Functional Characterization of Cellular Metabolism

U.S. Department of Energy (DOE)
Bioenergy Technologies Office (BETO)
2017 Project Peer Review

Advanced Algal Systems

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Los Alamos NATIONAL LABORATORY EST.1943

NNSA National Nuclear Security Administration

Goals and Outcomes

- **The goal of this project is to develop the capabilities for algae strain improvements to meet BETO's productivity goals through molecular engineering toolbox development and rapid, comprehensive single cell flow cytometry analysis and phenotyping.**
- **OUTCOMES**
  - Genetically improved strains for productivity and robustness
  - Expanded Molecular Engineering Toolbox for universal applications to new strain improvement
  - Rapid physiological characterization expands understanding of cellular responses
  - Individual cell characterization in a population to define and exploit variation

OVERVIEW

Timeline

- **Project start: June 2012**
- **Project end : September 2019**
- **Percent complete: 63%**

Budget

	Total Costs FY 12 – FY 15	FY 16 Costs	FY 17 Costs	Total Planned Funding (FY 18-Project End Date)
DOE Funded	$3 M	$1 M	$650K	$1.3 M
Project Cost Share (Comp.)*	$0	$0	$0	$0

Barriers

– **Aft-C. Biomass Genetics and Development**

– Engineer improved lines and expand the molecular tools available

- **Aft-E. Algal Biomass Characterization**

– Rapid phenotyping of intercellular physiology by flow cytometry for real-time characterizations

Partners/ Collaborations

- NREL (0%)

1-Project Overview

- **The objectives of this project are to integrate biotechnology and genetic developments for improved algae lines coordinating genetic engineering and flow cytometry phenotyping for universal applications to algae strain productivity and robustness.**

  -Focus on engineering nitrogen regulatory responses altering carbon dynamics to optimize biomass and product flux potentially uncoupling stress responses for concurrent biomass and lipid accumulation.

  -Developing flow cytometry tools for rapid physiological characterization and phenotyping robust strains

Algae Strain Characterization and Improvement

Characterization of Productivity and Robustness

DISCOVR

Genome Sequencing, Functional 'Omics, Metabolic Mapping

Greenhouse: Comprehensive Knowledge Base of Algal Feedstocks

Genetic Blueprint of Microalgae Carbon Productivity

Strain Improvement: Genetic Modification & Non-GM Strategies

Algae Biotechnology Partnership

Functional Characterization of Cellular Metabolism

Multi-scale Characterization of Improved Algae Strains

Breeding Algae for Long Term Stability& Enhanced Biofuel Prod'n

Strategy

Use a multi-lab consortium approach to establish a state-of-the-art platform for the deep *characterization of new strains under outdoor-relevant conditions*

Leverage the expertise at LANL and LBNL/JGI to deliver the *data and tools* required to understand algae metabolism and develop GM tools

Use a range of expertise & approaches across Labs to deliver *improved algae strains, and a suite of tools* that are effective in our top strains of interest and are also broadly applicable to new strains of interest (from DISCOVR or external stakeholders)

GOAL: *Deliver deeply characterized and improved strains, with accompanying data and tools, to stakeholders including industry, academics, and other BETO projects (e.g. BioFoundry)*

Summary of Past Results

- **Initial AOP focus on developing and transitioning NAABB technical advances**
  - **New projects with components developed from AOP**
    - The Greenhouse: A comprehensive knowledge base of algal feedstocks
    - Genetic blueprint of microalgae
    - Breeding algae for long term stability and enhanced biofuel production
    - Bioprocessing separations consortium
    - Advancing commercialization of algal biofuels through increased biomass productivity and technical integration (ABY1)
    - Producing algae for coproducts and energy (TABB)
    - Realization of algae potential (ABY1)
    - Algae lipid output for high-value applications (pending) (ABY2)

Capability Foundation Developments for Strain Improvements

- **Phenometrics ePBR arrays enhanced (38)**
  - Replicated environmental scripting
  - Improved CO_2 feed and pH control
- **Mbio 100 L raceways in greenhouse (6)**
  - GMO approved testing at larger scales
  - Improved CO_2 delivery systems
- **2 L Flask arrays (30)**
  - Regulated CO_2 feed
  - pH control, Continuous recording temperature and pH
  - LED light control
  - Air bubbling and mixing control
  - Sterile sampling ports in and out
- **5 L fermentor system (2)**
  - Labview custom software control
  - pH, DO, temperature, light, mixing, air, N_2, CO_2, O_2, photoperiod
  - Continuous headspace CO_2 analysis $^{13}C/\ ^{12}C$
  - Automated refrigerated sampling

2- Management Approach

- **Scott Twary –PI**
  - –Attelia Hollander -Technologist
  - –Munehiro Teshima -Technologist
- **Babetta Marrone -co-PI**
  - –Christina Tyler -Post Doctoral Researcher
  - –Claire Sanders –Technologist

  - –Milestones, technical progress and Go/ No go points reviewed weekly in team meetings
  - –Decision points based on strain improvement over 30% increase in productivity of biomass or lipids

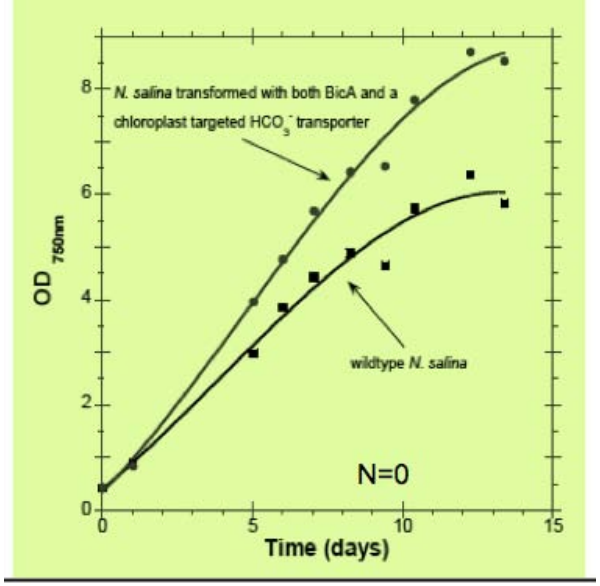
3- Technical Approach

- **A) Biotechnology Strain Improvement**
  - *Nannochloropsis salina* CCMP1776
- **B) High throughput Single Cell Analysis**
  - Novel physiological characterization applications across many algae strains to complement multiple project needs
  - *Nannochloropsis salina* CCMP1776
  - *Picochlorum soleocismus*
  - *Chlorella sorokiniana*
  - *Tetraselmis striata*
- **C) Algae Biotechnology Partnership multi-lab Hub**
  - Monthly telcon and quarterly BETO updates
  - Shared advances and capability developments
  - New AOP project spin-offs

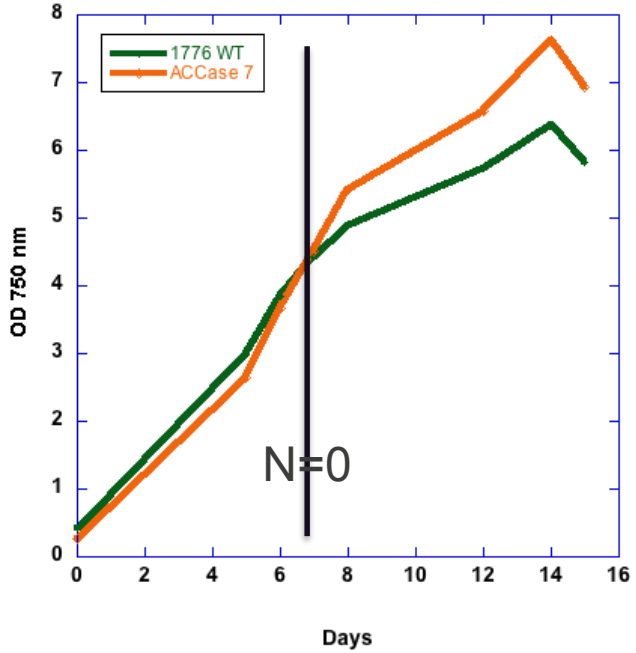
Engineered Productivity Increases in *Nannochloropsis*

Targeted genetic engineering for improving strain productivity including biomass and lipid accumulation.

OUTCOMES: Molecular genetic tools for *Nannochloropsis* engineering.
Improved carbon assimilation and partitioning to lipids

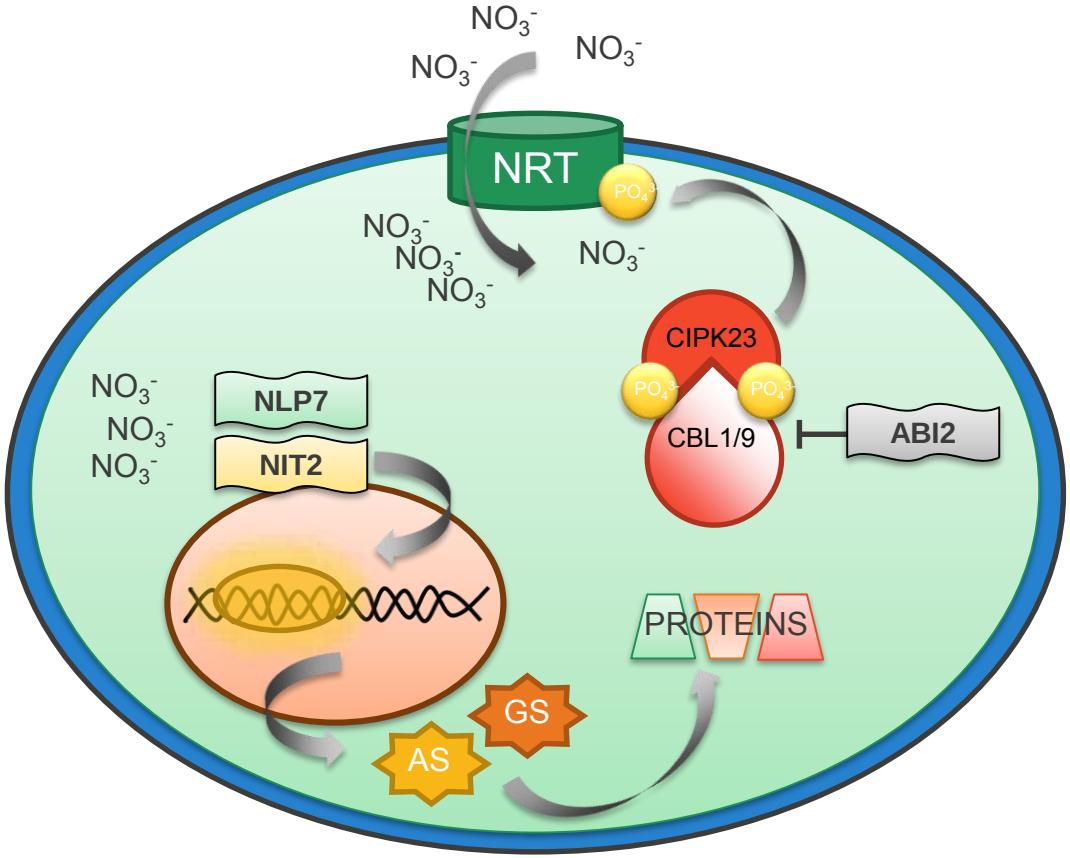


Overexpression of both chloroplast and plasma membrane bicarbonate transporters increased dry weight 46%



Overexpression of ACCase increased Lipid accumulation by 22%

Nitrate Sensing and Signaling: Understanding N stress Induction of Lipid Accumulation



-Overexpression cassettes driven by native lipid droplet protein promoter and translation elongation factor terminator. Transformation selections by Zeocin and Blasticidin antiobiotics.

-Overexpressed GS both chloro and cyto, AS, and PEPC

A) Biotechnology Strain Improvement

- **Expand the molecular engineering toolbox to include Cas9 knock-outs**
  - NO_3^- sensing and signaling pathway: N stress induction of lipid accumulation
  - ABI2 protein phosphatase of CIPK23
  - CIPK23 protein kinase of NO_3^- transporter
- **Overexpress functional target genes**
  - N assimilation and N use efficiency
  - Glutamine synthetase (both chloroplast and cytoplasm)
  - Asparagine synthase
  - Phosphoenolpyruvate carboxylase

OUTCOMES: Understand the genetic basis for nutrient stress regulation to increase productivity and robustness

Technical Approach: Cas9 protein uptake

CAS9 SV40 NLS from *S. pyogenes*

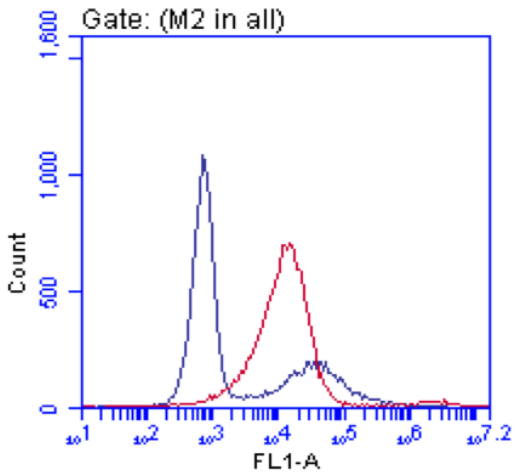
- -native NLS sequences will be explored also
- -Cpf1 alternative endonuclease from *Francisella*
- -Transient functional protein minimizes off-target toxicity

Protein expression system

- -His tag purification 15 uM
- -GFP fusion optional

Increase cell porosity: testing by FITC-dextrans -10k to150k MW

Lysozyme, cellulase, Tween 20,
Electroporation Xfect and Turbofect reagents

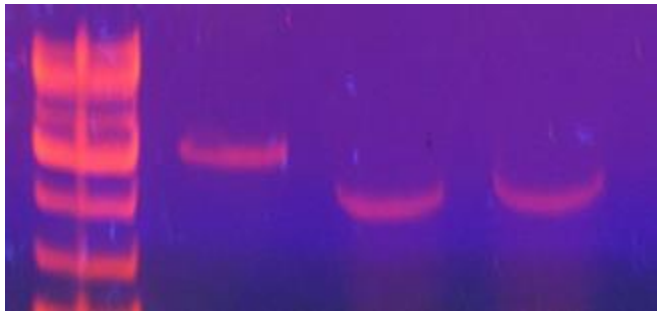


Lysozyme/ Tween 20 pre-treatment increases cell wall porosity to electroporation as determined by retention of FITC-dextran 150K mw

Technical Accomplishments: Cas9 Knock-out Approach

Guide RNAs designed to specifically target 5' exons with PAM site (nGG)

- ABI2
- CIPK23
- Scaffold 121 neutral site
- Scaffold 10 neutral site
  - Create targeted landing sites with stable gene expression



PCR of target *In vitro* Cas9 digest

Cas9 GFP labeled and guides Cy3 labeled for cellular tracking

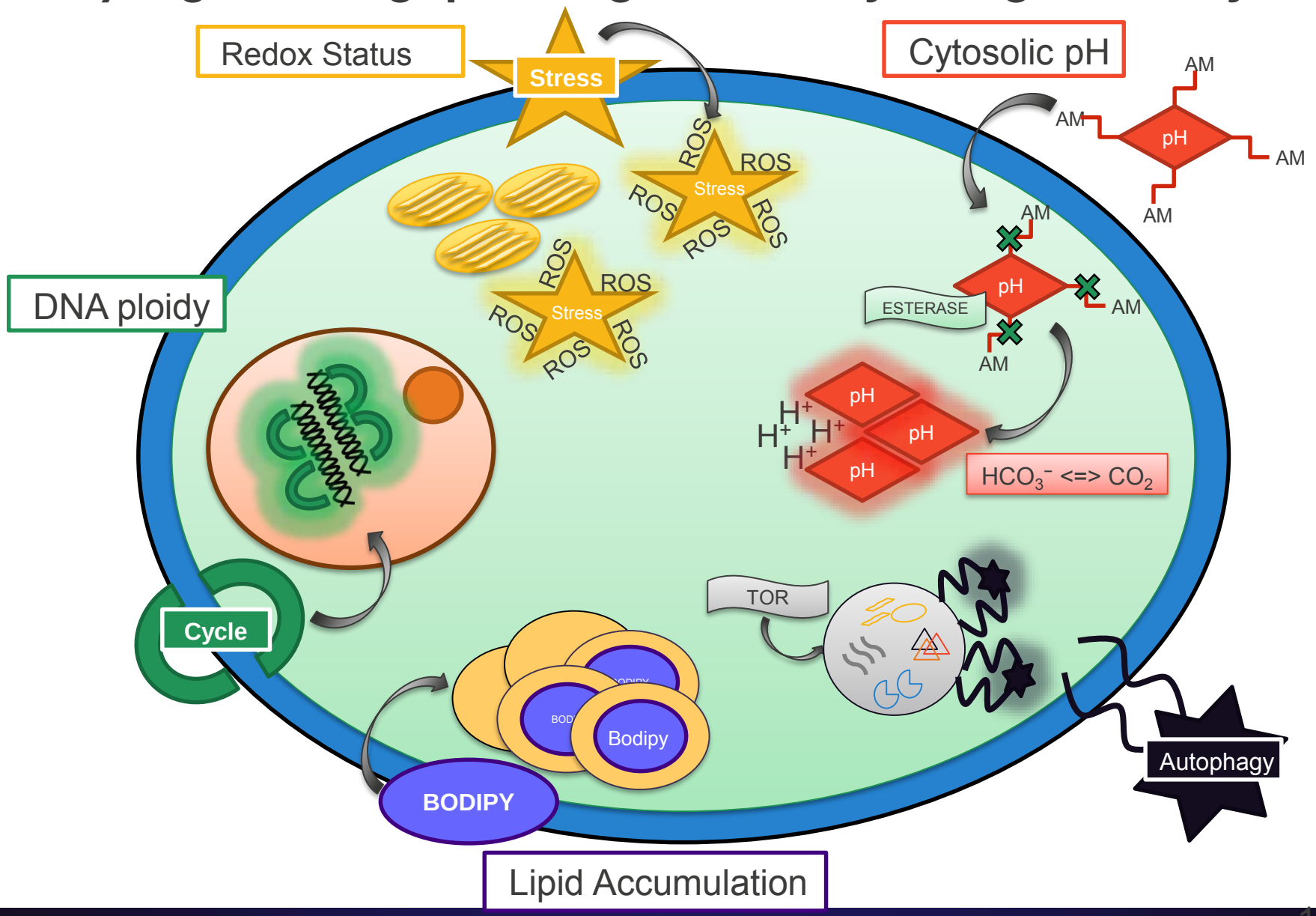
Gene editing selection with promoter/*sh ble*/terminator replacement cassette

- Homologous arm overlaps also investigated for HDR homologous joining repair
- Site-specific PCR identifies genetic insertion sites
  - Random vs targeted

Currently screening transformant colonies for positive targeted insertion

Outcome: An expanded molecular toolbox applicable to multiple algae species. Uncoupling N stress responses may alter lipid accumulation dynamics.

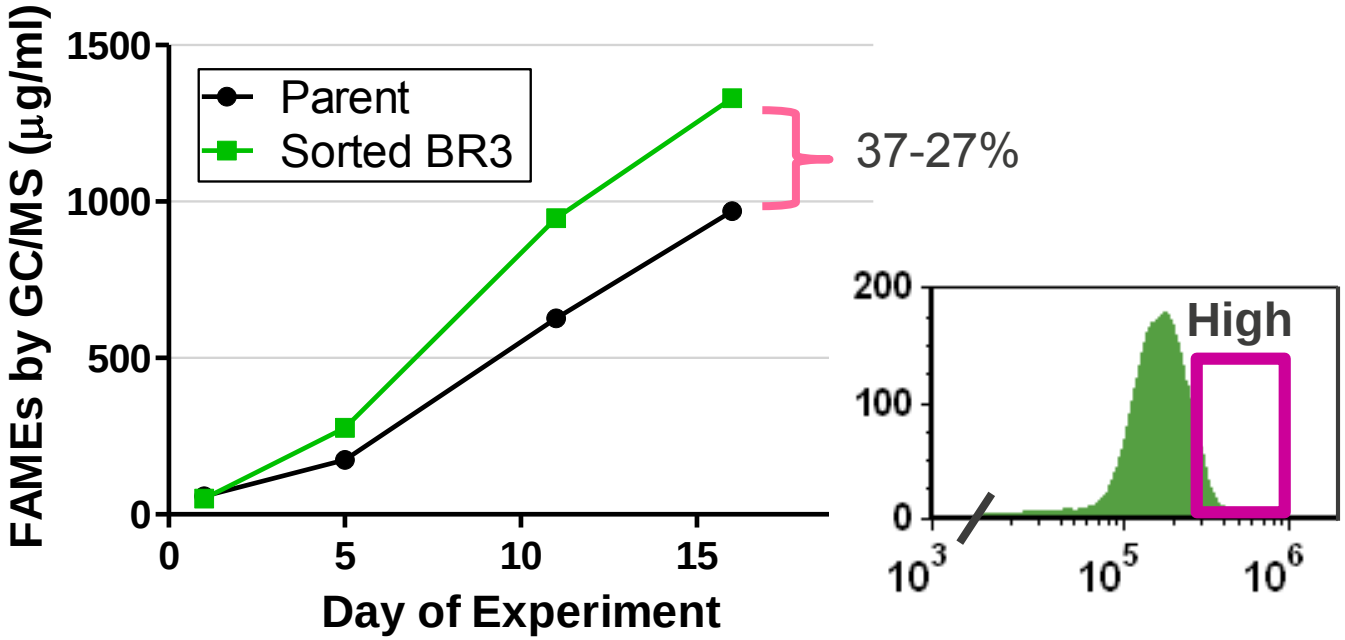
B) High Throughput Single Cell Physiological Analysis



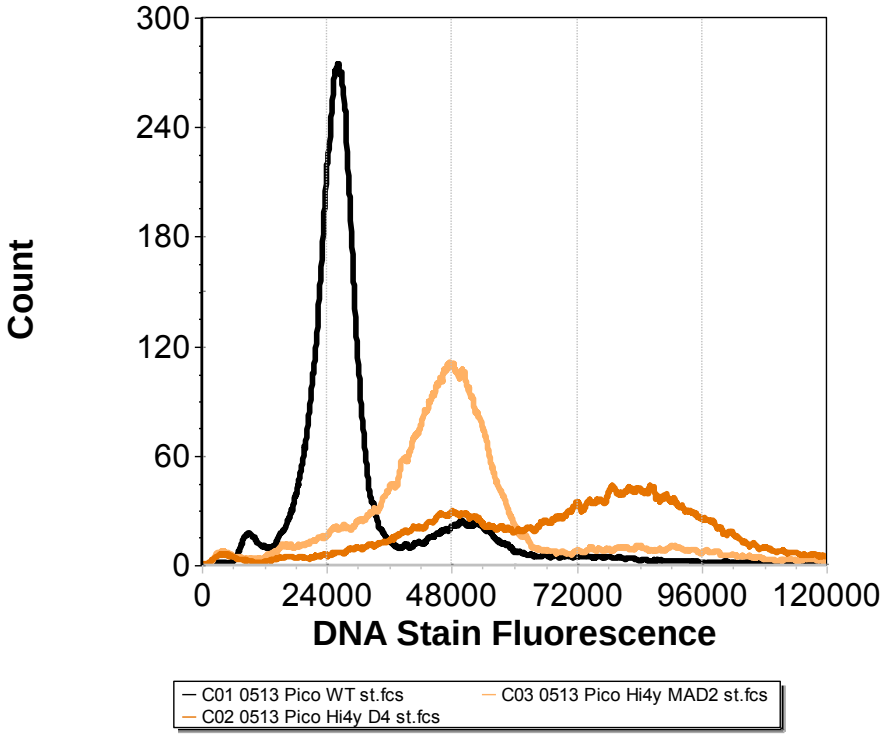
Technical Accomplishments: Application of flow cytometry for *Nannochloropsis* strain Improvement

- Utilize advancements made in other strains/ projects
- Developmental hand-offs of techniques for broader application
- Exploit population variation
- *BODIPY* neutral lipid staining

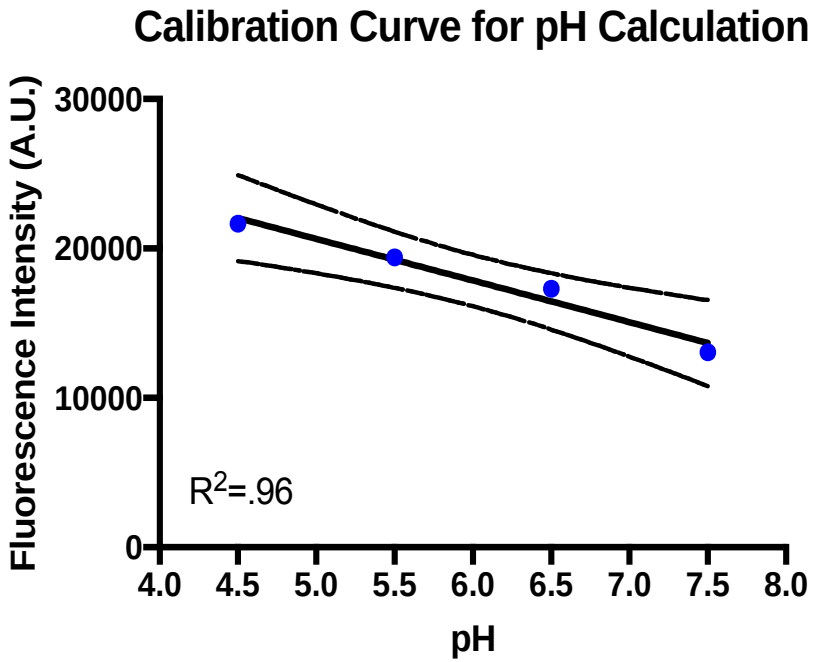
OUTCOME: BR3 FACS line produces more FAME during N stress
Validation testing at outdoor 1000 and 60000 L scale at Cellana ABY1



Technical Accomplishments: Developing Flow Cytometry Characterization of Algae



DyeCycle Orange staining of *Picochlorum* cell stages from haploid, diploid, and tetraploid



pHrodo Green AM staining of *Picochlorum* in a range of pH buffers reflecting altered intercellular pH

4-Relevance

- ***The goals of this project are to advance technical capabilities for rapid strain improvements in productivity and robustness.***
- **To BETO: Understanding complex nutrient signaling responses will allow for designing efficient nutrient management strategies and directed metabolism. Improved algae lines with greater biomass productivity will advance BETO goals of sustainable algae production systems.**
- **To industry: Rapid flow assays allow greater real-time diagnostic analysis for evaluating culture physiology and stress permitting preventative actions and culture optimization**

5-Future Work

- **Develop ReDOX and autophagy standardized assays**
- **Utilize flow cytometry assays for expanded algae phentotype characterization and sorting for improved lines including genetically engineered transformants**
- **Push technology out to larger scale for evaluation of application space**
- **Stack highest impacting engineered traits together to complement improvements**
- **Evaluate N use efficiency on C assimilation and competition**
- **Screen most productive lines outdoors at scale**
  - –Cas9 engineering potential for non-GMO status
  - –EPA TERRA

Summary

- **Past accomplishments have resulted in bioinformatic, technical, and analytical advances expanded into multiple new research portfolios**
- **Current research focused on understanding the basis for N sensing and signaling responses altering C allocation**
  - Genetic knock-outs with transient Cas9
  - Overexpression of key genes to drive C/N balance
- **Current research focused on applying flow cytometry assays for rapid characterization of algal physiology and phenotype**
  - Auotphagy Cyto-ID
  - ReDOX CellRox green
  - pHrodo green intracellular pH
  - DyeCycle orange DNA ploidy cell cycle analysis

- **ADDITIONAL SLIDES**

Responses to Reviewers Comments

- ***This project should be focused on the Algae Biotechnology Hub exclusively, because this is the strength of the overall project***
  - Efforts have been focused to two tasks for strain improvement: New genetic engineering tool development utilizing transient Cas9 gene editing and flow cytometry physiological phenotyping. Both of these tools will be applied towards advancing strain productivity and robustness.

Transition to scale. Evaluate improvements at relevant culture scale.

- Flow sorted improved *Nannochloropsis* line BR3 grown and evaluated at Cellana HI at 10000 L scale outdoors in hand-off to ABY1 project
- Ultrasonic harvesting expanded in scale in hand-off to both Cellana ABY1 and PACE TABB projects
- CO_2 cultivation system proposed scaled to 1000L for pending ABY2 project
- New improved strain testing outdoors at scale proposed as Milestone in year 3 for current AOP
- Hand-off of flow cytometry assay to larger scale production evaluation included as Milestone in current AOP

Publications, Patents, Presentations, Awards, and Commercialization

Publications:

1. Kumar A, Perrine Z, Stroff C, Postier BL, Coury DA, Sayre RT and Allnutt FCT (2016) Molecular Tools for Bioengineering Eukaryotic Microalgae. *Curr Biotechnol*. 5:93-108.

2. Negi S, Barry AN, Friedland N, Sudasinghe N, Subramanian S, Pieris S, Holguin FO, Dungan B, Schaub T and Sayre RT (2015) Impact of nitrogen limitation on biomass, photosynthesis, and lipid accumulation in *Chlorella sorokiniana*. *J. Appl. Phycol*. DOI 10.1007/s10811-015-0652-z.

3. Keeling et al. (2014) The Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP): Illuminating the functional diversity of eukaryotic life in the oceans through transcriptome sequencing, PLos Biology, June 24, 2014. DOI:10.1371/journal.pbio.1001889.

4. Neofotis, P.,Huang, A.,Sury,K.,Chang, W.,Joseph,F.,Gabr, A.,Twary, S.,Qiu, W.,Holguin, O.,Polle, J. 2016. Characterization and classification of highly productive microalgae strains discovered for biofuel and bioproduct generation, Algal Research, pp. 164-178 DOI: 10.1016/j.algal.2016.01.007

5. C.J. Unkefer, et al., Review of the algal biology program within the National Alliance for Advanced Biofuels and Bioproducts, Algal Res. (2016), http://dx.doi.org/10.1016/j.algal.2016.06.002

- 6. Lammers, P.J., M. Huesemann, W. Boeing, D.B Anderson, R.G Arnold, X. Bai, M. Bhole, Y. Brhanavan, L.Brown, J. Brown, J.K Brown, S. Chisholm, C.M. Downes, S. Fulbright, Y. Ge, J.E Holladay, B. Ketheesan, A. Khopkar, A. Koushik, P. Laur, B.L. Marrone, J.B Mott, N. Nirmalakhandan, K.L. Ogden, R.L Parsons, J. Polle, R.D. Ryan, T. Samocha, R.T. Sayre, M. Seger, T. Selvaratnam, R. Sui, A. Thomasson, A. Unc, W. Van Voorhies, P..Waller, Y. Yao, and J.A. Olivares. Review of the cultivation program within the National Alliance for Advanced Biofuels and Bioproducts. Algal Research, *Available online December 12, 2016.*
- *7.* Downes, C.M., J.W. Richardson, J.M. Abodeely, D.B. Anderson, P. Blowers, A.M. Coleman, E..Dunlop, E.D. Frank, M.C. Johnson, R. Handler, J.E. Holladay, M. Johnson, K. Coaldrake, B.L. Marrone, J.B. Mott, D..Newby, K.L. Ogden, R.T. Sayre, W.D. Seider, C.S. Silva, R..Shi, D. Shonnard, R. Skaggs, and J.A. Olivares. Review of the sustainability program within the National Alliance for Advanced Biofuels and Bioproducts. Algal Research, *Submitted, in revision*.
- 8. B.L. Marrone, R. Lacey, D.B. Anderson, B. Bonner, J. Coons, T. Dale, C.M. Downes, S. Fernando, C. Fuller, B. Goodall, J.E. Holladay, K. Kadam, D. Kalb, W. Liu, J.B. Mott, Z. Nikolov, K.L. Ogden, R.T. Sayre, B.G. Trewyn, and J.A. Olivares. Review of the harvesting and extraction program within the National Alliance for Advanced Biofuels and Bioproducts. Algal Research, *Submitted, in Revision.*
- *9.* Kalb, D.M. Ph.D. Dissertation: “High Throughput Acoustic Focusing for Separation and Interrogation of Biological Particles.” University of New Mexico, Department of Chemical and Biological Engineering (February 2017)

- 10. Dale, T., S. N. Twary, B. Fearey, C. K. Sanders, H. Tian, and B. L. Marrone. Development of a rapid flow cytometry assay for quantitative lipid detection in *Picochlorum* sp. *Manuscript in preparation*.
- 11. Tanhaemami, M., C. Sanders, E. Alzadeh, B.L. Marrone, and B. Munsky. Integrating flow cytometry and stochastic analyses to achieve label-free quantification of heterogeneous populations. In preparation.
- 12. Kalb, D.M., J.E. Coons and B.L. Marrone. Acoustic Focusing for Single Cell Impedance Spectroscopy. In preparation.
- 13. Twary, S., Tiasse-Yoder, P., Teshima, H., Alvarez, M., Unkefer, C. 2017. Increased triacyglycerol accumulation in *Nannochloropsis salina* through overexpression of acetyl co-A carboxylase, *Algal Research*, In Preparation.
- 14. Enid J. Sullivan Graham, Cynthia A. Dean, Thomas M. Yoshida, Scott N. Twary, Munehiro Teshima, Mark A. Alvarez, Tawanda Zidenga, Jeffrey M. Heikoop, George B. Perkins, Thom A. Rahn, Gregory L. Wagner, Paul M. Laur. 2017. Oil and gas produced water as a growth medium for microalgae cultivation: A review and feasibility analysis *Algal Research*, accepted.

- **Presentations**
- Acoustic Focusing for Single Cell Impedance Spectroscopy. CYTO 2016 Conference, Seattle WA, 2016 (Exceptional Poster Award, CYTO 2016) Kalb, D.M., Coons J.E., Marrone BL.
- Targeted metabolic engineering of *Nannochloropsis salina*, 2016 6th International Conference on Algal Biomass Biofuels and Bioproducts, S. Twary, M. Alvarez, M. Teshima.
- Property evolution of *Nannochloropsis species* in nitrogen-deplete conditions and its ramifications on overall process energetics. 6th International Conference on Algal Biomass, Biofuels, and Bioproducts, San Diego, CA June 2016 J.E. Coons and T. Dale.
- Ultrasonic harvester test results at Cellana's Kona Demonstration Facility. Algae Biomass Summit, Pheonix, Arizona, October 2016 J.E. Coons, D. Kalb, T. Dale, A. Kramer, and J. Anton.
- Integrating Cultural and Genetic Strategies for Maximizing Algal Biofuel Productivity, 2015 TechConnect World Innovation Conference, S. Twary.
- Optimizing CO_2 utilization in *Nannochloropsis* cultures, 2015 5th International Conference on Algal Biomass Biofuels and Bioproducts, S. Twary, J. Heikoop, M. Alvarez, M. Teshima, C. Unkefer.

- An Innovative Fundamental Approach towards Low-Energy Ultrasonic Harvesting of Microalgae. Algae Biomass Summit, Washington DC, September 2015 J.E. Coons, D. Kalb, T. Dale, S. Twary, and B.L. Marrone.
- Development of an Energy-Cost Parity Approach for Comparing Harvesting & Extraction Technologies. 4th International Conference on Algal Biomass, Biofuels, and Bioproducts, Santa Fe, NM June 2014 J.E. Coons, D. Kalb, T. Dale, and B.L. Marrone.
- The Influence of Physical Cell Properties on the Dewatering of Microalgae using Ultrasonic and Centrifuge Harvesters. Invited presentation to the 5th International Conference on Algal Biomass, Biofuels, and Bioproducts, San Diego, CA June 2015 J.E. Coons, D. Kalb, T. Dale, and B.L. Marrone.
- **Patents:**
- 1. J.E. Coons, et al., Acoustic Manipulation of Fluids Based on Eigen Frequency, S133320, January 2016.
- 2. J.E. Coons, et al., Acoustic-Driven and Optical-Driven Emulsion Destabilization, S129598, July 2015.