Robust genome engineering tools for the algal research community

March 5th, 2019
Advanced Algal Systems

Blake Hovde
Los Alamos National Laboratory

National Nuclear Security Administration
Managed by Triad National Security, LLC for the U.S. Department of Energy's NNSA

Goal Statement:
Release genome engineering tools for the algal research community

Goal:

To Identify and validate a range of diverse native promoters for use in genetic engineering applications of algae.

Outcomes:

The outcome of this project will be a library of ten promoters representing various gene expression strength (at least 100-fold expression level difference from lowest to highest expression) and three additional inducible promoters.

Relevance:

By augmenting the *variety* and *function* of promoter sequences available to researchers, advancements in genetic engineering of algae will be obtained.

Quad Chart Overview

Timeline

- Project start date: 10/1/2018
- Project end date: 9/30/2019
- Percent complete: 33%

	FY 18 Costs	FY 19 Costs	Total Planned Funding (FY 19-Project End Date)
DOE Funded	$0	$200K	$200K
Project Cost Share*	N/A	N/A	N/A

•Partners: None

MYP Barriers addressed:

Aft-C: Biomass Genetics and Development. Improved control and throughput of algal genetic engineering efforts.

Objective

Project Objectives

- Modern genome engineering techniques are currently under-utilized by algal research groups – we aim to improve genetic engineering tools for algae by developing variable strength, and inducible promoter libraries in a stable Cas9 algal cell line.

End of Project Goal

- Publish ten curated promoter sequences of various strength and three inducible promoter sequences.

1 - Project Overview - Background

- **Challenge**: Genetic engineering is currently slow and tools are limited across algal systems.

- **Question**: Can we rapidly generate a verified library of distinct promoters that can be used for generating specific gene expression levels?

- **Opportunity:** This project will provide researchers verified tools for algal engineering and will enhance the genetic engineering toolbox greatly. It will also aid in the development of stable Cas9 cell line development across algal species, which will great increase throughput of testing genetically engineered mutants.

- **Immediate applications:** New promoter libraries can be used for the development of new mutants in *Nannochloropsis salina*
- **Future applications:** Development of stable Cas9 cell lines.
- **Future applications:** (Fine)tuning target product and co-product pathways

1 - Project Overview – Background

Promoters drive applications genetic engineering/synthetic biology

Current:

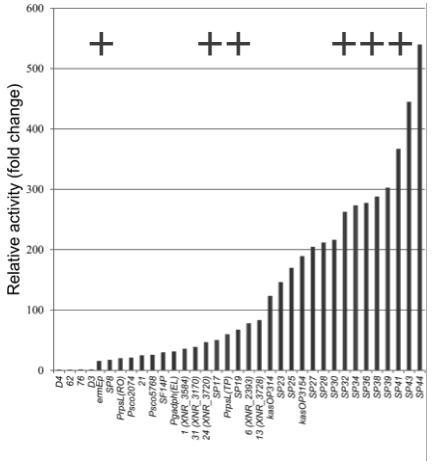
Strong constitutive promoters

+++

RBCS2
CaMV35S
PSAD
FCP

Goal:

Variety in promoter strength



E. coli promoter library from Myronovski and Luzhetskyy, *Nat Prod Rep*. 2016)

Inducible promoters:

"On" in the presence of nitrate

+++

NIT1

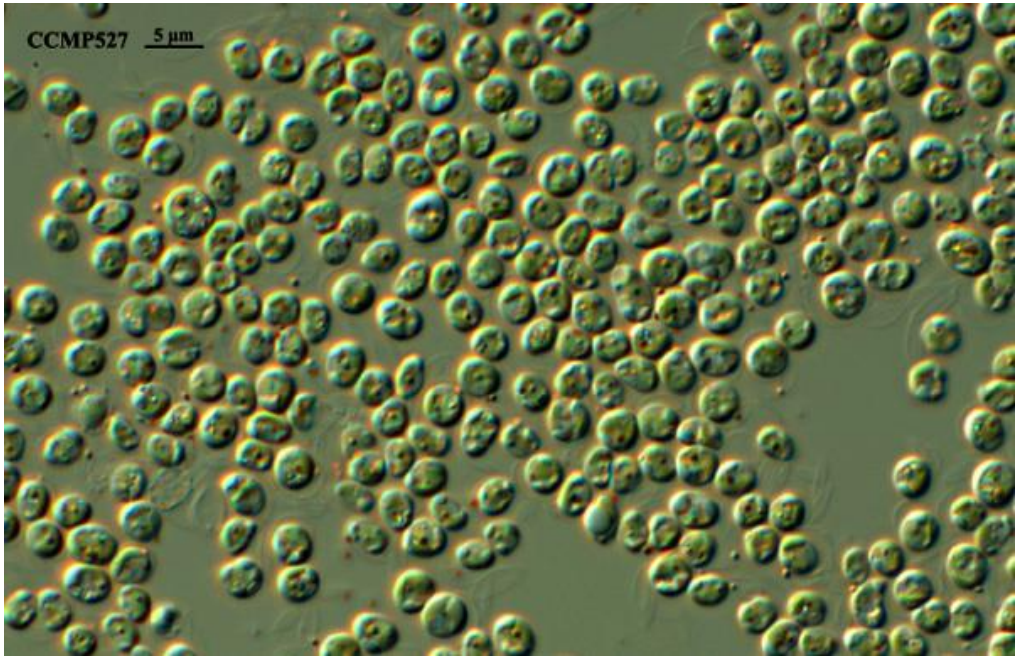
Additional conditions for activation:

Light
Temperature
Nitrogen starvation

- Engineering tools will drive improvements in **productivity** and **Co-products** applications

1- Project Overview - Objectives

- Technical objectives:
  - Characterization of *Nannochloropsis salina* gene expression
    - » Determine variable/inducible promoter strengths
  - Verification of promoter strength/inducibility in-vitro



NCMA: https://ncma.bigelow.org/ccmp527

We are using the production strain ***Nannochloropsis salina***

2 – Approach (Management)

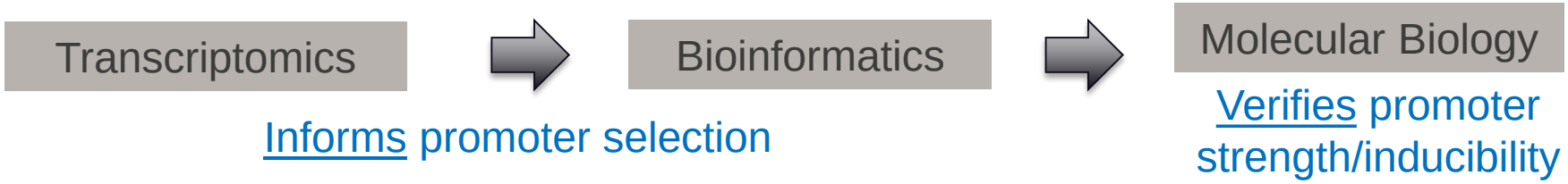
- Blake Hovde (PI – LANL) – Bioinformatics – Gene expression analysis/promoter identification
- Raul Gonzalez (Postdoc – LANL) – Molecular Biology – Gene Expression cloning strategies and promoter synthesis
  - Jackie Mettler (Graduate student – LANL/UNM)
  - Cassie Miller (Post-bac Student – LANL)

Regular progress updates:
-Weekly team meetings
-Monthly BETO AAS meeting
-Quarterly reporting on deliverables to BETO

Current synergistic activities:

Project	PI
Functional Characterization of Cellular Metabolism	Twary - LANL
Genetic Blueprint	Grigoriev and Starkenburg Berkeley/LANL
Robust Genome Engineering	**Hovde - LANL**
Multiscale Characterization of Improved Algae Strains	Dale - LANL

2 – Approach (Technical)



- Our team will use transcriptomic analysis to identify genes that are differentially expressed in *N. salina*.
- This information will identify variable strength and inducible promoters to be validated using molecular biology techniques

Critical success factors:

- Quantitation of gene expression is measurable/consistent
- Promoter sequences identified provide a reliable level of gene expression in practice

Challenges include:

- Successful isolation of high quality RNA for transcriptomic analysis and successful verification of highly diverse promoter strength sequences to build the library.

2 – Approach (Technical)

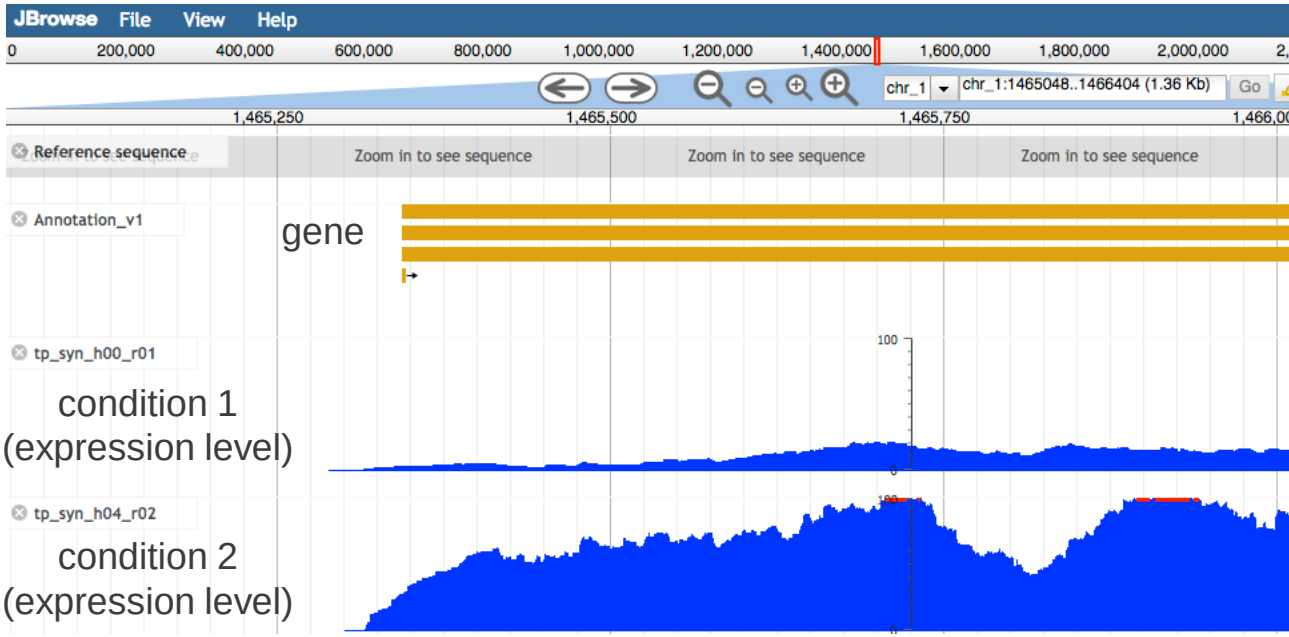
Generation of promoter libraries through transcript analysis

Transcriptomics → Bioinformatics → Molecular Biology

Testing for inducible promoters in the following culture conditions:

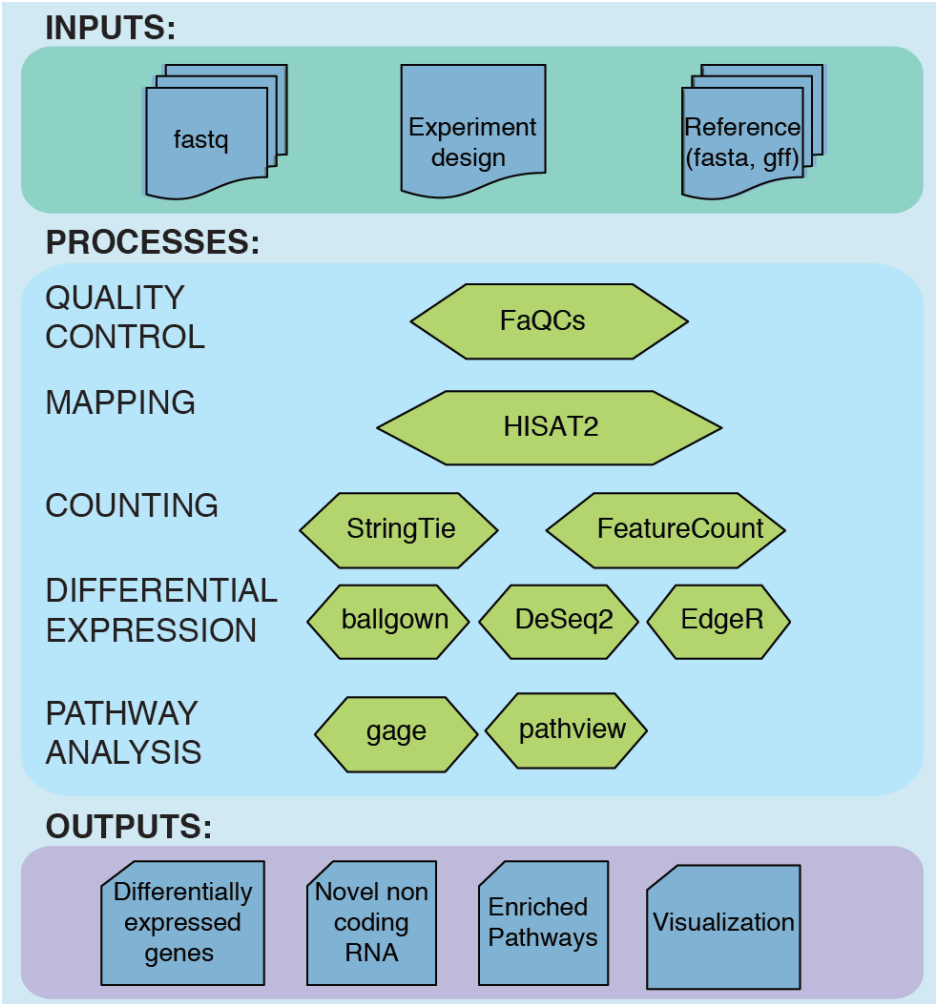
- Light/Dark
- Temperature
- Nitrogen starvation

Check gene expression levels between conditions:



2 – Approach (Technical)

PiReT: Pipeline for Reference based Transcriptomics



- PiReT is an RNA sequencing workflow that minimizes nuances of a complex analysis.
- It is put together by systematic arrangement of available and in-house RNAseq programs.
- It allow users to find differentially expressed transcripts (genes, sRNAs), co-expressed genes and pathways only from raw fastq, reference sequence, and experimental design.
- It can analyze prokaryotic, eukaryotic, or both of them together (dual RNAseq, e.g. Host-pathogen)

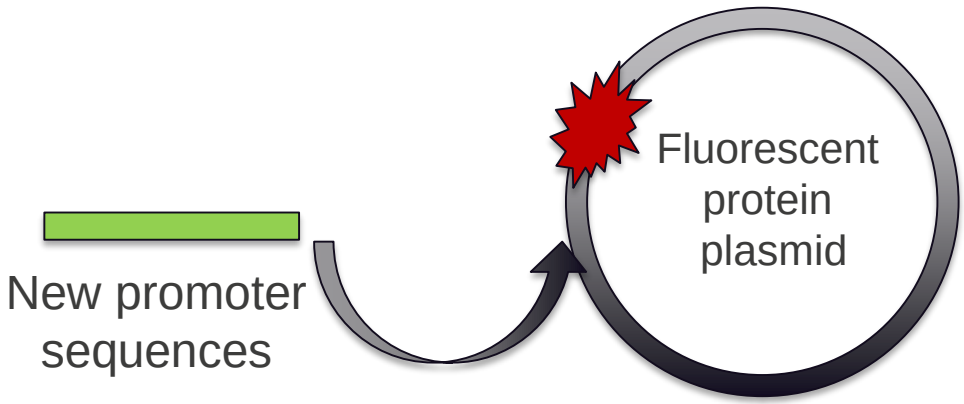
2 – Approach (Technical)

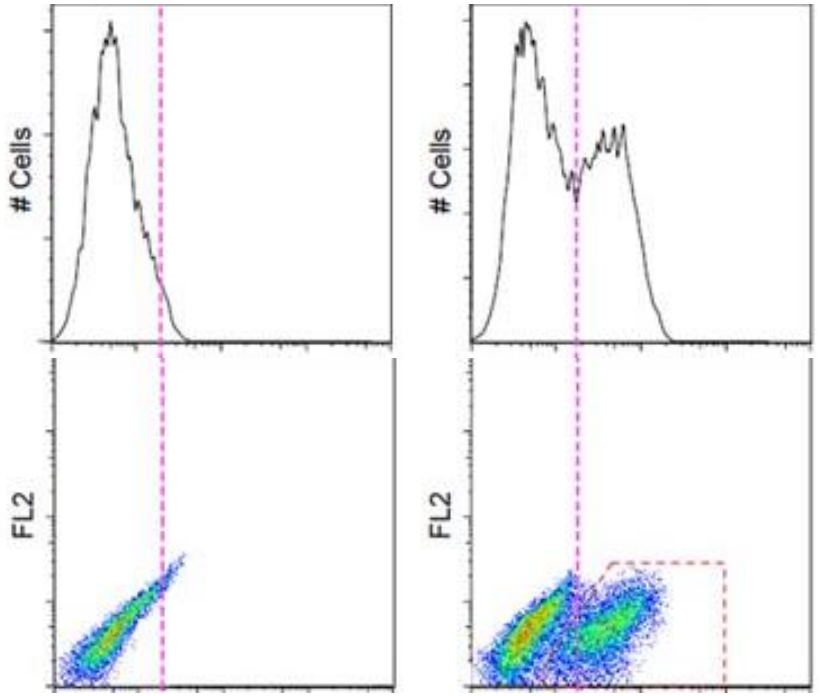
Verification of promoter strength using fluorescent signal

Transcriptomics → Bioinformatics → **Molecular Biology**

To validate promoter strength (Method 1):

- Newly identified promoter sequences will be integrated into our universal fluorescent protein expression vector. The intensity of the fluorescent signal will allow measurement of promoters of different strengths.





Fluorescent signal detection (via flow cytometry)

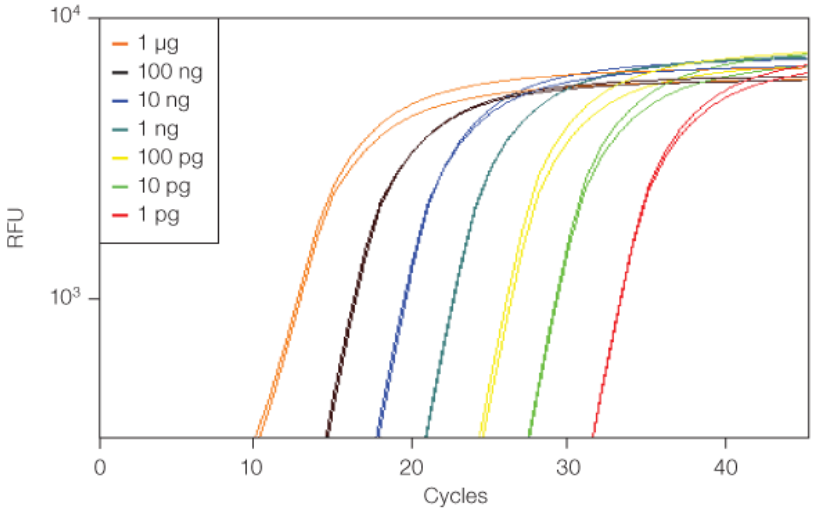
2 – Approach (Technical)
Verification of promoter sequences using qPCR

Transcriptomics → Bioinformatics → **Molecular Biology**

To verify promoter strength (Method 2):

- Quantitative PCR (qPCR) will be used to enumerate a more precise transcript number as a measure of promoter strength.

- Fluorescent signal detection is not as sensitive as qPCR and *N. salina* provides challenges due to natural pigment overlap with fluorescent signal detection



qPCR – quantitative PCR

(Example) Can quantify the amount of transcripts present

3 – Technical Accomplishments/
Progress/Results (New project)

- RNA extraction protocols are optimized and all growth experiments are complete:

  <u>Time courses over:</u>

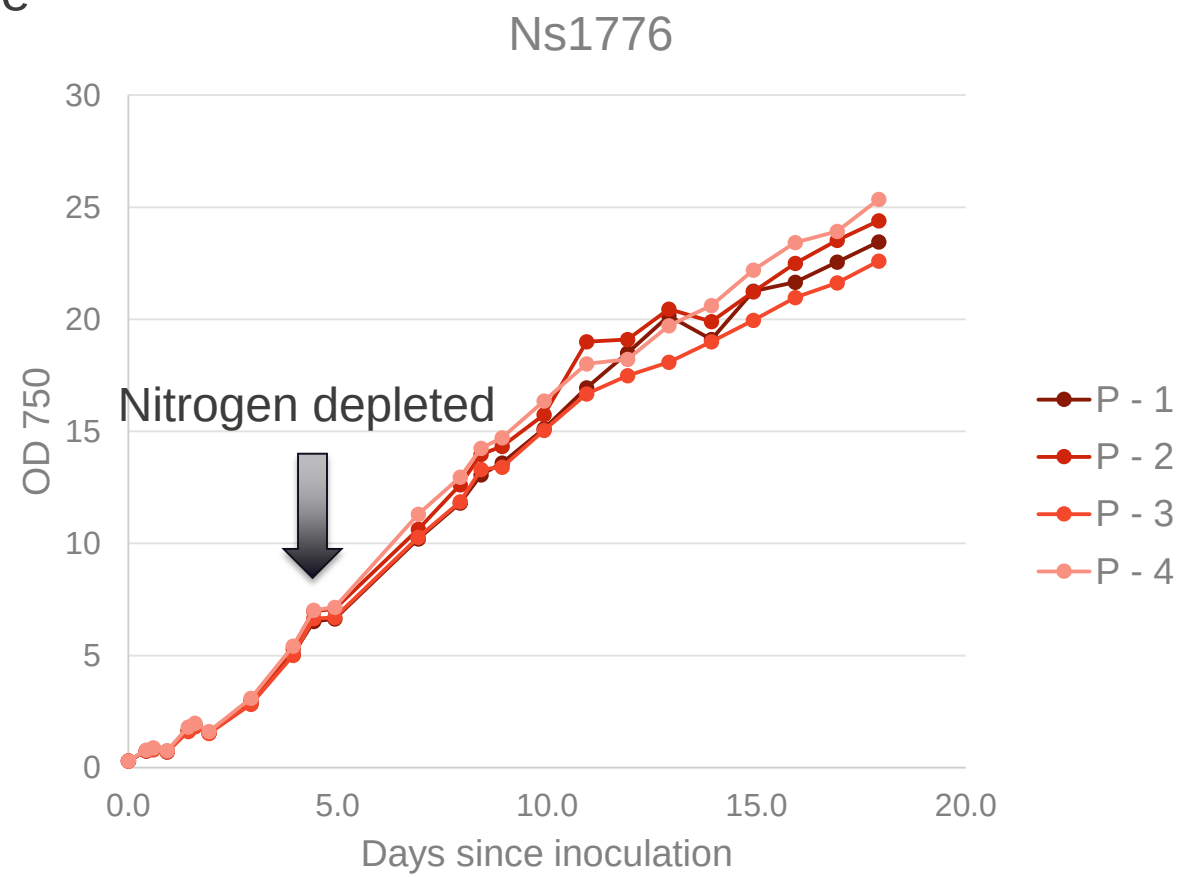
  – light/dark periods

  – Temperature change

  – Nitrogen depletion

  <u>are complete</u>

- The promoter verification construct (mCherry fluorescent protein) is designed and synthesized



OD of a *N. salina* culture over multiple weeks (Nitrogen depletion experiment)

4 – Relevance

Improving genetic engineering efficacy and toolboxes

- Currently, only a limited number of very strong promoters are used in algal genome engineering efforts (limited tools). This limits the capacity of genetic engineering in algae.
  - Stable Cas9 cell lines for example, can greatly improve throughput of genetic engineering experiments. By regulation gene expression of Cas9 in a cell line, more algal editing cell lines can be created.
  - As genetic engineering in algae expands, finer control of genetic elements will be required in both single and multiple gene engineering experiments

- Genetic engineering tools will drive improvements in
  - **Productivity**
  - **Co-products** applications

4 – Relevance to BETO mission

BETO and industry impact

- Goal: improve genetic engineering tools for algae by developing variable strength, and inducible promoter libraries in a stable Cas9 algal cell line.

- If successful in completing these objectives:
  - Expand the promoter library in *Nannochloropsis salina*
  - Follow on work would include development of these tools for additional BETO recommended algal production strains. This would allow for:
    - » Rapid development of promoter libraries across BETO algal strains
    - » Generation of stable Cas9 cell lines utilizing the developed promoter libraries
    - » Stable Cas9 cell lines lead to rapid genome engineering applications
  - BETO synergies:
    Strain improvement and strain development toolkits and technologies
    Production/incorporation of valuable co-products

- Industry impact
  - The promoter library is immediately useful to the academic and industrial algal research community utilizing *Nannochloropsis* species and is likely transferable to closely related species (*Picochlorum*, *Scenedesmus* etc.)

4 – Relevance to BETO mission

Partner impact

Project	PI
DISCOVR / State of Technology	PNNL (+ LANL, NREL, SNL)
Functional Characterization of Cellular Metabolism	LANL
Multiscale Characterization of Improved Algae Strains	LANL
Genetic Blueprint	Berkeley/LANL
Robust Genome Engineering	**LANL**
Algal Translational Genomics	LANL
CAP Process Research	NREL
Algae Biotech Partnership	NREL
ATP^3/AzCATI Testbeds	ASU

5 – Future Work

FY19 Deliverables

- Upcoming milestones:
  - Complete differential expression sequencing and analysis (Q2)
  - Amplification and insertion of 10 candidate promoter sequences into the mCherry reporter construct complete (Q3)
  - Measurement of mCherry expression of all promoters completed and promoter strength is ranked. Release of 10 promoter sequences (promoter library) with relative expression levels. Additional release of 3 inducible promoter sequences (Q4)
- Challenges include successful isolation of high quality RNA for transcriptomic analysis and successful verification of highly diverse promoter strength sequences to build the library.

5 – Future Work

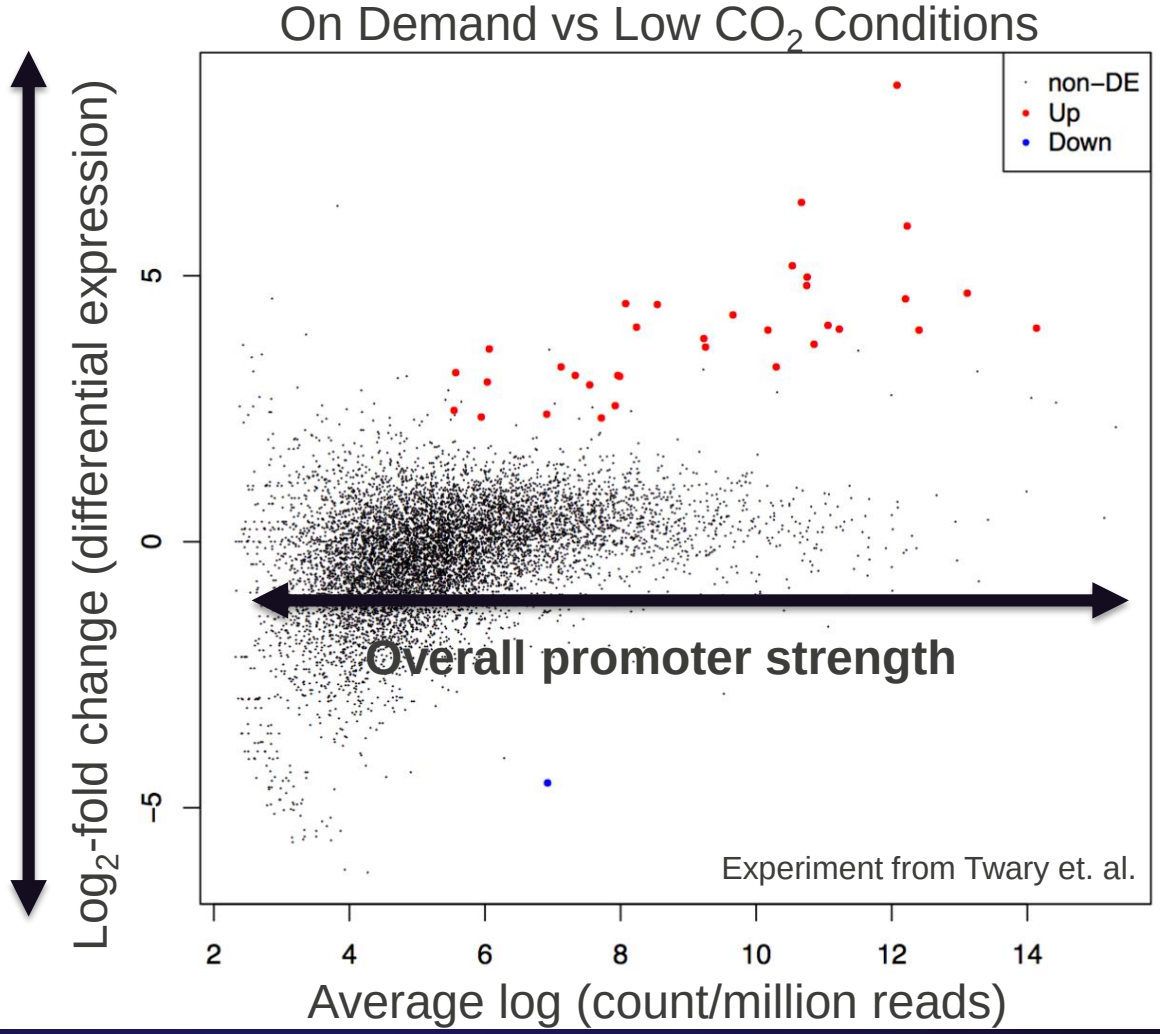
Gene expression analysis (transcriptomics) to inform promoter selection

Transcriptomics → **Bioinformatics**

Informs promoter selection

Testing for Inducible promoters:

- Light/Dark
- Temperature
- Nitrogen starvation



5 – Future Work

Follow-on work based on project success

- If the project is successful in generation and verification of a promoter library for *N. salina*: We now have a model process for rapidly characterizing and verifying beneficial promoter libraries to be used across algal genome engineering projects.

  – Development of promoter libraries in important algal production strains:
    - *Chlorella sorokiniana*
    - *Scenedesmus* spp.
    - *Picochlorum* sp.
    - *Chrysochromulina* sp.

  – Development of stable Cas9 cell lines in important algal production strains:
    - *Chlorella sorokiniana*
    - *Scenedesmus* spp.
    - *Picochlorum* sp.

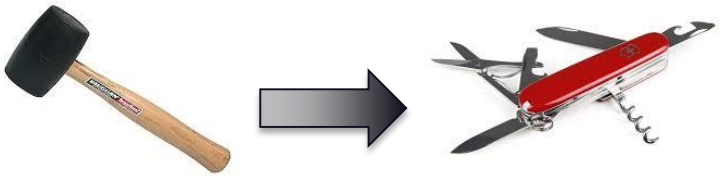
5 – Future Work

Follow-on work based on project success

There are many potential targets for algal genome engineering:

- **Lipid Biosynthesis** (diacylglycerol acyltransferase (DAGAT), Acetyl-CoA synthase)
- **Lipid Modification** (thioesterases, saturases and elongases)
- **Starch energy storage** (ADP-glucose pyrophosorylase, ADP-glucose isoamylase)
- **Lipid metabolism** (Triacylglycerol lipase, esterase-lipases)
- **Light harvesting proteins** (Phototropin **-** non-lethal, confers increase in growth rate)

But we need better tools!



Summary

Overview: Genetic engineering is currently slow and limited in algal systems. This project will provide researchers verified tools for algal engineering and will enhance the genetic engineering toolbox greatly.

Approach:

Technical Accomplishments: (New Project)

- Completed transcriptome experiment RNA collection
- Promoter verification construct designed

Relevance:

- Rapid development of promoter libraries
- Generation of stable Cas9 cell lines utilizing the developed promoter libraries
- Stable Cas9 cell lines lead to rapid genome engineering applications

Future work: Data analysis – identification of candidate promoters. Molecular biology – Verification and quantification of promoter strengths

Additional Slides

Publications, Patents, Presentations, Awards, and Commercialization

- List any publications, patents, awards, and presentations that have resulted from work on this project
  - –None to report - New start project

Note: This slide is for the use of the Peer Reviewers only – it is not to be presented as part of your oral presentation. These Additional Slides will be included in the copy of your presentation that will be made available to the Reviewers.