chemical transformations



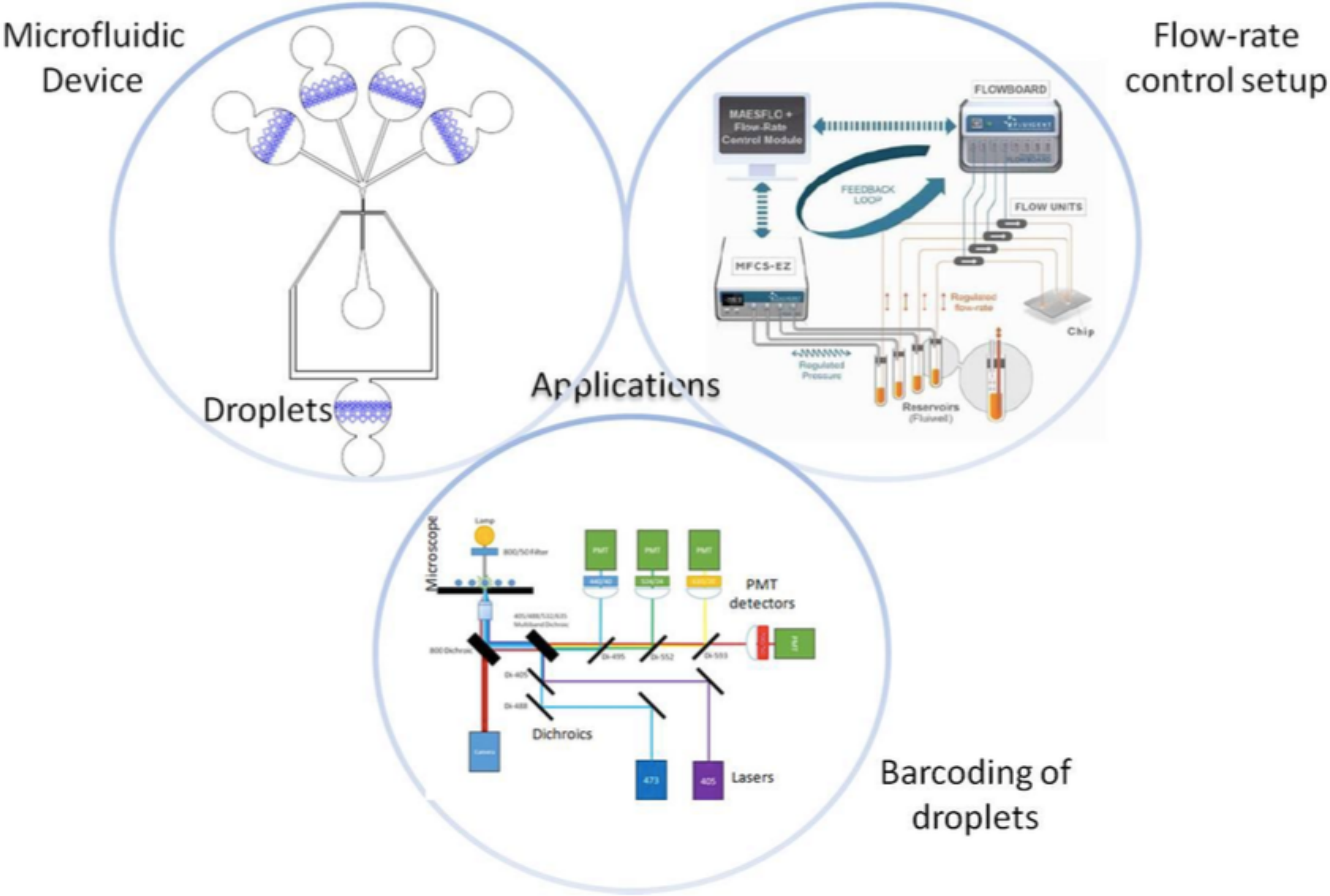
-By applying a standard heat-shock protocol, we can implement chemical transformation on-chip.

-Although growth rates are slower in the microfluidics environment, when transformed cells are plated onto selective media, they recover and sequence correctly.

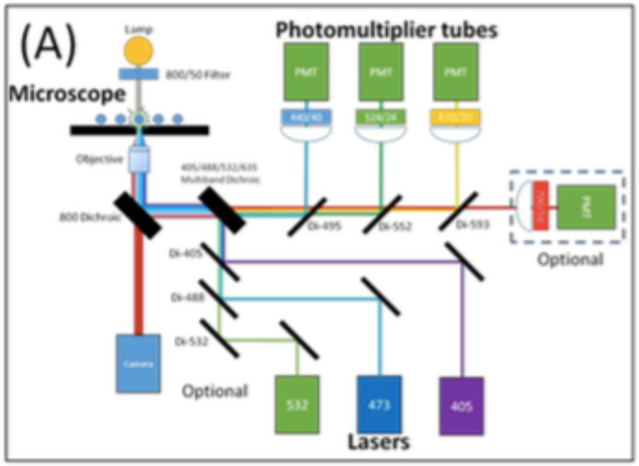


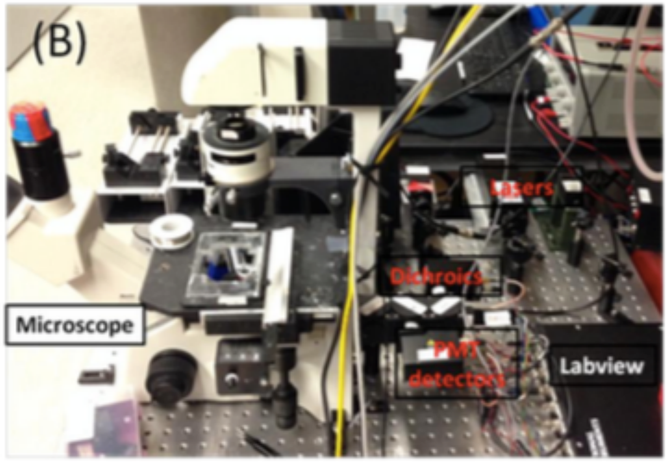
Currently, we're looking at protocols that combine TXTL prototyping with cell transformation and in vivo characterization .

Combinatorial Design Space Exploration

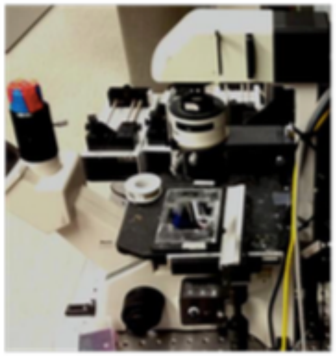


Dropometer

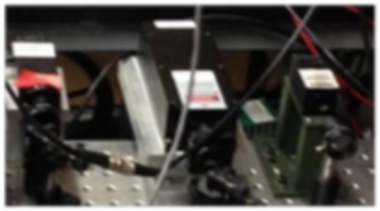




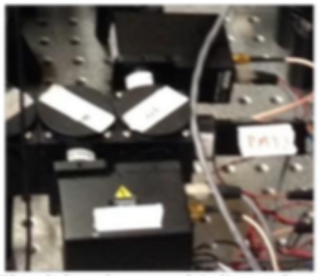
(C) Optical Components for microfluidic dropometer



Motic AE31 inverted microscope with 4X, 10X, 20X and 40X high NA objectives with two-way observation (eyepiece and camera) and lamp.



Three low noise colour lasers : violet (405nm), blue (473nm) and green (532nm); 50mW



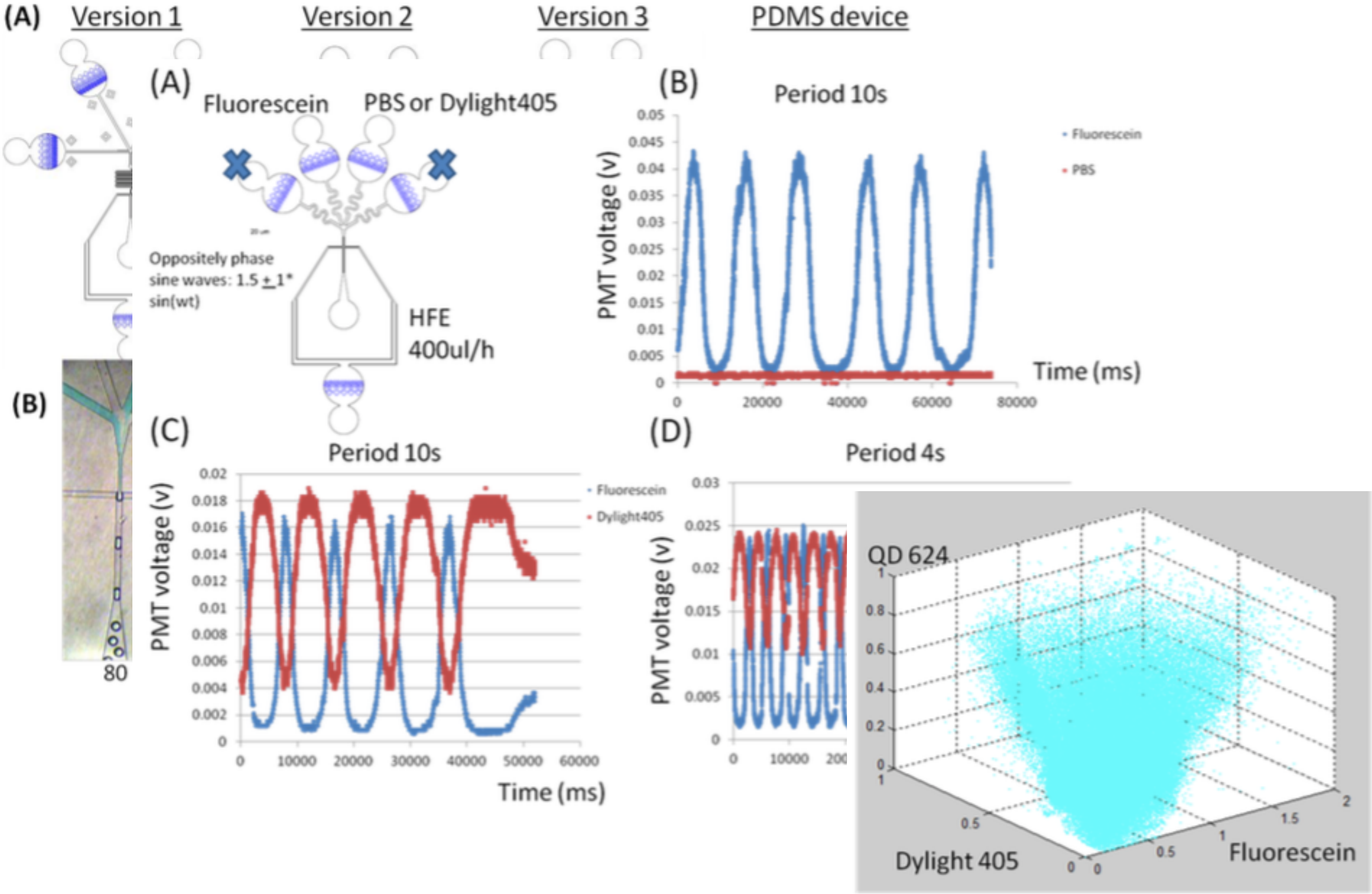
Thorlabs photomultiplier tubes-PMM02 with wavelength range response in 280-630nm

FPGA NI-7842R (200kHz, LX50) from National instruments

Dichroics and mirror assembly on the optical breadboard as per in the schematic

(A) We have built the microfluidic dropometer which can work with one laser and one detector. The system can be extended up to 3 PMTs and 3 lasers which were required for testing TX-TL circuits. We use Labview programming required for necessary data collection. The acquisition rate of 0.2MHz can be achieved by NI FPGA as per the milestone. Optical setup of the dropometer can be seen. (B)&(C) We have chosen FPGA (NI-7842R) which has acquisition rate of 200 KHz and built the optical system surrounding it using lasers and PMTs as shown. The selection of optical components (lasers, PMTs, dichroics, bandpass filters) was carried out to suit the emission and excitation spectra of these dyes: fluorescein, GFP, Cy5, Dylight 405, BV510, BE605, PE610, QD624 etc. The system is modular and can be possibly adapted to the most of the fluorescent dyes which are excited by specified lasers (violet, blue, green).

Combinatorial Mixer



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[3] D. Siegal-Gaskins, Z. A. Tuza, J. Kim, V. Noireaux, and R. M. Murray. Resource usage and gene circuit performance characterization in a cell-free 'breadboard'. *ACS Synthetic Biology*, 2014.

[4] Z. Z. Sun, C. A. Hayes, J. Shin, R. M. Caschera, F.and Murray, and V. Noireaux. Protocols for implementing an *escherichia coli* based tx-tl cell-free expression system for synthetic biology. *Journal of Visualized Experiments (JoVE)*, (e50762), 2013.

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[7] Z. A. Tuza, V. Singhal, J. Kim, and R. M. Murray. An *In Silico* modeling toolbox for rapid prototyping of circuits in a biomolecular "breadboard" system. In *Conference on Decision and Control*, 2013.