

# ***Synechococcus* sp. PCC 7002: A Cyanobacterial Chassis for Advanced Biofuel Production**

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March 5, 2014

BSAP Annual Review

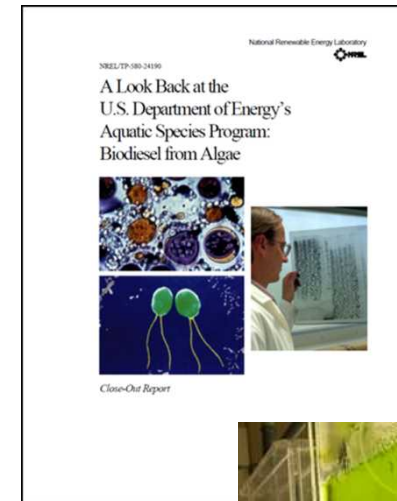
# Cyanobacterial-Based Biofuel Production

## Desirable strain traits

- Easily transformed
- Homologous recombination – targeted genome integration
- Gene expression not complicated by RNAi
- Established genetic tools
- Fast growth rates and strain robustness

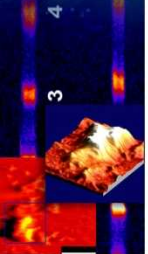
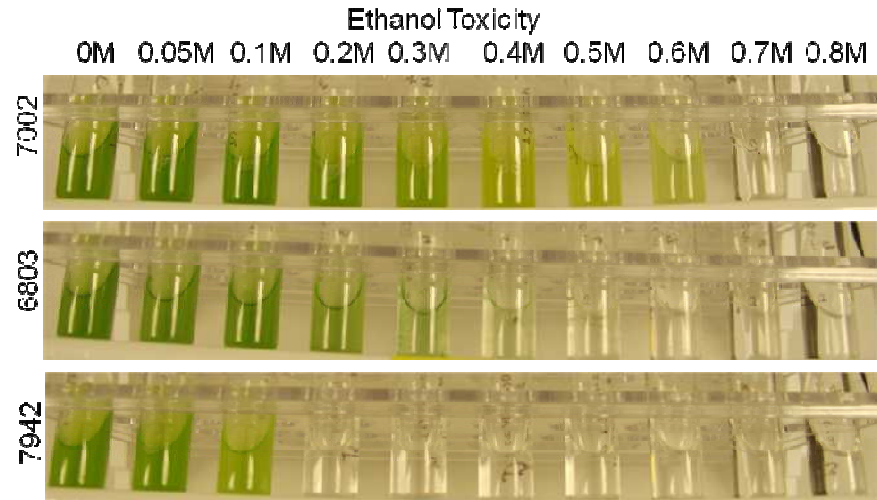
## Process design advantages

- Product excretion enables continuous production
- Biomass harvesting not required
- Lower nutrient requirements (N&P)



# *Synechococcus* sp. PCC 7002

- Model cyanobacterium
  - Genetic tools available
  - Genome sequence
- Rapid doubling time (2.6 – 4 hrs)
- Temperature tolerance
  - Grows under 22-40°C
- Salt tolerance
  - Survives > 1.5 M NaCl, 2.5x sea water)
- High light tolerance
  - Survives light intensities > 4.5 mE m<sup>-2</sup> s<sup>-1</sup> (2x peak sunlight)
- Biofuel tolerance
  - Grows in ethanol concentrations up to 0.6 M

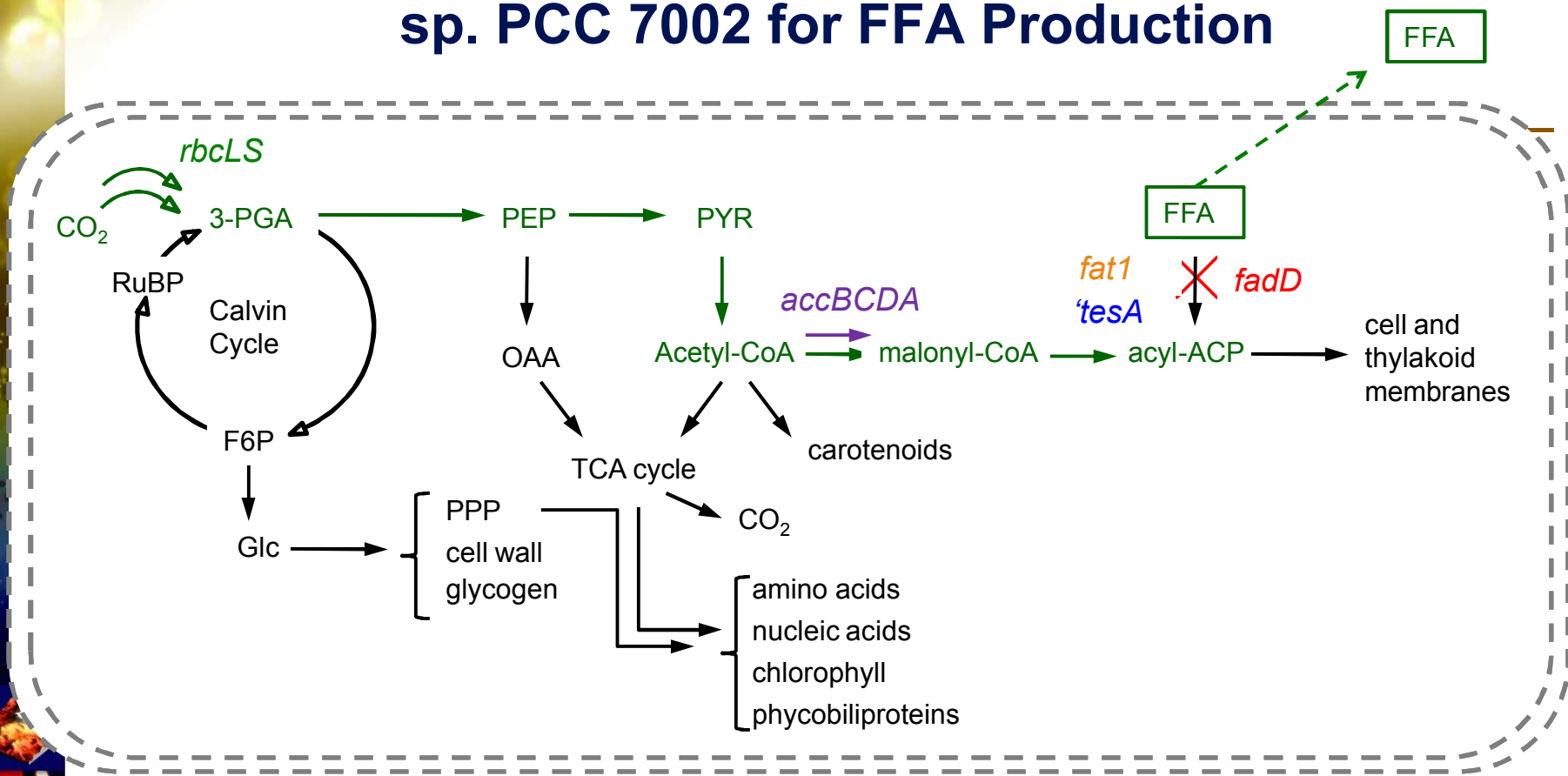


# Engineering *Synechococcus* sp. PCC 7002 for Advanced Biofuel Production

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- Traditional Metabolic Engineering of *Synechococcus* sp. PCC 7002 for Free Fatty Acid Production (Funding: Truman Fellowship)
- Systems-Level Metabolic Engineering of *Synechococcus* sp. PCC 7002 for Alkane Production (Funding: Early Career LDRD)

# Traditional Metabolic Engineering of *Synechococcus* sp. PCC 7002 for FFA Production



7002: wild type; S01:  $\Delta fadD$ ; S02:  $\Delta fadD$ , *'tesA*; S03:  $\Delta fadD$ , *fat1*; S05:  $\Delta fadD$ , *'tesA*, *rbcLS*; S06:  $\Delta fadD$ , *'tesA*, *rbcLS*, *accBCDA*; S07:  $\Delta fadD$ , *'tesA*, *PpsbA1-rbcLS*

*fadD* – acyl-ACP synthetase / long-chain-fatty-acid CoA ligase

*'tesA* – truncated thioesterase from *Escherichia coli*

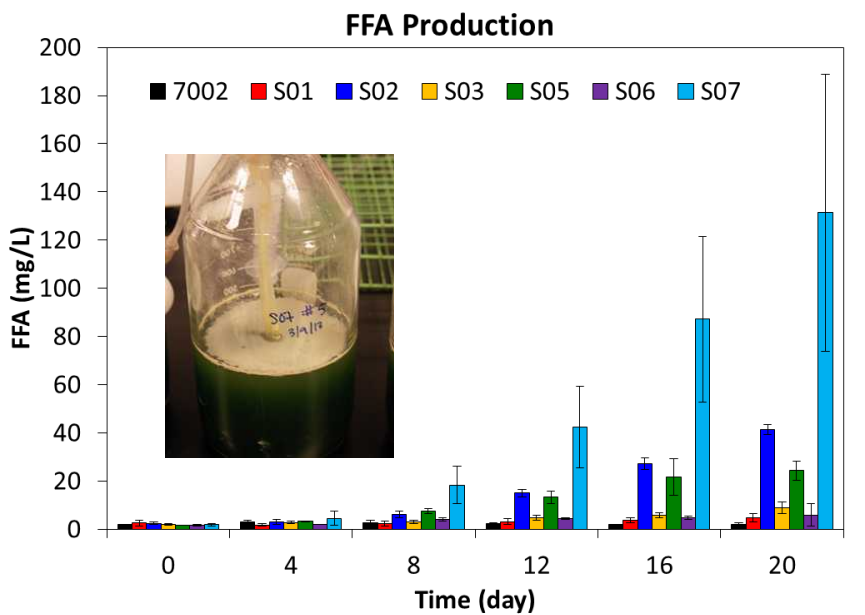
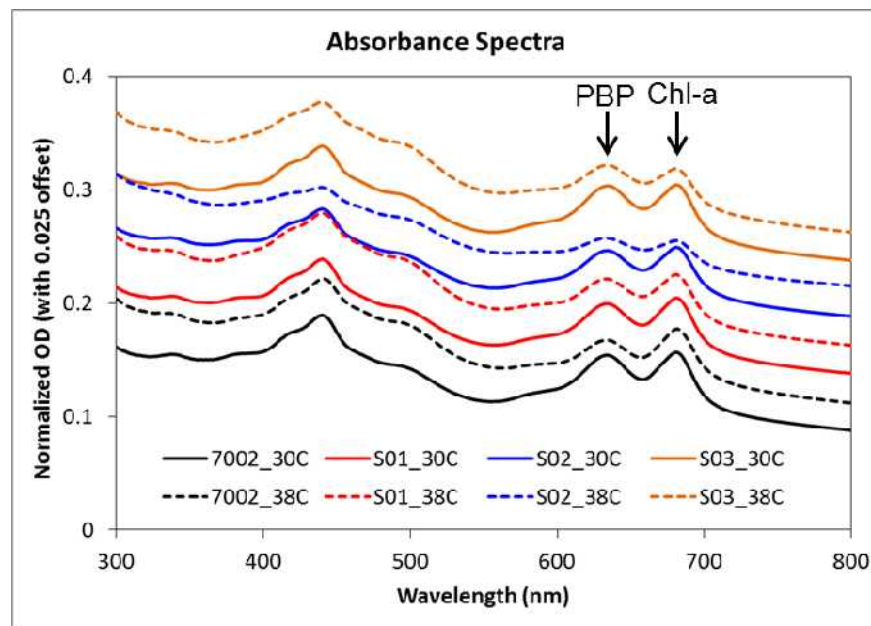
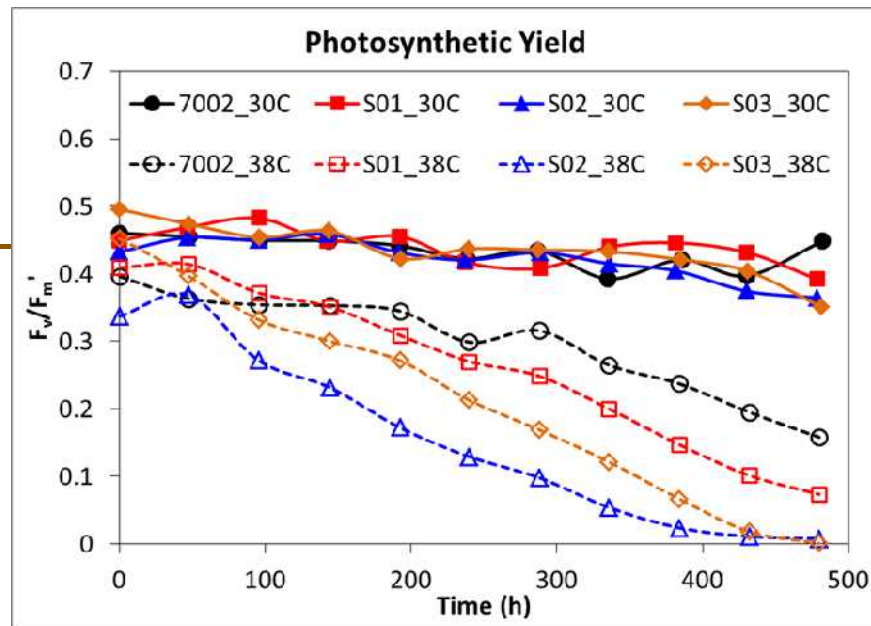
*fat1* – acyl-ACP thioesterase from *Chlamydomonas reinhardtii*

*rbcLS* – native RuBisCO

*accBCDA* – multi-subunit acetyl-CoA carboxylase from *C. reinhardtii* (chloroplast associated)

# FFA Production

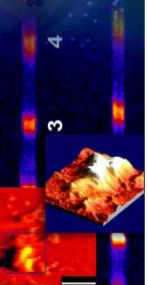
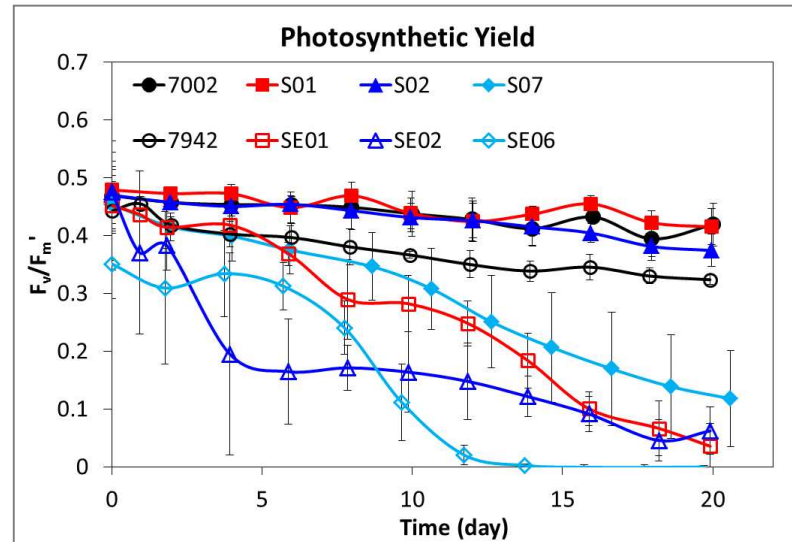
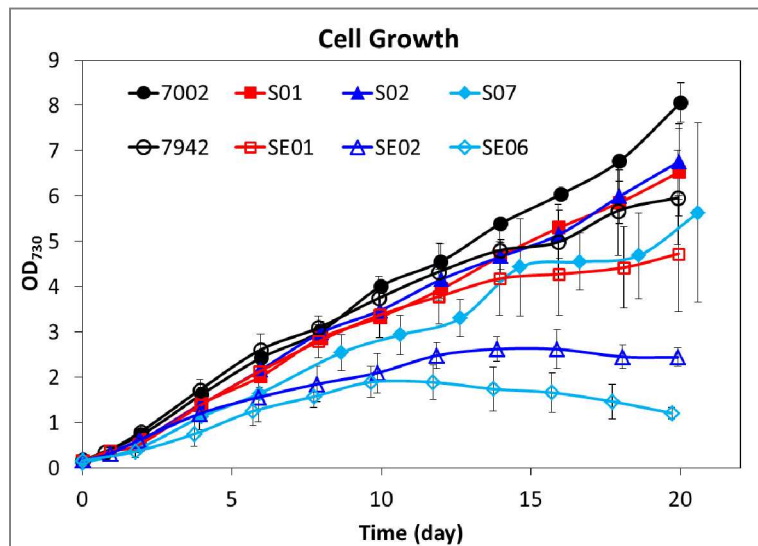
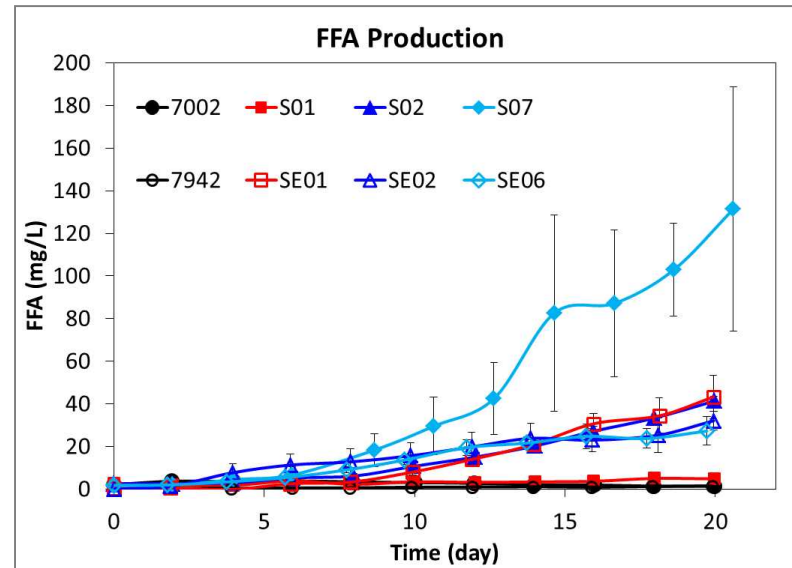
- Maintenance of high photosynthetic yields and photosynthetic pigments (PBP, Chl-a) is temperature dependent
- Significant increase in FFA production with overexpression of RuBisCO from *psbA1* promoter



7002: wild type; S01:  $\Delta fadD$ ; S02:  $\Delta fadD$ , *'tesA*; S03:  $\Delta fadD$ , *fat1*; S05:  $\Delta fadD$ , *'tesA*, *rbcLS*; S06:  $\Delta fadD$ , *'tesA*, *rbcLS*, *accBCDA*; S07:  $\Delta fadD$ , *'tesA*, *PpsbA1-rbcLS*

# Host Selection: *Synechococcus* sp. PCC 7002 vs *Synechococcus elongatus* PCC 7942

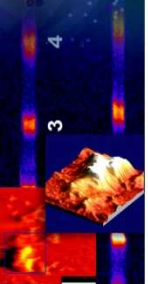
- Highest FFA producing strains:
  - 7002: S07 (131 mg/L)
  - 7942: SE01 (43 mg/L)
- Improved growth and photosynthetic yields for 7002 strains



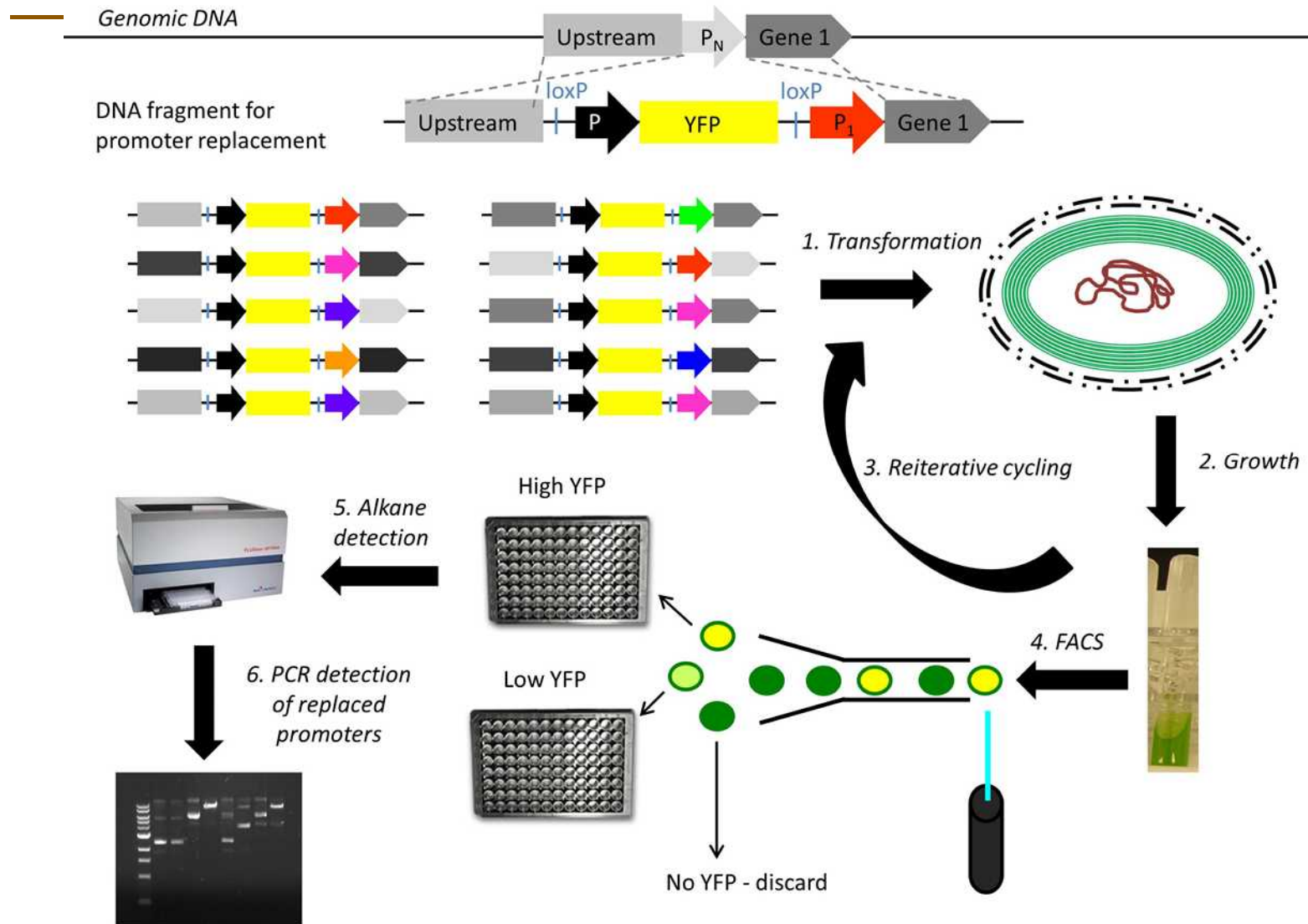
# Traditional Metabolic Engineering: Conclusions

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- *Synechococcus* sp. PCC 7002 is a better host for FFA production compared to *S. elongatus* PCC 7942
- Carbon fixation (RuBisCO) limits FFA biosynthesis in *Synechococcus* sp. PCC 7002
- 'Predicted' rate-limiting steps (i.e. ACCase) do not appear to be rate-limiting
- Gene expression is critical for optimizing FFA production
- FFA production has complex, systems-level interactions (growth, photosynthetic yield, pigments) that cannot be optimized by traditional pathway engineering

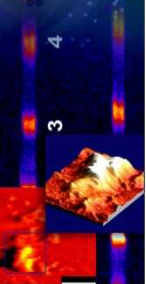
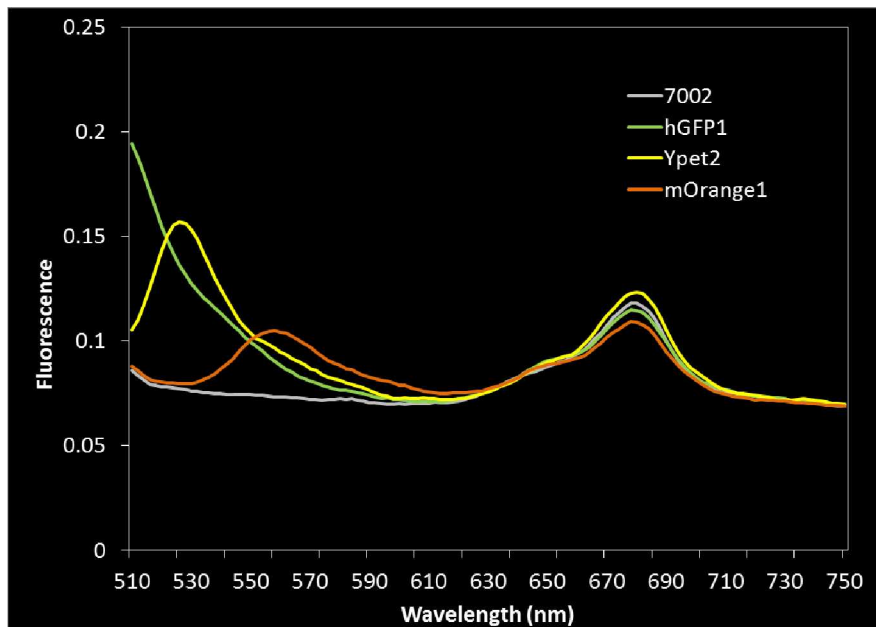
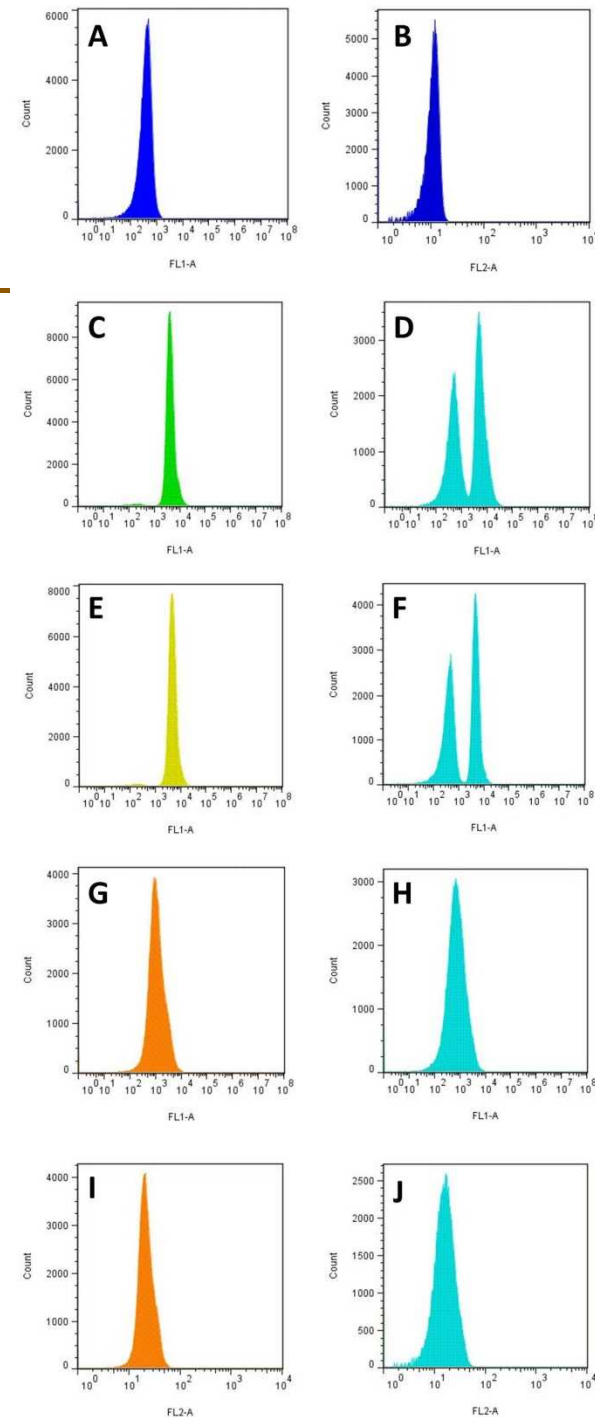


# Development of a Systems-Level Metabolic Engineering Technique for *Synechococcus* sp. PCC 7002



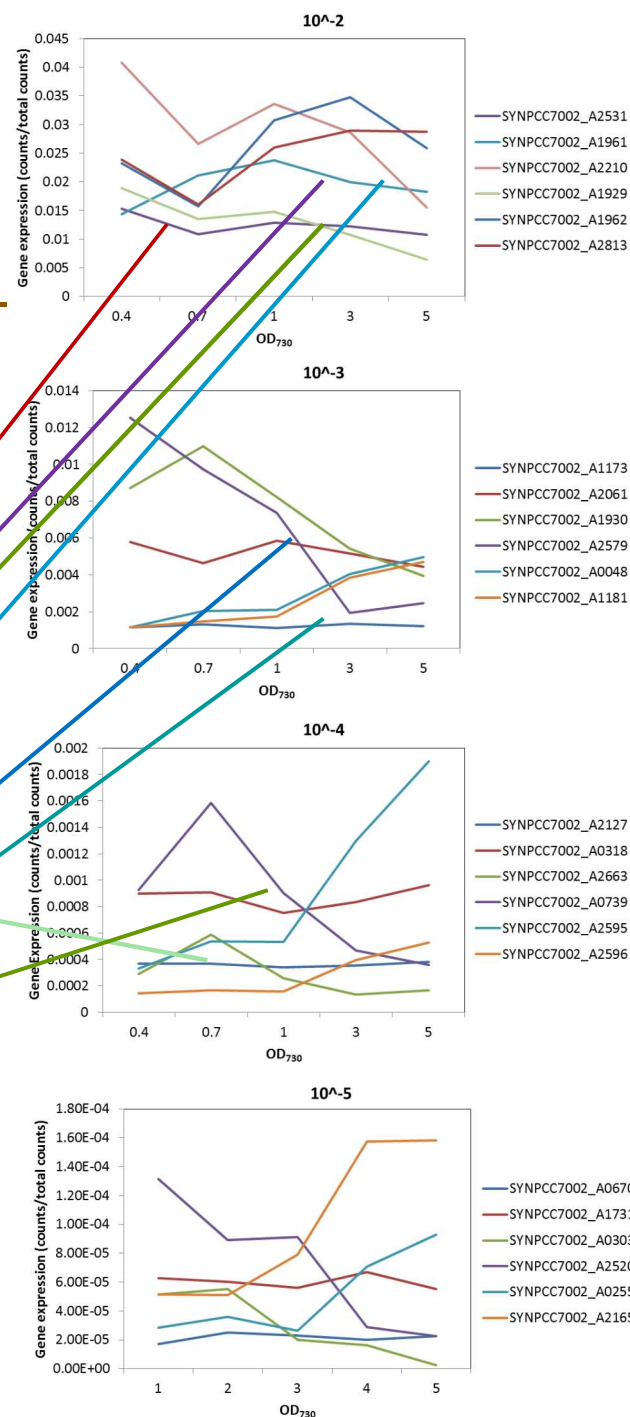
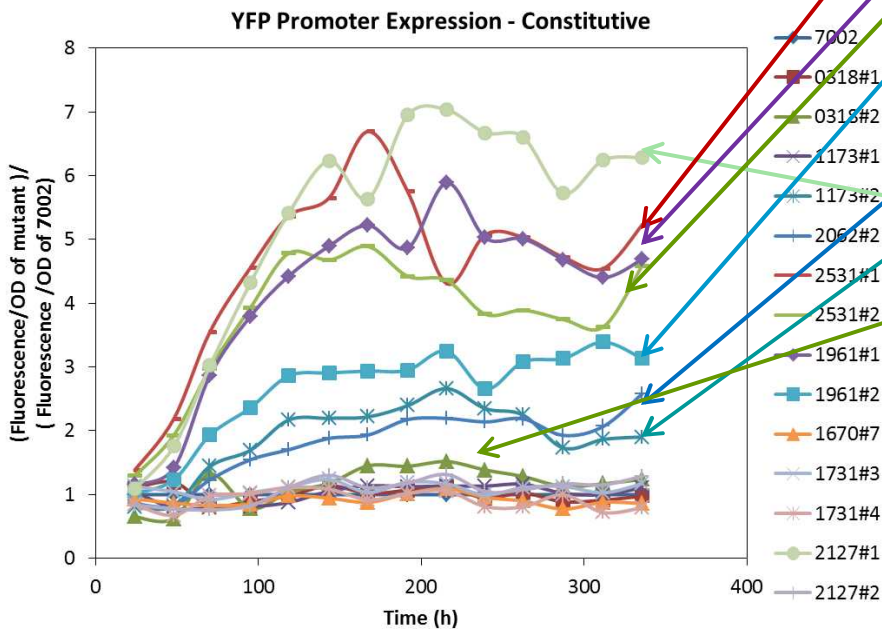
# Fluorescent Protein Reporter Selection

- 3 fluorescent proteins investigated: GFP, YFP, and OFP
- YFP has the best combination of :
  - high signal intensity
  - minimal overlap with cyanobacterial photosynthetic pigments
  - good flow cytometer peak separation (488nm excitation laser)
  - excitation (514nm) and emission (527nm) peaks



# Promoter Characterization in *Synechococcus* sp. PCC 7002

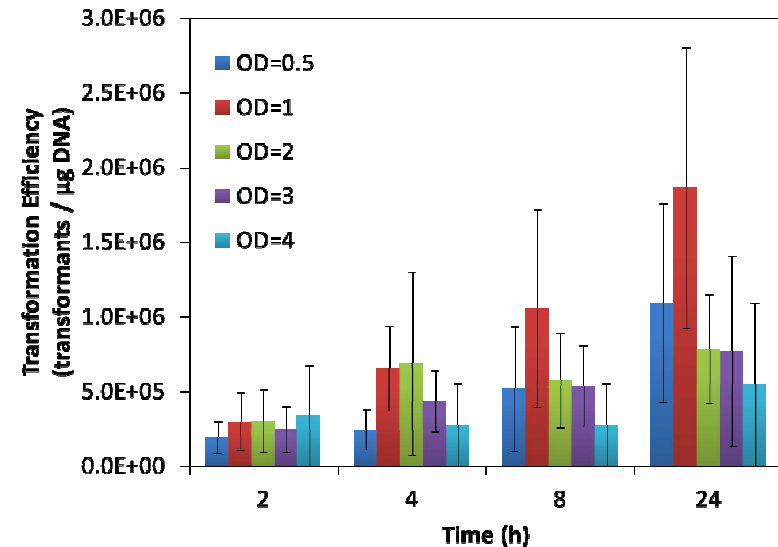
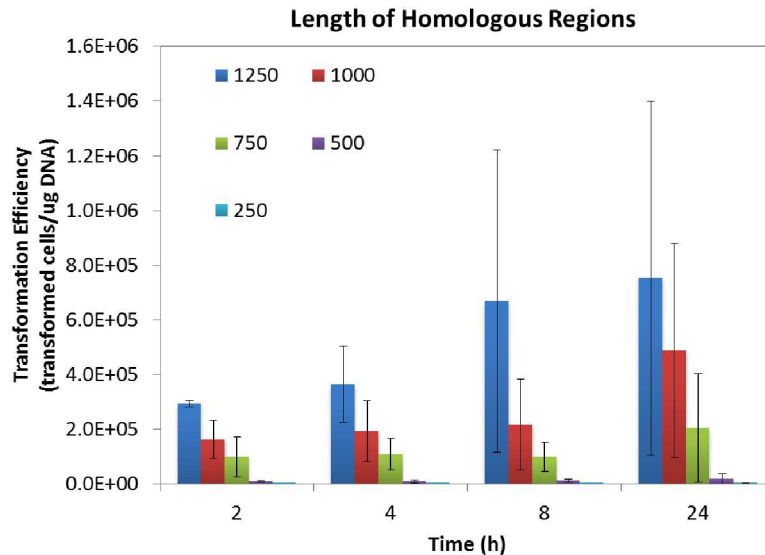
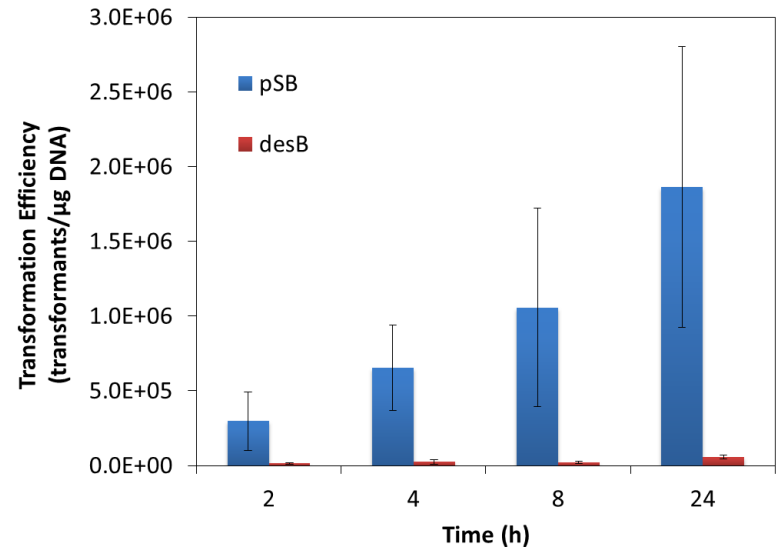
- Promoters selected from previously published RNAseq data<sup>1</sup>
  - Expression levels:  $10^{-5}$  to  $10^{-2}$
  - Regulatory patterns: constitutive, linear growth phase, and stationary phase
- Linked promoters to YFP for characterization under continuous light and diurnal conditions



1. Ludwig M, Bryant DA. 2011. Transcription profiling of the model cyanobacterium *Synechococcus* sp. strain PCC 7002 by Next-Gen (SOLiD™) sequencing of cDNA. *Frontiers in Microbiol.* 2:41.

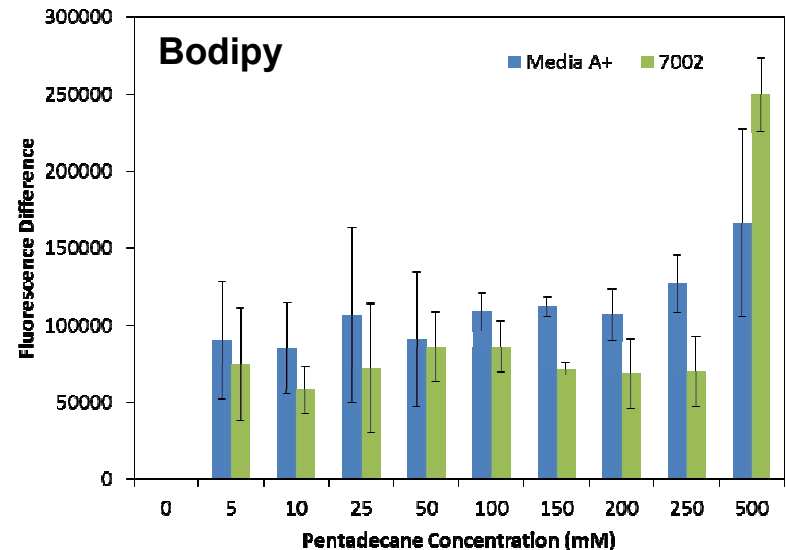
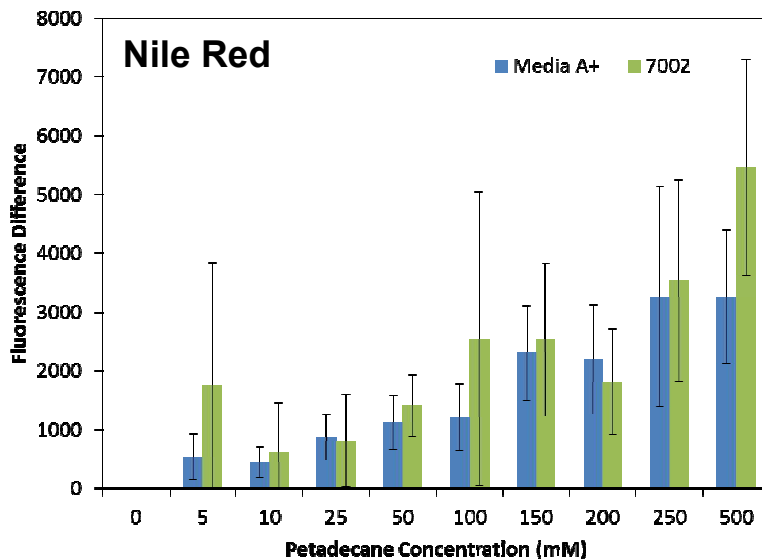
# Optimizing Transformation in *Synechococcus* sp. PCC 7002

- Exonuclease activity present in *Synechococcus* sp. PCC 7002
- Cell concentration:  $OD_{730} = 1.0$
- Length of homology for homologous recombination: 1kb
- Genome copy number (ploidy): lowest at  $34^{\circ}\text{C}$ ,  $60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ,  $OD_{730} > 3.0$

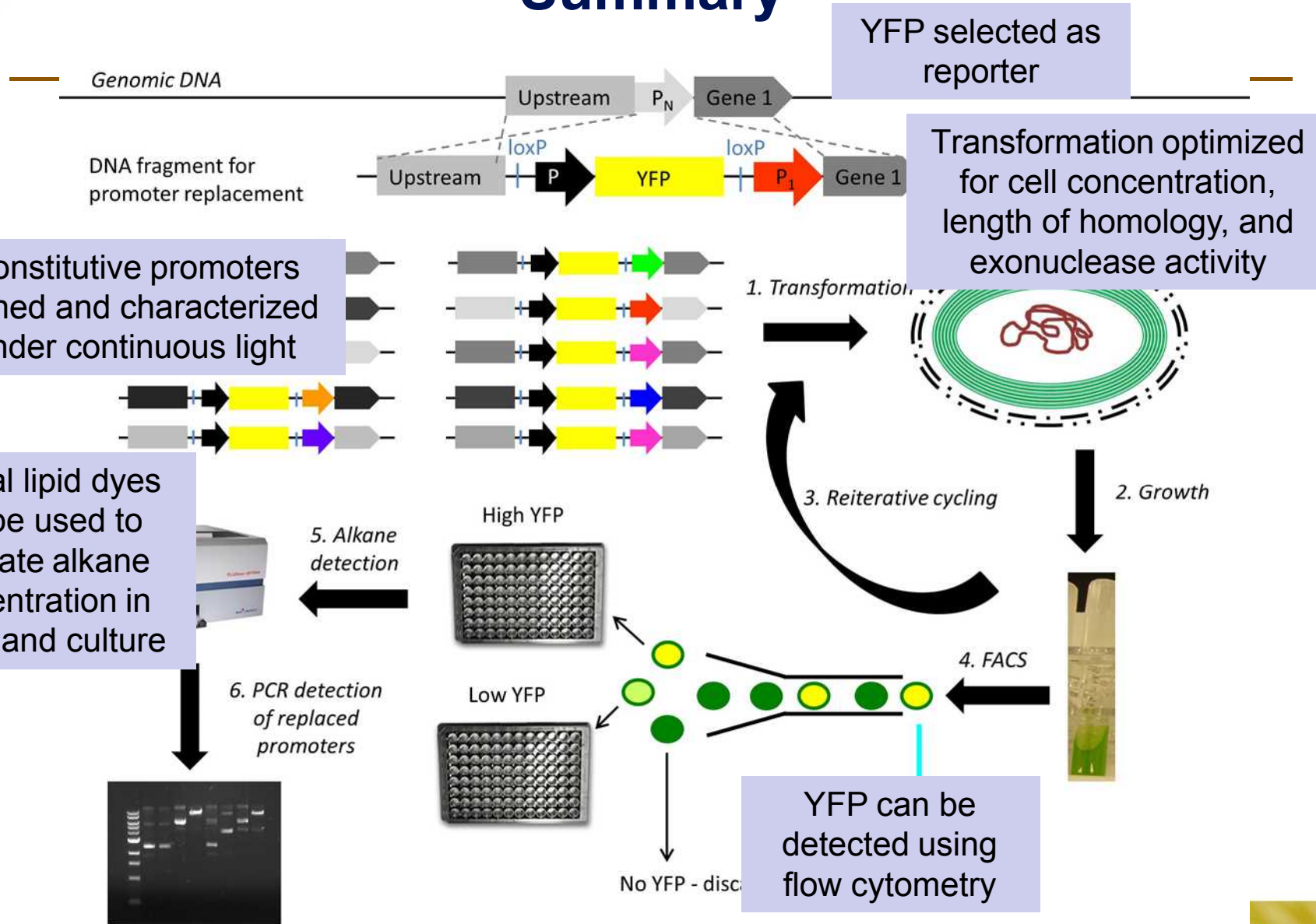


# High-Throughput Alkane Screening

- 2 neutral lipid dyes: Nile red and bodipy
- Exogenous addition of pentadecane to media or dilute culture ( $OD_{730} = 0.1$ )
- Nile red is more quantitative
- Bodipy is more sensitive



# Systems-Level Metabolic Engineering: Summary



# Systems-Level Metabolic Engineering: Future Work

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- Clone and characterize linear growth phase and stationary phase expression promoters
- Determine conditions for minimal genome copy number
- Analyze fluorescence detection of multiple YFP copies
- Characterize neutral lipid stains for intracellular alkane production
- Design synthetic DNA constructs for systems-level metabolic engineering (JGI DNA synthesis proposal pending)
- Demonstrate systems-level metabolic engineering for improving alkane production in *Synechococcus* sp. PCC 7002

# Acknowledgements

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## Staff Members

Howland D.T. Jones (entrepreneurial leave)  
Omar F. Garcia (now in 5448)

## Student Interns

Christine Trahan  
Travis Jensen  
Michelle Raymer

## Managers

Anthony Martino (now in 6124)  
Eric Ackerman (8635)  
James Carney (8622)

## Funding

President Harry S. Truman Fellowship in  
National Security Science and Engineering  
(LDRD): 01/25/2010 – 01/24/2013

Early Career LDRD: 04/01/2013 - present



# Publications

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Anne M Ruffing and Christine A Trahan. Biofuel Toxicity and Mechanisms of Biofuel Tolerance in Three Model Cyanobacterial Strains. To be submitted to *Algal Research* (June 2014).

Anne M Ruffing. Improved Free Fatty Acid Production in Cyanobacteria with *Synechococcus* sp. PCC 7002 as Host. To be submitted to *Frontiers in Bioengineering and Biotechnology* (March 2014) – Research Topic: Cyanobacteria, the green *E. coli*.

Anne M Ruffing. RNA-Seq analysis and targeted mutagenesis for improved free fatty acid production in an engineered cyanobacterium. *Biotechnology for Biofuels*. 2013. 6:113.

Anne M Ruffing. --Borrowing genes from *Chlamydomonas reinhardtii* for free fatty acid production in engineered cyanobacteria. *Journal of Applied Phycology*. 2013. 25(5): 1495-1507.

Anne M Ruffing. Metabolic Engineering of Hydrocarbon Biosynthesis for Biofuel Production. Book chapter in *Liquid, Gaseous and Solid Biofuels – Conversion Techniques*. Intech, Rijeka, Croatia. 2013.

Anne M Ruffing and Howland DT Jones. Physiological effects of free fatty acid production in genetically engineered *Synechococcus elongatus* PCC 7942. *Biotechnology and Bioengineering*. 2012. 100(9): 2190-2199. (Cover, Spotlight)

Anne M Ruffing. Engineered Cyanobacteria: Teaching an old bug new tricks. *Bioengineered Bugs*. 2011. 2(3).