

Final Technical Report
9/1/09-5/31/13

a. DOE Award # and name of the recipient (Institution)
DE-SC0001216 (University of California, Berkeley)

b. Project title and name of the PI
Engineering the Interface Between Inorganic Materials and Cells
PI: David Schaffer

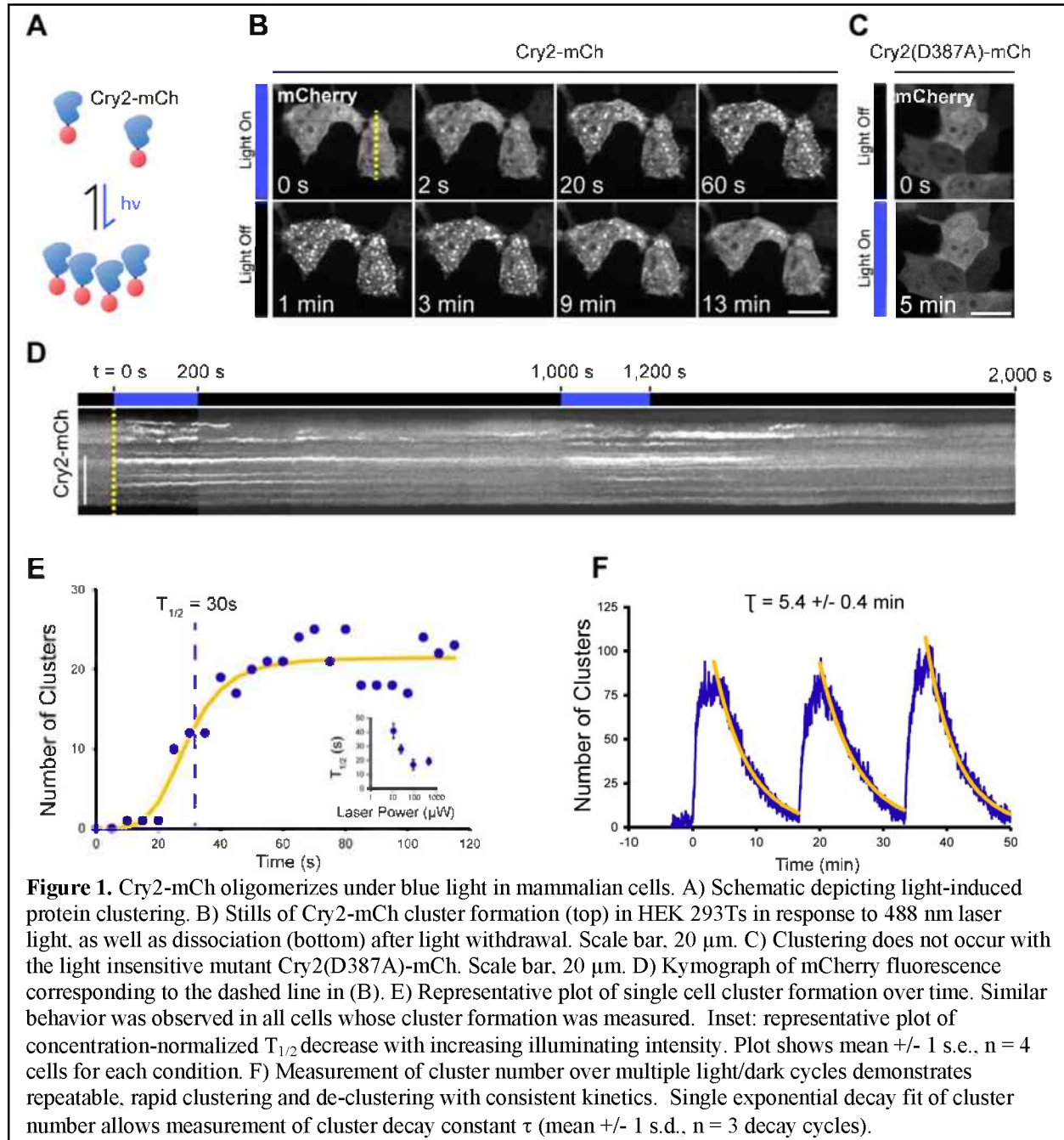
c. Date of the report and period covered by the report
Period covered by the report: 9/1/09-5/31/13

d. A Brief Description of Accomplishments.

To further optimize cell function in hybrid “living materials”, it would be advantageous to render mammalian cells responsive to novel “orthogonal” cues, i.e. signals they would not ordinarily respond to but that can be engineered to feed into defined intracellular signaling pathways.

I. Inducible Protein Clustering and Cytoskeletal Remodeling in Cells in Response to Light

We recently developed an optogenetic method, also based on *A. thaliana* Cry2, for rapid and reversible protein oligomerization in response to blue light (Fig. 1). We also demonstrated the ability to use this method to channel the light input into several defined signaling pathways. A manuscript based on this work was published in *Nature Methods*, and some key findings are



summarized below.

Protein oligomerization – the assembly of proteins into multimeric structures – is a particularly powerful mechanism for modulating cellular signaling and is used to activate broad classes of proteins including receptors, ligands, kinases, transcription factors, and numerous other cytosolic signaling effectors. Within such systems, protein oligomerization enables ultra-sensitive “all or nothing” signaling responses and connects nanoscale protein assembly and activity to microscale control of cellular structures and behavior¹. Upon transfection of Cry2 (fused to mCherry) alone into HEK 293T cells we observed that Cry2-mCh rapidly formed distinct fluorescent puncta within 10 seconds of blue light illumination (Figure 1A,B). Upon blue light withdrawal, the clusters dispersed to their original state within minutes (Figure 1B,D,F). Clustering was not observed with a light-insensitive Cry2 mutant harboring a D387A mutation² (Figure 1C). From a diffuse initial condition, Cry2-mCh fluorescence could be repeatedly clustered and unclustered with similar kinetics (Figure 1D,F). These results demonstrate light-mediated assembly of protein structures inside cells in an inducible and reversible manner.

Light-Induced Activation of Rho GTPases

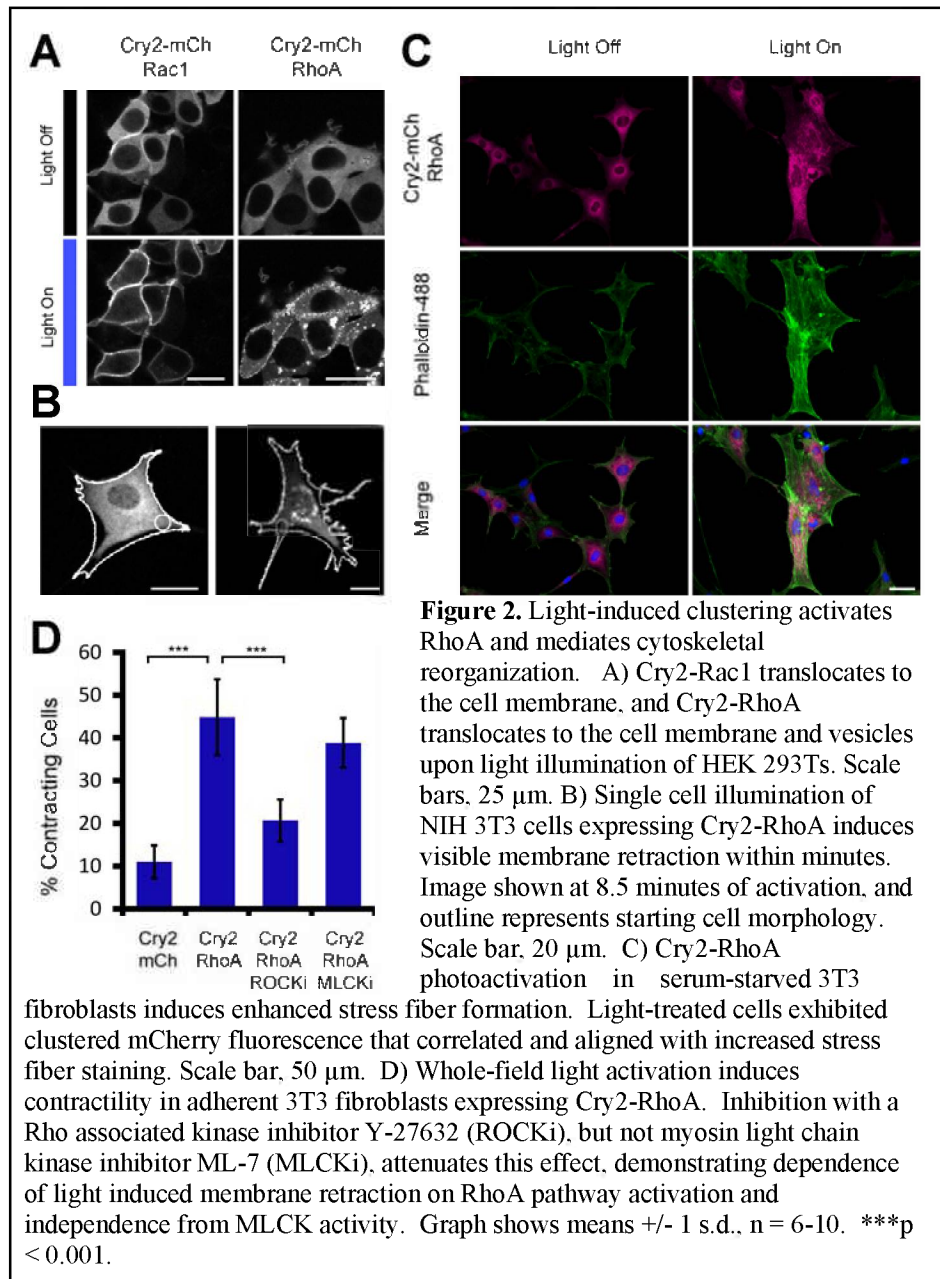
Next, motivated by the longer-term goal of controlling cell morphology and migration on surfaces using light, we explored the ability to use the Cry2-mCh system to activate Rho GTPases, small proteins involved in actin polymerization, cell motility, and mechanotransduction³⁻⁶. Oligomerization of the Rho GTPase Rac1 has been reported to enhance its enzymatic activity and effector activation within *in vitro* assays⁷, though the functional significance of Rac1 oligomerization in living cells has to our knowledge not been studied. Within 10 seconds of illumination, HEK 293T cells expressing a fusion of Cry2-mCherry with Rac1 (Cry2-Rac1) demonstrated translocation of mCherry fluorescence from the cytoplasm to the membrane, accompanied by lamellipodial extension, suggesting Rac1 activation⁸ (Figure 2A). To our knowledge, this is the first indication that clustering wild-type Rac1 may be sufficient to induce its membrane translocation in living cells.

Since a different strategy has recently been utilized to generate a light-activated Rac1⁹, we investigated whether our inducible clustering approach could be harnessed to activate a distinct Rho GTPase family member. RhoA is a small GTPase primarily responsible for mediating cellular tension and cytoskeletal contraction¹⁰. However, photocontrol of RhoA has not previously been achieved, and oligomerization has not been linked to RhoA activity. Cry2-mCh-RhoA (Cry2-RhoA) was initially diffuse but rapidly translocated to membranes and vesicles within 10 seconds of activation with 458 nm or 488 nm laser light (10 μ W) in HEK 293Ts (Figure 2A) and NIH 3T3 fibroblasts (data not shown). Furthermore, a laser was used to illuminate a single spot within individual cells in a confocal microscope (shown as a circle in Figure 2B), and within minutes cellular membrane retraction was evident (Figure 2B), suggesting that the RhoA translocation coincided with RhoA activation and cellular contractility. Finally, both membrane retraction and Cry2-RhoA membrane localization were reversible within minutes, and cells could be repeatedly activated and inactivated for hours (data not shown).

We also examined the induction of stress fibers – bundled structures of filamentous actin that mediate cytoskeletal contractility and that are formed downstream of RhoA activation¹¹.

Illumination of fibroblasts expressing Cry2-RhoA showed a strong increase in both the number and intensity of stress fibers over unilluminated controls (Figure 2C). Interestingly, in highly-expressing cells, visible light-induced Cry2-RhoA clusters orient along – and in many cases colocalize with – the stress fibers. To further investigate the functional specificity of the photo-clustered RhoA response, fibroblast contractility was observed under wide field microscopy by

assessing cell membrane retraction in the presence of pathway inhibitors. Illuminated cells expressing Cry2-RhoA contracted at a higher frequency than cells expressing Cry2-mCh ($p < 0.001$, Tukey-Kramer test, $n = 6$ and 8 fields of view, Figure 2D). Furthermore, inhibition of the RhoA downstream effector Rho-associated protein kinase (ROCK) with the small molecule Y-27632 reduced the percentage of contractile cells ($p < 0.001$, Tukey-Kramer test, $n = 8$ and 8 fields of view), but inhibition of myosin light chain kinase – which mediates cytoskeletal contractility independently of RhoA and ROCK – did not ($p = 0.3$, Tukey-Kramer test, $n = 8$ and 10 fields of view), indicating that the light-induced contractile response is in large part specific to RhoA activity. Taken together, these data strongly suggest that Cry2-mediated, light-inducible



clustering of RhoA activates the RhoA pathway and induces cytoskeletal remodeling within adherent cells.

II. Inducible Control over Translation in Response to Light

Light-Activated mRNA Translation

As a demonstration of the ability to control the cellular machinery using light, we initially focused on designing a system to activate mRNA translation in response to light. The ability to synthesize a protein of interest “on demand” in a controlled fashion would provide a useful handle to control many cellular functions. An important step in translation initiation is the recruitment of the small ribosomal subunit to an mRNA^{12, 13}, a process involving the 5'-cap-binding subunit of the eukaryotic initiation factor (eIF), eIF4E. Previous studies have shown that tethering of the subunit eIF4E to a target mRNA alone is sufficient to initiate mRNA translation¹².

Our approach to activate protein-RNA assembly and translation in response to light draws on this prior work and makes use of the photoreactive PHR domain from the cryptochrome 2 protein (Cry2PHR or Cry2), which has been shown to heterodimerize with the protein CIB1 upon illumination with blue light¹⁴. Specifically, we designed two fusion proteins (Figure 3): 1) λ -CIBN, a fusion of the RNA-binding domain of the λ phage antiterminator protein N¹² and CIBN – a truncated mutant of CIB1¹⁴; and 2) Cry2PHR-eIF4E, in which the eIF4E subunit was fused to the C-terminus of Cry2PHR.

We also designed a reporter plasmid coding for a bicistronic mRNA (Figure 3) that contains a green fluorescent protein (GFP) upstream open reading frame (ORF), a firefly luciferase downstream ORF, and six copies of the specific binding site for the λ phage protein N¹² (boxB). We hypothesized that illumination with blue light would induce Cry2-CIBN heterodimerization,

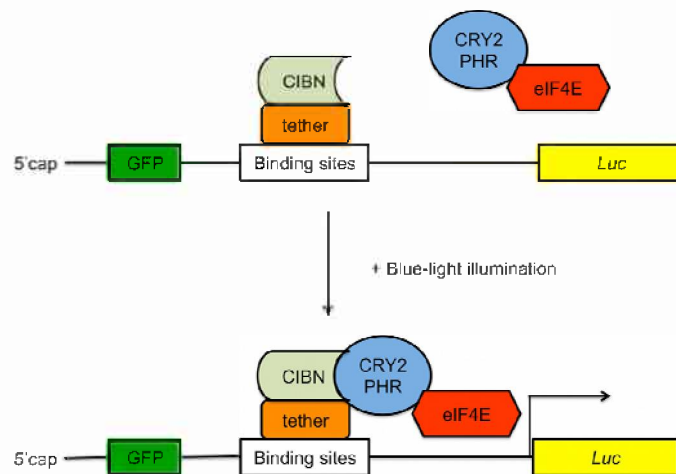


Figure 3. The λ -CIBN fusion protein localizes to the boxB aptamer upstream of the reporter protein on mRNA. Cry2PHR-eIF4E fusion protein binds to λ -CIBN fusion protein upon blue-light illumination through the Cry2PHR-CIBN dimer and eIF4E recruits other related proteins to initiate the translation of downstream gene.

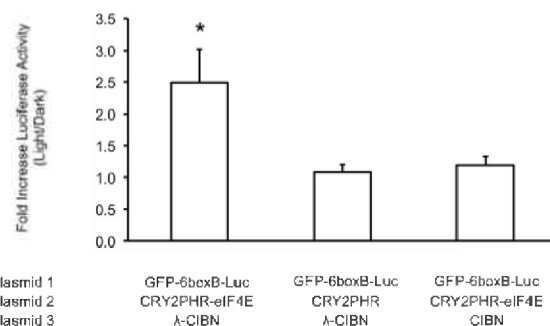


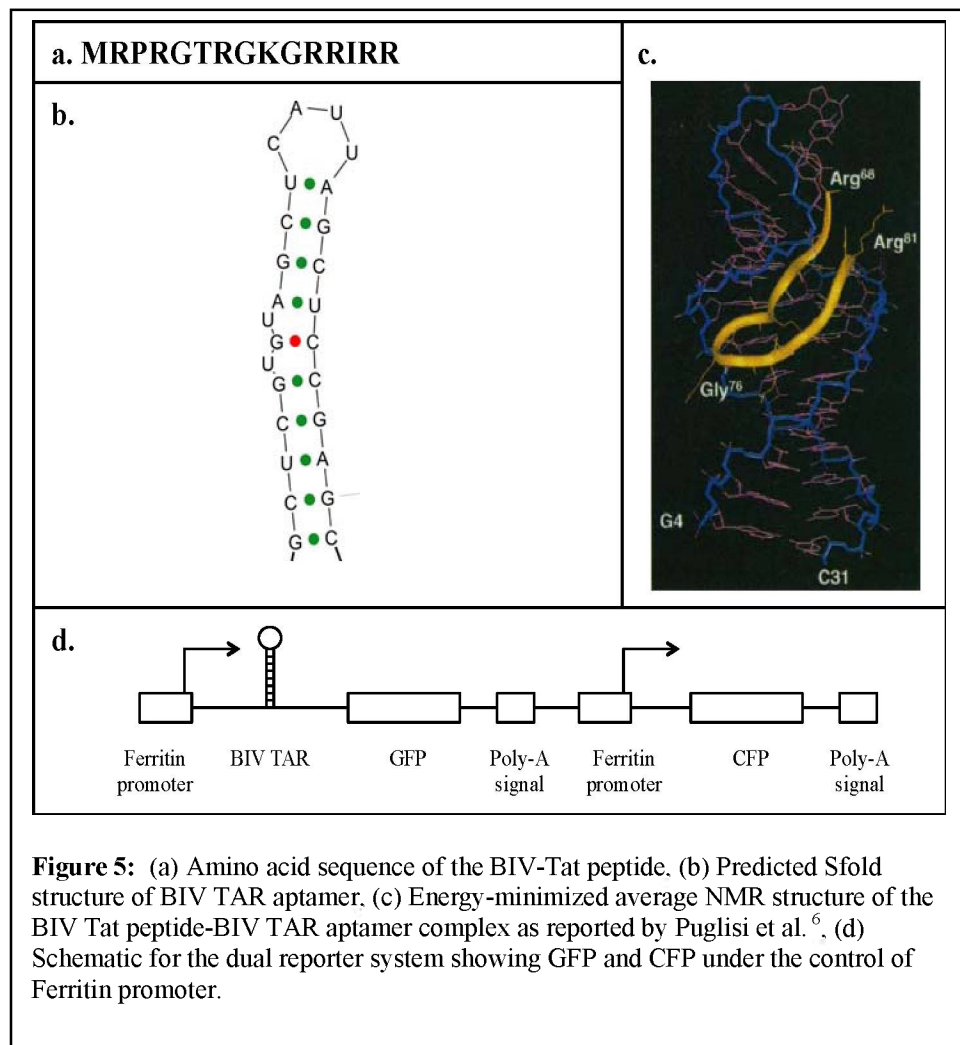
Figure 4. Activating translation of luciferase in response to blue light. HEK 293T cells were transfected with the luciferase reporter and i) Cry2PHR-eIF4E and λ -CIBN; ii) Cry2PHR and λ -CIBN; or iii) Cry2PHR-eIF4E and CIBN. Transfected cells were placed in the dark or exposed to blue light, and luminescence was measured after the addition of the substrate for luciferase. A 2.5-fold increase in luminescence was seen upon illumination with blue light when both Cry2PHR-eIF4E and λ -CIBN were expressed.

thereby recruiting Cry2PHR-eIF4E to the target mRNA (Figure 3) and activating the translation of luciferase.

We transfected HEK 293T cells with a mixture of the reporter plasmid, an effector plasmid coding for Cry2PHR-eIF4E, and an effector plasmid coding for λ -CIBN. As seen in Figure 4, blue light illumination resulted in an almost 2.5-fold increase in the translation of luciferase. The presence of effector plasmids containing both fusion proteins was necessary; relatively insignificant changes in the amount of luciferase were seen in control experiments where Cry2PHR-eIF4E was replaced by Cry2PHR, or where λ -CIBN was replaced by CIBN.

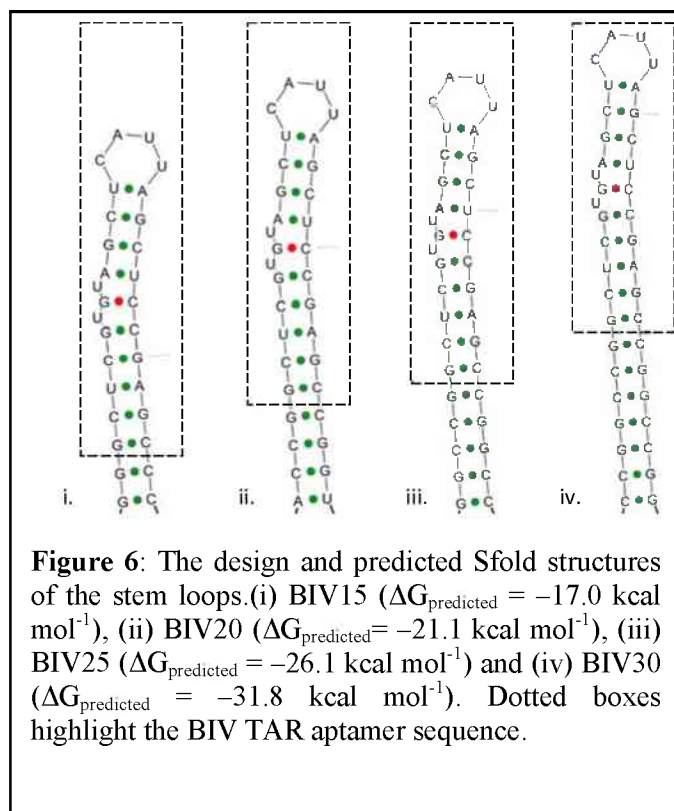
Engineering Translational Repression

Our goal was to create a light-sensitive, aptamer-based, translational repressor to control translation of a desired cellular protein. This design involved the insertion of aptamers in the 5'-UTR of a target mRNA. The binding of a ligand to its aptamer would impart increased thermodynamic stability and consequently result in the repression of translation from that mRNA.



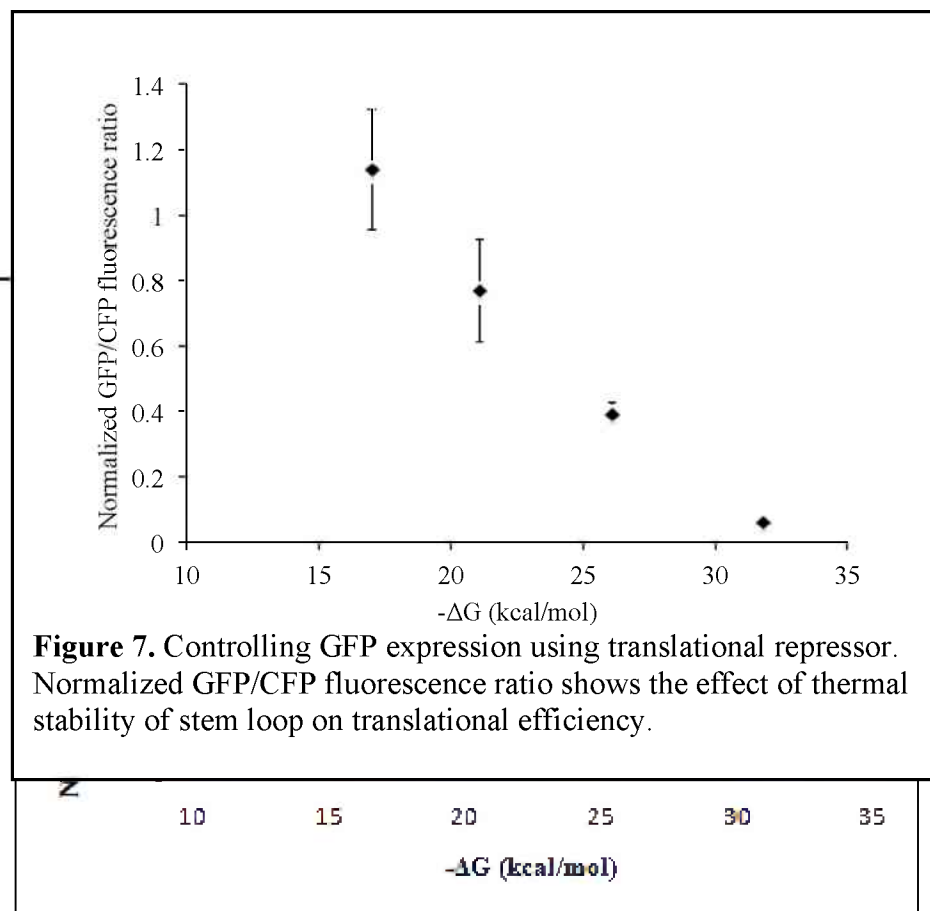
We picked BIV Tat peptide, which binds specifically and with high affinity to the BIV TAR RNA aptamer. The amino acid sequence of BIV Tat peptide, predicted Sfold structure of BIV TAR aptamer and the energy-minimized average NMR structure of the BIV Tat peptide-BIV TAR aptamer complex are shown in Figure 5a-c (Puglisi et al.¹⁵). To demonstrate the above concept, we chose GFP as the desired target protein whose translation was to be controlled. We incorporated BIV

TAR aptamer-based stem loops in the 5'-UTR of GFP mRNA. The plasmid also contained CFP as a marker of transfection in a second multiple cloning site (MCS). Both GFP and CFP were under the control of Ferritin promoter. A schematic for this construct is shown in Figure 5d.



Babendure et al.¹⁶ have shown that the presence of thermodynamically stable structures in the 5'-UTR of an mRNA may result in a decreased translational efficiency. Guided by these results, we designed four BIV TAR aptamer-based stem loops with increasing thermodynamic stabilities as shown in Figure 6. These stem loops were placed 13 nucleotides downstream of the 5'-cap of GFP mRNA. The GFP expression from these stem loops was quantified 48 hours post-transfection in HEK 293T cells, by FACS. As can be seen in Figure 7, increasing the stability of stem loops results in a decrease in the translation of GFP relative to that of CFP.

Next, we tested the hypothesis that binding of a suitable ligand (BIV Tat) would result in a further increase in the thermodynamic stability of the stem loop, which should similarly result in translational repression. We decided to test this hypothesis



with the dual reporter construct (Figure 5d), containing the BIV20 stem loop (Figure 6) in the 5'UTR. The BIV20 stem loop consisted of a BIV TAR aptamer with additional complementary bases added to the stem to increase the thermal stability to $\Delta G = -21.1 \text{ kcal/mol}$ (Figure 6). In the BIV20 construct, the stem loop was located 13 nucleotides downstream of the 5'-cap of GFP mRNA. The plasmid also contained CFP as a

Figure 7. Controlling GFP expression using translational repressor. Normalized GFP/CFP fluorescence ratio shows the effect of thermal stability of stem loop on translational efficiency.

marker of transfection (Figure 5d). Both GFP and CFP were under the control of the Ferritin promoter (Figure 5d). We also made additional constructs expressing either CIBN-BIV Tat or CIBN-His peptide fusions (His peptide sequence: MRGSHHHHHHG) and DsRed as a marker of transfection (see schematic in Figure 8a). CIBN-His served as a negative control.

The dual reporter construct with the BIV20 stem loop was co-transfected with the CIBN-ligand constructs in HEK 293T cells. The ratio of GFP and CFP fluorescence from double transfected cells was quantified by FACS, 48 hours post-transfection. As can be seen in Figure 8b, expression of the control CIBN-His fusion resulted in a ratio of GFP and CFP fluorescence that was 3.6 times that obtained with the expression of the CIBN-

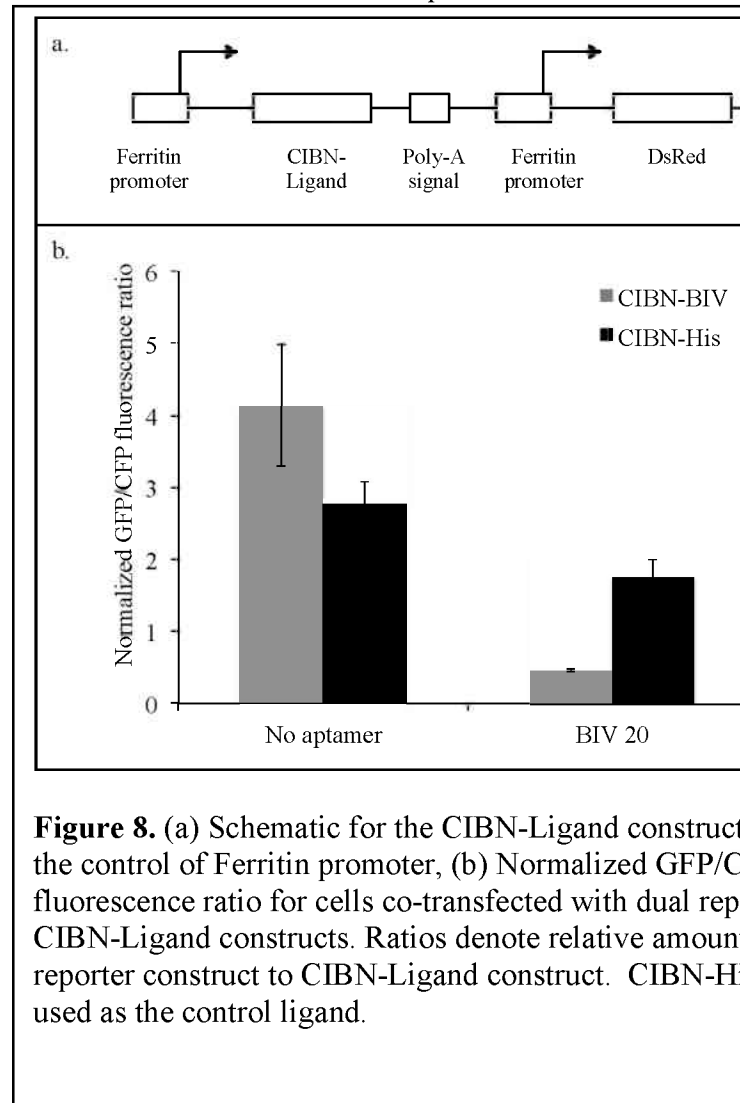


Figure 8. (a) Schematic for the CIBN-Ligand construct the control of Ferritin promoter, (b) Normalized GFP/CFP fluorescence ratio for cells co-transfected with dual reporter constructs. Ratios denote relative amount of reporter construct to CIBN-Ligand construct. CIBN-His used as the control ligand.

BIV Tat fusion protein, consistent with ligand-induced translational repression.

In future work, to make this translational repressor light sensitive, we propose to use the light-sensitivity of the interaction between CRY2 PHR and

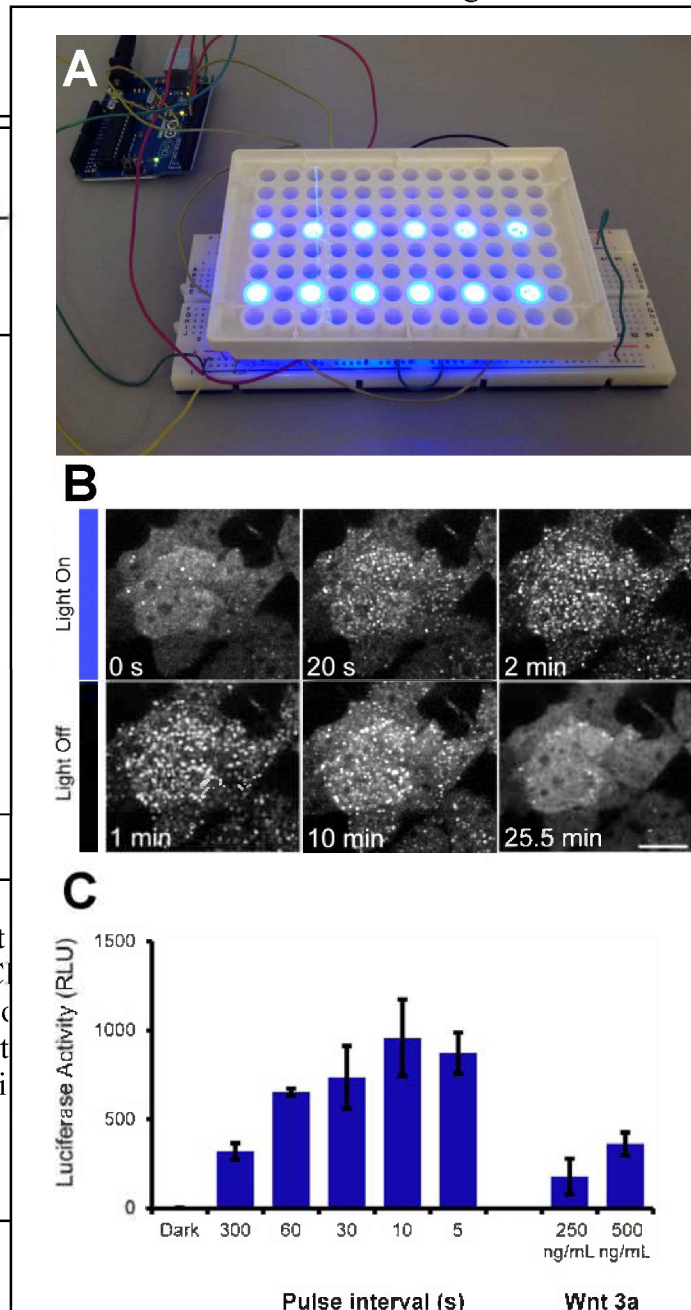
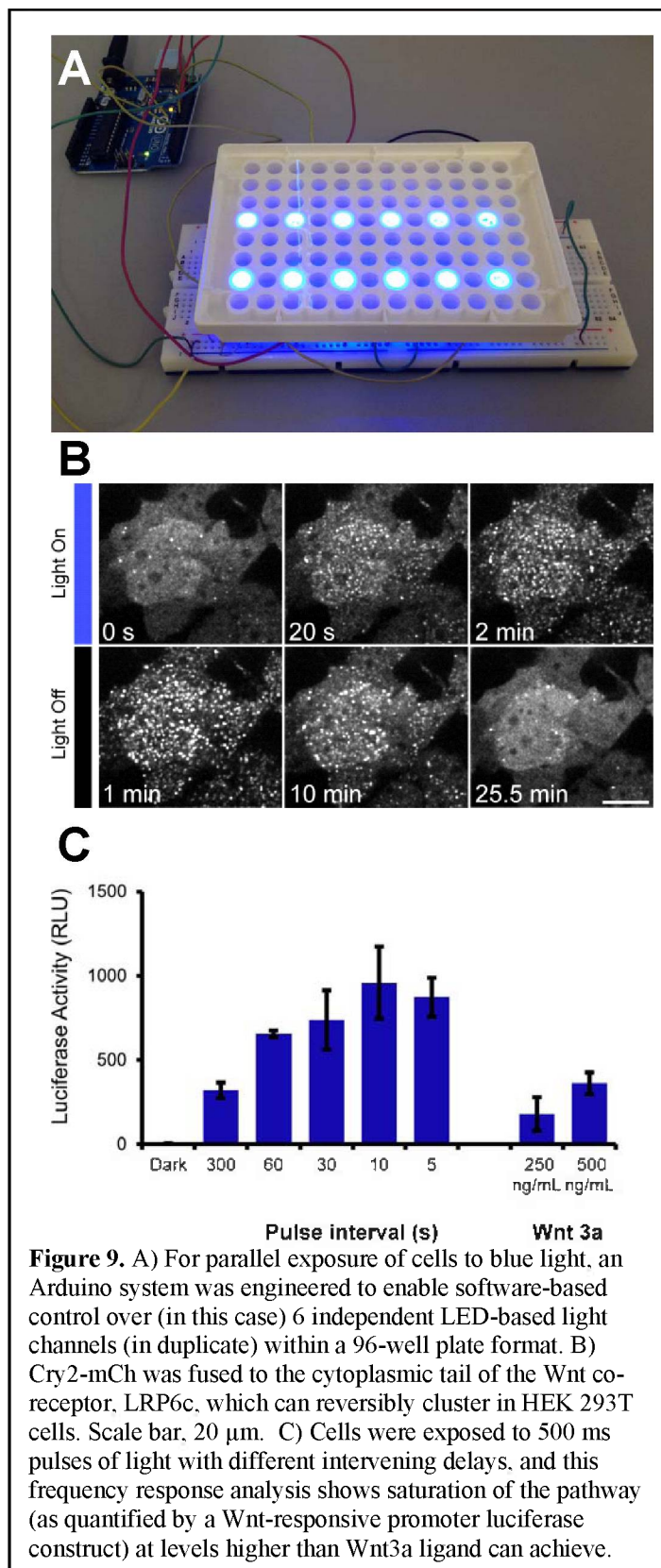


Figure 9. A) For parallel exposure of cells to blue light, an Arduino system was engineered to enable software-based control over (in this case) 6 independent LED-based light channels (in duplicate) within a 96-well plate format. B) Cry2-mCh was fused to the cytoplasmic tail of the Wnt co-receptor, LRP6c, which can reversibly cluster in HEK 293T cells. Scale bar, 20 μ m. C) Cells were exposed to 500 ms pulses of light with different intervening delays, and this frequency response analysis shows saturation of the pathway (as quantified by a Wnt-responsive promoter luciferase construct) at levels higher than Wnt3a ligand can achieve.



computer control over 6 independent channels (in duplicate) such that the pulse frequency and

CIBN¹. Specifically, as described earlier in the report, we will use a fusion protein wherein the CRY2 PHR domain is fused to a peptide linker that can mediate degradation via the cellular protein degradation machinery. On shining blue light, the dimerization between CIBN-BIV Tat and the CRY2 PHR fusion protein would facilitate the degradation of CIBN-BIV Tat, thereby eliminating translational repression. We would therefore have a system where the extent of translation would be increased in response to illumination with blue light.

III. Engineering Devices for Illumination of Cells

A central goal of this proposal is to engineer systems where light is utilized to interrogate and control the behavior of cells on materials and in devices. For some applications, it would be advantageous to stimulate multiple cellular samples in parallel, for example to investigate the effects of “communicating” different waveforms (e.g., frequencies, intensities, etc.) to cells with higher throughput than light microscopes. To take a step in this direction, we assembled a computer-controlled light emission device based on the Arduino platform (www.arduino.cc). Arduino is an open-source microcontroller board that can receive inputs from multiple sources, including open-source software that runs on a PC, and in response can actuate a number of outputs, including lights. We interfaced the Arduino board with 5 mm blue light-emitting diodes (LEDs) arranged in a breadboard to fit into wells of an inverted, clear-bottom, 96 well plate. In the apparatus shown in Figure 9A, for example, the system was designed to enable

pulse width of light can readily be controlled with a script. For experiments, cells were seeded in the appropriate wells in a clear-bottom, black-walled plate, which was placed on top of the depicted assembly to align the LED light with the wells and subsequently illuminate the cells therein. The resulting device could readily fit into a standard cell culture incubator for long-term illumination of living cells. By changing the LED placement, this approach could be used for illuminating cells in non-96-well cell culture formats as well.

In addition to creating photo-inducible forms of RhoA and Rac1, we engineered a photo-activatable agonist of the canonical Wnt signaling pathway. Canonical Wnt/ β -catenin signaling is triggered by Wnt ligand binding to Frizzled and LRP6 co-receptors, which then form higher order clusters¹⁷ and activate β -catenin. Upon activation, β -catenin enters into the nucleus and functions as a transcriptional cofactor to activate key targets that play roles in cell shape, division, differentiation, and other responses. Recently, constitutive clustering of just the LRP6 C-terminal domain (LRP6c) was shown to robustly activate β -catenin signaling¹⁸. To determine whether LRP6c endodomain could be oligomerized in response to light, and thus offer inducible control over Wnt signaling, we fused it to the C-terminus of Cry2-mCh (Cry2-LRP6c). After introduction of this construct into HEK 293T cells, visible puncta appeared within two seconds of blue light illumination, and these clusters dissociated upon light withdrawal (Figure 9B). To assess whether this clustering could induce signal activation, cells carrying a β -catenin responsive luciferase reporter¹⁹ were transfected with Cry2-LRP6c. To harness the capabilities of the Arduino system, these cells were exposed for 16 hours to 500 ms light pulses, with the interval between pulses varying from 5-300 s. The cells demonstrated a strong dose responsiveness to increased blue light pulse frequency ($p = 0.0051$, Jonckheere-Terpstra trend analysis, Figure 9C), whereas cells kept in the dark yielded a negligible readout. Maximal light-induced signaling increased luciferase expression ~ 200 -fold over unilluminated controls and even greatly exceeded reporter expression induced by high levels of pathway agonist Wnt3a (Figure 9C).

These results collectively demonstrate both light-mediated control over ubiquitously important signaling pathways and the versatility of a customized system that integrates cells into a device.

References:

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e. A list of papers (already published, in press, submitted) in which DOE support is acknowledged.

Bugaj, L. J., Choksi, A. T., Mesuda, C. K., Kane, R. S. and Schaffer, D. V., *Optogenetic protein clustering and signaling activation in mammalian cells*. *Nature Methods* 2013, 10 (3), 249-252.

Sudrik, C., Arha, M.; Cao, J., Schaffer, D. V. and Kane, R. S., *Translational repression using BIV Tat peptide-TAR RNA interaction in mammalian cells*. *Chemical Communications*, 2013, 49 (67), 7457-9.

Cao, J., Arha, M., Sudrik, C., Bugaj, L. J., Schaffer, D. V. and Kane, R. S., *Light-inducible activation of target mRNA translation in mammalian cells*. Chemical Communications, 2013, 49 (75), 8338-8340.

Bugaj, L. J., Kane, R. S. and Schaffer, D. V., *Development of a modular optogenetic system to cluster cell surface receptors*. Manuscript in preparation.

Bugaj, L. J., Kane, R. S. and Schaffer, D. V., *Elucidation of mechanisms for clustering-mediated activation of Rho GTPases*. Manuscript in preparation.

f. A list of people working on the project - graduate students, postdocs, visitors, technicians, etc. Indicate for each whether receiving full or partial support. In case of partial support indicate percentage of support.

Year 1: 9/1/09- 5/31/10

Lukasz Bugaj, Graduate Student (49% support from 9/1/09 to present)

Laura Quock, Graduate Student (49% support from 1/1/2010 to present)

Year 2: 6/1/10-5/31/11

Lukasz Bugaj, Graduate Student (100% support from 6/1/2010-5/31/2011)

Siddharth Dey, Graduate Student (100% support from 1/1/2011-5/31/2011)

Laura Quock, Graduate Student (100% support from 8/1/2010-8/31/2010, thereafter received a graduate student fellowship)

Year 3: 6/1/11-5/31/12

Lukasz Bugaj, Graduate Student (100% support from June through August, then 25% support with remaining support from departmental funds)

Siddharth Dey, Graduate Student (100% support from 6/1/2011-4/30/12)

Albert Keung, Graduate Student (50% support from 8/01/2011-4/30/2012, with remaining support from departmental funds)

Year 4: 6/1/12-5/31/13

Anthony Conway, Graduate Student (85% support from 1/1/2013-5/31/2013)

Dawn Spelke, Graduate Student (100% support from 9/1/2012-12/31/2012, with the remaining support from NSF fellowship)

Lukasz Bugaj, Graduate Student (100% support from 6/1/2012-7/31/2012, with remaining support from departmental funds)

Marjan Varedi, Postdoc (50% support from 10/1/2012-1/31/2013, with remaining support from departmental funds)

Sid Dey, Graduate Student (100% support from 6/1/2012-9/30/2012)

g. Estimate of the unexpended funds at the end of the budget period; If funds exceed 10% of the annual budget, provide a detailed explanation.

There are no unexpended funds.

h. Planned activities for next year (which can be a short paragraph).
Not applicable.

i. An updated list of other support (current and pending, federal and non-federal). For each, indicate briefly the overlap, if any, or distinctiveness with respect to the DOE-supported project.

No current project overlaps with the DOE-supported project.
Other support is listed below:

R21 (Co-PIs Maharbiz, Schaffer) 8/1/12-7/31/14 0.5 month
NIH/NIBIB \$171,791 Annual Direct
Precise Surface Patterning to Investigate Cell-Cell Interactions that Regulate Neural Stem Cell Fate Decisions
The major goal of this project is to develop bioactive surfaces that can spatially pattern cell attachment with high precision, as a means to investigate the role of cell-cell contact in stem cell fate decisions.
OVERLAP: None

R01 NS074831 (Co-PIs Kumar, Schaffer) 5/1/12-4/30/17 1.0 month
NIH/NIBIB NIH \$218,750 Annual Direct to Schaffer and Kumar
Mechanisms of Neural Stem Cell Mechanoregulation
The major goal of this project is to understand mechanisms through which biomechanical inputs from the cellular microenvironment influence neural stem cell neurogenesis.
OVERLAP: None

R01 ES020903 (Co-PIs Dordick, Schaffer, Clark) 12/23/11-10/31/15 0.5 month
NIH/NIEHS \$127,500 Annual Direct to Schaffer and Clark
Engineering High-Throughput Platform for Identifying Stem Cell Toxicity
The major goal of this project is to develop and implement a high throughput platform for analyzing and screening small molecule toxicity on stem cells.
OVERLAP: None

R01 GM 073058-04 (Co-PIs Arkin, Schaffer) 4/1/11-3/31/15 1.0 month
NIH/NIGMS \$205,000 Annual Direct
Investigation of Promoter Sequence-Function Relationships in a Model Retrovirus
The major goal of this grant is to use study the effects of variability in the HIV promoter sequence on viral gene expression and replication dynamics.
OVERLAP: None

CL1-00519 (Schaffer) 1/1/10-12/31/15 1 month
CIRM \$200,000 Annual Direct for Facility
Berkeley Human Embryonic Stem Cell Shared Research Laboratory

The major goal of this grant is to build and establish a laboratory facility on the Berkeley campus that will serve as a general resource for scientists to conduct human embryonic stem cell research.

OVERLAP: None

T32 GM098218-01 (Co-PIs Schaffer, Healy) 7/1/2011-6/30/2016 0.5 month
NIH/NIGMS \$138,590 Annual Direct for Program

Stem Cell Engineering Training Program

The major goal of this grant is to implement a formal training program to bridge research efforts in the fields of stem cell engineering and biology.

OVERLAP: None

PN2 EY018241 (Isacoff) 9/30/2010-9/29/2014 0.5 month
NIH/NEI \$75,000 Annual Direct to Schaffer

NDC for the Optical Control of Biological Function

The goal of the center is to analyze the data and model the results of experiments due to the creation of light inducible inputs to perturb cells in a controlled manner to design new light driven switches.

Role: Senior Investigator

OVERLAP: None

RT2-02022 (PI Schaffer, Co-Inv. Healy) 4/1/2011-3/31/2014 2.4 month
CIRM \$317,624 Annual Direct

Engineering Defined and Scaleable Systems for Dopaminergic Neuron Differentiation of hPSCs

The goal of this project is to develop defined, scaleable, and versatile systems for human pluripotent stem cell expansion and differentiation.

OVERLAP: None

RT2-02055 (Conolly) 4/1/2011-3/31/2014 0.5 month
CIRM \$38,987 Annual Direct to Schaffer

Magnetic Particle Imaging: A Novel Ultra-sensitive Imaging Scanner

The goal of this project is to engineer a human embryonic stem cell based culture model to investigate specific disease models for the source of the Alzheimer's disease state.

Role: Co-Investigator

OVERLAP: None

P01 HL051670 (Zabner) 8/1/2009-7/31/2014 1.0 month
NIH/NHLBI \$52,834 Annual Direct to Schaffer

Subproject: Directed Evolution of AAV Capsid for Cystic Fibrosis Gene Therapy

The goal of this project is to engineer lentiviral gene delivery vehicles for enhanced efficiency delivery to the lung epithelium, with application to cystic fibrosis.

Role: Senior Investigator

OVERLAP: None