Second Annual Report
May 8, 2017
Robust Microalgal Production Strains for High Yield Growth on Fossil Flue Gas

Principal Investigator: G. Charles Dismukes, Rutgers University, USA
Co-Principal Investigator: Jun Cheng, Zhejiang University, China
GCEP activity number 96

Sponsored by:
Strains & Growth: This report describes studies of three algae and one cyanobacterium: *Nannochloropsis oceanica*, *Nannochloropsis gaditana*, *Chlorella* sp., and *Arthrospira (Spirulina) maxima*. Optimal growth conditions for the wild strain of *Nannochloropsis o.* were established (temperature, nutrients, preferred form of dissolved inorganic carbon, and agitation method).

CO₂ Controls Light Energy Conversion: Growth data for *N. oceanica* and *N. gaditana* were obtained at a fixed temperature and nutrient media at three CO₂ concentrations. This revealed that *N. oceanica* is a significantly more robust growing strain. The light energy conversion by PSII is higher when elevated CO₂ is supplemented, consistent with kinetic control by the downstream electron acceptor (RuBisCO). Further improvement in energy conversion by PSII is achieved by adding an exogenous electron acceptor that supplements the native PQ pool. This establishes the presence of a blockage in electron transport between the PQ pool and RuBisCO.

Random Mutants: 1200 random mutants, obtained as described in the first year report were screened by two different methodologies for better growth. Four mutants were selected for further characterization. Based on quantitative growth rate and 3 rounds of screening at differential pHs, 5 mutants that can tolerate lower pH were selected for characterization. Both wild type and mutants were able to grow at 5% CO₂. All selected mutants exhibited higher lipid content (as estimated by Nile Red) than wild type. Future growth studies at 10% and 15% CO₂ in planning.

Targeted Mutants: Three site-directed mutants were constructed that target metabolic pathways that intersect with fatty acid production. Genomic analysis verified integration of transgenes in the mutants, while transcriptomic analysis verified expected mRNA phenotypes. Robust methods for lipid extraction and gravimetric quantification were tested and demonstrated to be highly reproducible. Physiological performance of each mutant strain is reported for growth rate, lipid production, and light energy conversion by PSII.
GCEP funded publication: “The Multiplicity of Roles for Bicarbonate in Photosystem II Operation in the Hypercarbonate-requiring Cyanobacterium *Arthrospira maxima*” Gennady Ananyev, Colin Gates and G. Charles Dismukes *Photosynthetica* (submitted). The role of dissolved inorganic carbon in stimulation operation of the PSII-WOC was investigated and three distinct targets that improve operation of PSII and the WOC cycle were identified.

**Chlorella mutant strain MS700**: isolated from gamma irradiated mutagenesis screening, this mutant exhibits faster growth rate, faster O2 evolution rate and faster CO₂ fixation rate.

Two methods for detection of lipids were refined. In vivo detection with Nile Red and in vitro quantification by extraction and gravimetric yield.
Public Presentations of GCEP funded research, 2016

- Oral presentations,
  - 2016 launch of the Rutgers-Zhejiang joint project for Global Climate and Energy Project and tour of the Yantai bioreactor facility. Zhejiang University, Hanzhou, China, May 13-14.
- Poster presentations
- Annual retreat of the Waksman Institute of Microbiology, September, 2016:
  - Mutate microalgae by nuclear irradiation to fix high concentration of CO2
  - Robust Microalgal Production Strains for High Yield Growth on Fossil Flue Gas: Toward Cost Effective Biofuels and CO2 Mitigation.
- Annual conference of Vietnam Education Foundation-Phoenix-Arizona, February 2017
  - Robust screening for high lipid production at high CO2 concentration in a mutant library of microalga Nannochloropsis oceanica CCMP 1779

Honors Received

- 2017 Elected AAAS Fellow, American Association for the Advancement of Science, Charles Dismukes
- 2017 BASF Catalysis Division, special recognition letter, Iselin, New Jersey, Charles Dismukes
- 2017 International cooperation and research program for exchange PhD student (subsidized by Zhejiang University), Hongxiang Lu
- 2016-2017 Vietnam Education Foundation fellowship, Hoa Vu
- 2016 C2B2 summer fellowship from Colorado consortium for Biofuels, Jonah Williams
GCEP Investigators

Co-workers at Rutgers
Principal Investigator: Charles Dismukes
Senior Scientist: Gennady Ananyev
Postdoctoral associate: Yunbing Ma
Ph.D. students: Yuan Zhang, Hoa Vu, Khue Tu Ho-Nguyen
Undergraduate student: Jonah Williams, Jeffery Luo,
Visiting Ph.D. student: Hongxiang Lu

Collaborators not funded by GCEP
Michigan State University: Prof. Christoph Benning, Eric Poliner
Zhejiang University, China: Prof. Jun Cheng, Hongxiang Lu
Progress made in each of the numbered steps given below is linked in the Table of Contents.
0. Summary

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2-3. Random and Targeted mutagenesis and mutant selection for high oil producing strains

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4. Physiological characterization

   1. Physiological characterization in Nannochloropsis oceanica wild type
      
      1. Medium selection for Nannochloropsis oceanica cultivation
      2. Temperature and light intensity dependent growth in Nannochloropsis oceanica at air and 2% CO2

   2. Physiological characterization in other candidate strains
      
      1. For high lipid production: Nannochloropsis oceanica and Nannochloropsis gaditana
      2. For biomass production in high DIC: Arthrospira maxima
      3. For biomass production: Chlorella sp. KMMCC C185 produced by 60Co gamma irradiation

5. Metabolic characterization

   1. Optimization of Lipid Extraction Protocols for Nannochloropsis oceanica
   2. Optimization and standardization of lipid quantification using Nile Red

6. Genomic characterization (will be carried out in coming year)

7. Transcriptomic characterization (will be carried out in coming year)

8. Cultivation: bench-, pilot and field trials (will be carried out in coming year)

9. Conclusion to each section (listed next page)
2-3. Random and Targeted mutagenesis and mutant selection for high oil producing strains

1. Random mutagenesis strains conclusion
2. Targeted mutagenesis conclusion

4. Physiological characterization

1. Physiological characterization in *Nannochloropsis oceanica* wild type
   1. Medium selection for *Nannochloropsis oceanica* cultivation conclusion
   2. Temperature and light intensity dependent growth in *Nannochloropsis oceanica* at air and 2% CO2 conclusion

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   2. For biomass production in high DIC: *Arthrospira maxima*
   3. For biomass production: *Chlorella* sp. KMMCC C185 produced by $^{60}$Co gamma irradiation

5. Metabolic characterization

1. Optimization of Lipid Extraction Protocols for *Nannochloropsis oceanica* conclusion
2. Optimization and standardization of lipid quantification using Nile Red conclusion
1_Strains selection

a) For high lipid production: *Nannochloropsis oceanica*

*Nannochloropsis oceanica* CCMP1779 was purchased from NCMA at Bigelow Laboratory

a) For biomass production in high DIC: *Arthospira maxima*

*Arthospira (Spirulina) maxima* (CS-328) was obtained from the Tasmanian CSIRO Collection of Living Microalgae

c) For biomass production: *Chlorella sp.* KMMCC C185 mutant produced by $^{60}$Co gamma irradiation

*Chlorella sp.* KMMCC C185 was obtained from the Freshwater Algae Culture Collection of Hydrobiology, Chinese Academy of Sciences, China.
2.1_Mutagenesis screening in *Nannochloropsis oceanica* CCMP 1779 for high CO$_2$ tolerating strain

Hoa Vu, Yunbing Ma,
Yuan Zhang, Eric Poliner
Screening for Random Mutagenesis in *N. oceanica*

- Random mutagenesis
- DNA cassette
- Nanno

Hygromycin resistant colonies

Mutant library

More physiological, metabolomic, biochemical and genetic characterization

Screening for biomass production

Winner strains
Mutant selection based on phenotype

**Hygromycin test**

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
</table>

**Absorbance Intensity at 750 nm**

![Graph showing absorbance intensity over time for WT and Mutant samples.]

**Doubling time of Nanno and Mutants**

- **Light Intensity**: 40 mE
- **Temperature**: 22°C
- **Duration for growth**: 8 days

Δ: Maximum doubling time observed after 2 day of inoculation. **G2 mutant has shortest doubling time: 22 hours in 2nd day at around 0.9 day (=22hr)**
Mutant selection based on growth rate - First Round of Screening

Library of Mutants

**Inoculate in 96 well plate**

- A+, 2% CO2, 22°C, 40 μE

**OD 750 nm measurement**

**Mutants in 96-well plate**

**Fluorescence Intensity Measurement**

**Data analysis**

- 10% (120) mutants were selected

**Microplate Reader Neo2 Biotek**

**Counting growth rate and doubling time**

**OD 750 nm**

- Wild Type
- C1
- E2
- G2

**Measurement**

**Fluorescence Intensity**

**Microplate Reader**
- Second Round of Screening: pH tolerance

$\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{HCO}_3^- + \text{H}^+$

300 ml flask with flow rate 30 mL/min

<table>
<thead>
<tr>
<th>CO$_2$ concentration</th>
<th>pH at equilibrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 %</td>
<td>6.4</td>
</tr>
<tr>
<td>10%</td>
<td>6.1</td>
</tr>
<tr>
<td>15%</td>
<td>5.5</td>
</tr>
</tbody>
</table>

- Temperature: 22 °C
- Light intensity: 40 μE
- CO$_2$ chamber: 2%
- Incubate for 10 days

25 mutants were transferred to solid media for further selection.

Phenotypic screening based on pH tolerance of the *N. oceanica* CCMP 1779 mutants. From left to right the growth phenotype of the mutants on A+ medium with pH at 8.2, 7.2, 6.4, 6.1 and 5.5 respectively.
- Third Round of Screening- Quantitative Screening

The mutants were grown in a liquid 96-well plate: A+ medium with varying pH.

Quantitative screening on different pH-liquid media

Data analysis combined with phenotypic observation

Incubate in 2% CO₂ chamber

Phenotype screening on different pH-solid media

25 selected mutants
final selection

- Doubling time (DT) of 25 mutants were calculated
- DT was normalized with WT
- Set DT of positive control as cut off point
- 5 mutants that have DT less than positive control were selected for further research
Confirmatory screening

5% CO2 experiment

The growth rate shown that the WT has the fastest growth rate and highest biomass production up to 0.78 g/L (dried weight). The five mutants have biomass production between 0.30 to 0.36 g/L.

The Nile red fluorescence intensity of the mutants and the Nile red fluorescence normalized to the biomass shows that the five selected mutants have higher Nile Red fluorescence compared with the WT. The mutant # 11 has the highest lipid content (3 times) than the WT and the mutant # 6 has two times higher than the WT.

Nile red fluorescence can reflect the lipid content in live cell but due to the fact that Nile Red does not specifically bind only to lipid therefor gravimetrically or FAME- GC-MS for lipid quantification and qualification will be next step.
Conclusions

1. Two different methodologies were applied to screen for better growing mutants.

2. Based on gross phenotype, 4 mutants were selected that exhibited higher potential for biomass production.

3. Based on quantitative growth rate and 3 rounds of screening at differential pHs, 5 mutants that can tolerate lower pH were selected for downstream characterization.

4. Both wild type and mutant were able to grow at 5% CO$_2$.

5. All selected mutants exhibited higher lipid content (as estimated by Nile Red) than wild type.
2.2 Metabolic engineering by targeted mutagenesis

Yuan Zhang, Yunbing Ma, Eric Poliner, Jonah Williams, Gennady Ananyev

April, 2017
Motivation

- *Nannochloropsis oceanica* CCMP1779 has very high lipid production (20-50 % dry cell weight), mainly in the form of TAG, which are important biofuel precursors.

- Knowledge of lipid metabolism in Nannochloropsis species are mainly based on genome predictions.

- With the advanced genetic engineering tools developed in *Nannochloropsis* 1779, not only the lipid metabolic pathways can be engineered to boost the lipid productivity, but also the functional nature of most potential key enzymes can be further studied and experimentally verified.

- The resulting technology could be applied to other oleaginous algae for metabolic engineering.
Questions to be studied:

1. Molecular Characterization:
   a) Does the transgenic lines screened out have the transformation vector steadily integrated?
   b) Are the transcriptional and translational level of the target genes up or down regulated in the transgenic lines as expected?
   c) Does the changes of mRNA and protein level in the targeted mutants even more substantial in the N- conditions?
   d) How does the target genes respond to N deprivation conditions in WT?

2. Physiological Characterization:
   a) Does any of the targeted mutants show enhanced lipid productivity over WT?
   b) Are the growth or cell development affected in the targeted mutants?
   c) What are the actual yield of biomass, neutral lipid and TAG in the targeted mutants compared to WT?
   d) How does elevated CO₂ in purging air affect grow rate?
The presence of the integrated genes in the 6 Gene X overexpressors was verified by PCR. Genomic DNA from above mentioned strains were extracted, and PCR were performed using primers targeting the coding region of Gene X.

**Gene X Overexpressor: pnoc stacked Gene X A/S**

1 kb

1  2  3  4  5  6
PCR verification of the transgene integration in the Gene X ox/ Gene Y RNAi stacking mutants.

The presence of the integrated genes in the 10 pnoc stacked-Gene Y X/A-Gene X A/S clones was verified by PCR. Genomic DNA from above mentioned strains were extracted, and PCR verified.

![PCR verification](image)
PCR verification of the transgene integration in the *Gene Y* RNAi mutants.

Insertion of the *Gene Y* RNAi inverted repeat in all the selected transgenic lines was verified by PCR. Genomic DNA from A) single pnoc 411 *Gene Y* ex2 sas line; B) 12 p noc stacked *Gene Y* AS lines and C) 8 p noc stacked *Gene Y* XA lines were extracted, and PCR verified.
Lipid content of *No. 1779* WT and mutants determined by Nile Red fluorescence

Three (G2, 4, 6) pnoc stacked *Gene X* A/S lines, five (G+C1, 3, 4, 6, 10) pnoc stacked-*Gene Y* RNAi X/A-*Gene X* A/S lines and the single pnoc 411 *Gene Y* RNAi ex2 sas line showed higher final NR fluorescence which were statistically significant in comparison to the NR fluorescence of the wild type culture. The phenotypes were confirmed by two rounds of replica experiments (Round 1 and Round 2).
All these robust lipid producers showed no compromised growth compared to wild type besides G+C6. The phenotypes were confirmed by two rounds of replica experiments (Round 1 and Round 2).
Cellular lipid content of No. 1779 WT and mutants determined by NR/OD750.

By normalizing the total Nile Red fluorescence determined in Figure 6 to the OD750 measured in Figure 7, we can estimate the cellular lipid production for each transgenic lines. Three (G2, 4, 6) Gene X overexpressors, two (G+C1, 6) stacked-Gene X OX-Gene Y RNAi lines and the single Gene Y RNAi line showed higher NR/OD750 which were statistically significant in comparison to that of the wild type culture.
### Selection of winning transgenic lines for downstream analysis

**Table 1.** Selection of winning transgenic lines for downstream analysis.

Three (G2, 4, 6) Gene X overexpressors, one (G+C1) stacked-Gene X OX-Gene Y RNAi lines and the single Gene Y RNAi line were selected for showing both higher total lipid productivity and higher cellular lipid content with no compromised growth rate. Another two (G+C3, 6) stacked-Gene X OX-Gene Y RNAi lines were also selected for showing higher total lipid productivity with different behavior in growth and cellular lipid content.

<table>
<thead>
<tr>
<th>Mutant Genotype</th>
<th>Mutant ID #</th>
<th>Growth (OD750)</th>
<th>Cellular Lipid Productivity (NR/OD)</th>
<th>Overall Lipid Productivity (NR Fluor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene Y RNAi line (1)</td>
<td>ClSi</td>
<td>Normal</td>
<td>Robust</td>
<td>Robust</td>
</tr>
<tr>
<td>Gene X OX Lines (6)</td>
<td>2, 4, 6</td>
<td>Normal</td>
<td>Robust</td>
<td>Robust</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>3, 5</td>
<td>Slow</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Gene X OX-Gene Y RNAi Stacking Lines (10)</td>
<td>2</td>
<td>Robust</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Normal</td>
<td>Robust</td>
<td>Robust</td>
</tr>
<tr>
<td></td>
<td>5, 7, 8, 9</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>3, 4, 10</td>
<td>Normal</td>
<td>Normal</td>
<td>Robust</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Slow</td>
<td>Robust</td>
<td>Robust</td>
</tr>
</tbody>
</table>
Transcriptional analysis showed the expression level of *Gene X* is higher in the overexpressors, and *Gene Y* level is knocked down in the RNAi mutant.

**Figure 9.** Transcriptional level of *Gene X* in No. 1779 WT and selected mutants determined by Semi-quantitative RT-PCR. PCR amplifying the 174 bp histone fragment was used as a positive control as well as internal reference to approximately verify that the input RNA/cDNA was equal across all samples (lower).

**Figure 10.** Transcriptional level of *Gene Y* in No. 1779 WT and selected mutants by Semi-quantitative RT-PCR. The absence of amplification signal in *Gene Y* RNAi mutant (lane 2) is evident for the efficient knock down by the RNAi.
In vivo variable chlorophyll-a fluorescence of *N.o* 1779 WT and selected mutants at different flash frequencies.

FRRF was performed on both wild type and three targeted mutant strains of *N.o* 1779 (CISi, G2 and G+C1) at 1, 100 and 250 Hz. Fv/Fm, denoting the quantum efficiency of PSII charge separation is lower in the mutants than in the WT.
A less efficient WOC cycling is indicated in the mutants.

Table 2. Kok parameters of *N.o* 1779 WT and selected mutants at flash frequency 1 and 100Hz. The *in vivo* Chla fluorescence $F_v/F_m$ determined by FRRF were fitted to the VZAD model. The obtained WOC cycling period is longer in the mutants than in the WT, indicating a less efficient WOC cycling for the mutants.
Summary:

- One knock out mutant (Gene Y), one overexpression mutant (Gene X) and the double mutant were constructed and genomic integration was verified for each mutant.
- Among several strains of each mutant, differing in genomic integration locus, the most robust growing transgenic lines were selected for further analysis.
- Lipid extraction protocol and gravimetric quantification methods were developed and demonstrated to be highly reproducible.
- Gene expression levels of winning transgenic strains were measured by quantitative semi-RT-PCR and found to be consistent with observed phenotype of each strain.
- The photosystem II quantum efficiency of the mutants was determined by FRRF and found to be less than that of the wild-type.

Future Work:

- Lipidome: Quantify the amount and types of lipids (fatty acids, TAGS) in mutants.
- Determine biomass and lipidome of mutants under N deprivation conditions.
4.1.1_Medium selection for Nannochloropsis Oceanica cultivation

Yuan

December 2016
Nanno grown in A+ medium showed substantially better growth as compared to grown in other medium.

<table>
<thead>
<tr>
<th>Components</th>
<th>A+</th>
<th>F/2</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>12 mM</td>
<td>2.5 mM</td>
<td>4.8 X</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.37 mM</td>
<td>0.18 mM</td>
<td>2 X</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>0.014 mM</td>
<td>0.024 mM</td>
<td>1.7 X</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.8 mM</td>
<td>4.9 mM</td>
<td>2.7 X</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.554 mM</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Conclusions

1. A+ medium is better than the traditionally used F/2 for laboratory Nanno cultivation, probably due to the high nitrate content in the A+ medium.
2. The addition of bicarbonate in the medium has inhibitory effect on its growth.
3. Doubling time of *Nannochloropsis* could be as short as 10 hours and as long as 50 hours.
4.1.2_Temperature and light intensity dependent growth in *Nannochloropsis oceanica* at air and 2% CO$_2$

KT, Yunbing, Hoa

November 2016
Motivation

• Assemble and establish standard operational procedure (SOP) for the newly purchased MC-1000OD

• Assess the temperature and light intensity effect on the growth and lipid content of the Nannochloropsis culture.
  • Current light intensity used in the lab for Nanno culture is 30-50uE. Will higher light intensity (150uE) stimulate growth for CCMP 1779, similar to other reported Nanno strains?
  • Lab condition for Nanno optimal growth is 22C. Can Nanno tolerate 33C degree as in field trials in Yantai?
Multichannel Cultivator

- Thermostated waterbath
- Independently illuminated by LEDs (light intensity and photoperiod)
- Parallel sampling

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1, 22°C</td>
<td>150 µE</td>
<td>150 µE</td>
<td>150 µE</td>
<td>50 µE</td>
<td>50 µE</td>
<td>50 µE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 2, 33°C</td>
<td>150 µE</td>
<td>150 µE</td>
<td>150 µE</td>
<td>50 µE</td>
<td>50 µE</td>
<td>50 µE</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Day 0: OD 0.15

Day 7: 22°C

Day 7: 33°C
**Exp. 1 Growth at 22C**

1. At 22C, cell growth is better under 150uE illumination.
2. With equal volume of cell culture, 150uE grown cells showed significantly higher Nile Red fluorescence than 50uE grown counterpart.
3. When normalize Nile Red reading to OD750, 150uE grown cells also showed higher fluorescence intensity than 50uE counterpart.
4. When normalize Nile Red reading to Chlorophyll a fluorescence, the same trend is observed.

Overall, at 22C, 150uE grown cell showed more robust growth (biomass) and more lipid content when compared to its 50uE grown counterpart. **Hence 150uE illumination can be recommended for Nanno growth.**
Exp.2 Growth at 33C

5. At 33C, a small, if any, cell growth is only overserved during the first three days. Growth is generally inhibited afterwards at both light intensities.

6. With equal volume of cell culture, 150uE grown cells showed significantly higher Nile Red fluorescence than 50uE grown counterpart.

7. When normalize Nile Red reading to OD750, 150uE grown cells also showed higher fluorescence intensity than 50uE counterpart.

8. When normalize Nile Red reading to Chlorophyll a fluorescence, the same trend is observed.

Overall, at 33C, cells under both light conditions grow poorly. However, cells under this condition appear to exhibit higher lipid production capability especially for the 150uE grown cells.
Pooled data from 22C and 33C

9. Based on the OD750 reading, 150uE light intensity and 22C temperature resulted in best growth comparing to other tested conditions. Cells grown at 33C grow poorly.

10. With equal volume of cell culture, 150uE (both 22C and 33C) grown cells showed significantly higher Nile Red fluorescence than 50uE (both 22C and 33C) grown counterpart.

11. When normalize Nile Red reading to OD750, 150uE light intensity and 33C temperature grown cells also showed highest fluorescence intensity than other conditions.

12. When normalize Nile Red reading to Chlorophyll a fluorescence, the same trend is observed.
Further temperature studies at 2% CO$_2$

- In open pond cultivation eventually in Yantai, flue gas with higher CO$_2$ concentration will be used as carbon supply for Nannochloropsis.
- Also higher than 22°C temperature will be likely a challenge for Nannochloropsis especially during the summer time.
- We question how Nannochloropsis behave during moderately elevated CO$_2$ (2%), and higher temperature (30°C and 37°C)
At 2% CO2, 22°C temperature is favorable for N. oceanica than 30°C.

Growth condition:
• WT
• Light Intensity: 40μE
• Medium: A+
• Humidity 60%
• Diurnal cycle 16/8 (light/dark)
• InitialOD_{750nm}=0.05 (100 μL on Neo2 microplate Reader)

Fig.3 N. oceanica grow exponentially on 2% CO₂ at 22 °C and grow slower 2% CO₂ 33 °C.

Fig.4 Chlorophyll fluorescence intensity strictly flow the same rate with growth rate.

Fig. 5 The doubling time of N. oceanica grown at 22 °C is much shorter than at 30 °C. The shortest doubling time at 22 °C is around 30 hours at days three. And at 30 °C it is started at 44 hour after two days and keep increasing up to 120 hours after 11 days.

Over all: N. oceanica with initial OD 0.05 grows on medium A+ 2%, CO₂ at 22 and 30 °C. At 2% CO₂ at 22 °C N. oceanica grows much faster comparing with at 30 °C. Higher than 30C temperature suppresses the growth of N.O.
At 2% CO₂, *N. oceanica* cannot survive at 37°C

1-4), Light: 40μE
Every OD measured with 100 μL on Neo2 microplate Reader

**Over all:** After 4 times of attempting to grow *N. oceanica* at 2%, CO₂ at 37 °C has no success. It might needed bigger initial cell number a/o preadapted with lower temperature for example at 33, 35 °C a/o modifying the medium to help the cell grow in high temperature
Conclusion

Overall,
• At air level CO₂, Nanno culture grows best under 150uE at 22C temperature (Figure 1,9), while at 33C Nanno growth is generally inhibited (Figure 5,9).
• However, high lipid production is observed when Nanno is grown at elevated temperature (33C, Figure 11,12 ), where cells are presumed to undergo stress and growth is inhibited.
• Therefore, a strategy can be proposed, in which cells are allowed to reach maximum density under 150uE/22C condition, then switched to 150uE/33C condition which appeared to have a stimulatory effect on lipid production.
• With this strategy, high biomass and high lipid production can both be achieved. Comparing to the typical Nitrogen depletion method, this method has the advantage of not needing to change growth medium.

• At elevated CO₂ (2%), Nannochloropsis also grows faster at 22C. 37C completely inhibits the growth.

• Growth studies laid the foundation, and standardize the approach for further detailed characterization of selected mutants.
4.2.1_Strain selection for high lipid yield production.

We have selected the marine microalga *Nanochloropsis oceanica* CCMP1779 as the strain for random and targeted mutagenesis. This decision has been reinforced by data we obtained comparing it to the marine microalga *Nanochloropsis gaditana*. 
Motivation

• *Nannochloropsis* sp. are tolerant to temperature in range from 5 to 40°C and light intensity up to 400 μE/m²/s;

• Unique pigment composition: includes *Chl* a and lack of *Chl* b and c;

• β-carotene, violaxanthin and vaucheriaxanthin are the most abundant carotenoids;

• Xanthophyll cycle induction through exposure of cells to high irradiance and at 40°C, conversion of violaxanthin into zeaxanthin may attain up to 70% of the violaxanthin content;

• Cell density reaches more than $10^8$ cells/mL at abundant nutrients and light intensity up to 400 μE/m²/s;

• We may expect outstanding regulation of State Transitions at acclimation to fluctuating light and various environmental conditions.
Questions studied:

1. How does elevated CO₂ content in purging air affect grow rate?

2. What are the relative CO₂ tolerance of *N. oceanica* and *N. gaditana*?

3. What is the effect of elevated CO₂ on linear electron flow from water (measured by flash O₂ yield)?
Effect of CO₂ concentration on growth of Nanochloropsis strains
Relative Growth Rate at 6, 15 and 30 % CO₂

*N. oceanica* grows fast and reaches a higher biomass yield than does *N. gaditana*.
15% CO₂ stimulates O₂ yield in both strains, indicating that PSII operates better with downstream electron acceptor for RuBisCO +DMBQ stimulates O₂ yield even further in the presence of 15% CO₂, indicating that PSII operates below optimal even at such high CO₂.

Activation occurs over time. Origin to be determined.
Effect of 15% CO$_2$ on PSII operation via flash O$_2$ yield

**N. oceanica, no DMBQ**

- CO$_2$: 15%
- No DMBQ
- Alpha: 0.110
- Beta: 0.032
- Delta: 0.011
- Epsilon: 0.030
- S0Norm: 0.332
- S1Norm: 0.627
- S2Norm: 0.041
- S3Norm: 0
- TheorPeriod: 4.254
- FitPeriod: 4.248
- FitFT_area: 0.898
- ExptPeriod: 4.242
- ExptFT_area: 0.901
- RTD = 0.027

**N. oceanica, + DMBQ**

- CO$_2$: 15%
- + 250 uM DMBQ
- Alpha: 0.186
- Beta: 0.031
- Delta: 0
- Epsilon: 0.010
- S0Norm: 0
- S1Norm: 0.911
- S2Norm: 0.089
- S3Norm: 0
- TheorPeriod: 4.570
- FitPeriod: 4.515
- FitFT_area: 0.880
- ExptPeriod: 4.335
- ExptFT_area: 0.902
- RTD = 0.053

Although adding DMBQ increases O$_2$ yield even in presence of 15% CO$_2$, it has almost no effect on the resting S state populations of the WOC and minimal effect of the WOC photocycle measured at low flash rate (0.5 Hz). Note small increase in misses (alpha) and decrease in photoinactivation (Epsilon). The response in *N. gaditana* is like that of *N. oceanica* but less extreme (not shown). These responses are not observed with DMBQ in low CO$_2$ conditions and must result from cellular response to elevated CO$_2$.  

4.2.1
Conclusions

• Growth rate data for *N. oceanica* and *N. gaditana* were obtained at a fixed temperature and nutrient media at 6, 15 and 30% CO₂/air bubbling. This revealed that *N. oceanica* is a significantly more robust growing strain by two fold.

• 15% CO₂ stimulates O₂ yield in both strains, indicating that PSII operates at higher flux with elevated CO₂, the downstream electron acceptor for RuBisCO.

• Adding an exogenous electron acceptor that supplements the native PQ pool (DMBQ) stimulates the PSII light driven O₂ yield even in the presence of 15% CO₂. This establishes a kinetic blockage in electron transport between the PQ pool and RuBisCO. Hence, PSII operates below optimal even at such high CO₂.

• Activation of electron transport occurs over time. Unknown origin to be determined.

• Operation of the PSII-WOC cycle in both strains was characterized using the VZAD model.
4.2.2_Strain selection for biomass production.

We have selected the hypercarbonate-requiring cyanobacterium *Arthrospira maxima* for comparative studies of high growth rate and biomass yield at elevated CO$_2$. 
In contrast to most oxygenic phototrophs, the cyanobacterium *Arthrospira maxima* (formerly *Spirulina*) is capable of sustained growth under the highest possible DIC levels, ranging from atmospheric conditions to 1.2 M total DIC in its native alkaline lake habitats (Vonshak and Tomaselli 2000). In evolving this strong alkaline tolerance (growth at pH 11.5 can occur), *A. maxima* shed the genes for carbonic anhydrases involved in carbon-concentrating mechanisms (Hillier et al. 2006). It has adapted a specificity for (bi)carbonate uptake rather than carbon dioxide, utilizing bicarbonate transporters to control its distribution in cells and store energy as ion gradient. *A. maxima* is widely used for biomass production, as it grows quickly, reaching yields up to 3 g/L in open ponds, can be grown on flue gas-enriched carbonate media, exhibits robust stress tolerance, and the pH/medium is toxic to many other parasites and fungi (Carrieri et al. 2007). It is a unique prokaryotic photoautotroph for biomass production that is mainly protein and glycogen. It has low lipid production below 8%.

Abstract

The alkalinophilic cyanobacterium Arthrospira maxima is unique among phototrophs for its growth conditions- it lacks carbonic anhydrase but can grow in more than 1M dissolved inorganic carbob (DIC). Herein we investigate the consequences of DIC depletion from whole cells of A. maxima, by measurements of photosystem II (PSII) using oximetric and fluorometric techniques. We developed an improved method for complete DIC depletion using a bicarbonate chelator and Mg$^{2+}$ to shift the equilibrium to CO$_2$ for removal. The method inhibits 99% of O$_2$ evolution and is reversible. DIC depletion was found to influence three reaction sites near the water-oxidizing complex (WOC), the plastoquinone electron carriers $Q_A$Fe(II)$Q_B$, and a new site that abolishes PSII primary charge separation (P$^+$Q$^-_A$- formation) as seen by loss of chlorophyll variable fluorescence yield. Light + NaHCO$_3$ are both necessary for restoration of normal function, consistent with energy-dependent transport of bicarbonate by A. maxima cells. The role of DIC near the WOC appears to involve free energy stabilization of S-states and kinetics of the WOC cycle.

* Available on request, 7 figures and 2 schemes
Scheme 1. Current map of HCO$_3^-$ binding sites within PSII and their involvement in electron and proton transfer steps, cofactor electrochemical potentials and photoassembly of the WOC. Acceptor-side reactions: (A) and (B), donor-side reactions: (C) and (D), and arginine-mediated (bi)carbonate binding and exchange with small anions (X$^-$) distributed throughout PSII: (E).

4.2.3 Physiological characterization of a *Chlorella* species mutant produced by gamma irradiation.

Hongxiang Lu (Zhejiang Univ.),
Gennady Ananyev (Rutgers)
Random mutagenesis of Chlorella sp. produces a growth gain*

*from Zhejiang University collaborators.

Chlorella sp. KMMCC C185 was mutated by $^{60}$Co gamma irradiation, single colonies were isolated from agar plates. Selection was done by growth rate in liquid media and by stationary biomass yield.

1. A hypothesis was proved by our experiment that the photosynthetic oxygen evolution rate of Chlorella mutant was higher than the wild type (oxygen evolution rate can reflect how fast an organism can split water into oxygen and its capability to utilize carbon dioxide).

2. Fv/Fm value can reflect the photosynthetic potential in some certain parts of photosynthesis, such as the PSII primary charge separation efficiency. Relationship between average Fv/Fm value and growth rate in microalgae was explored in the Chlorella wild type and mutant. Similar correlation between Fv/Fm and growth rate is repeatedly observed in both strains under various growth conditions.
Faster O2 evolution & CO₂ fixation rates for *Chlorella* mutant MS700 vs. wild strain

O₂ evolution rate of *Chlorella* wild type and mutant MS700 cells bubbled with 5% CO₂ gas. When normalized to dried biomass weight, *Chlorella* mutant MS700 produced O₂ at faster rate than the wild type, 13.7% and 23.6% higher O₂ evolution rate on day 8 and day 10, respectively.

PSII charge separation yield (Fv/Fm) using CO₂ as terminal electron acceptor is 25% greater in the MS700 mutant vs. wild strain.

**Black** and **Green**: Fv/Fm of wild and mutant strains, respectively, after DIC depletion.

**Red** and **Blue**: + 50mM NaHCO₃.
Conclusions

• **Chlorella mutant strain MS700**: isolated from gamma irradiated mutagenesis screening, this mutant exhibits faster growth rate, faster O\textsubscript{2} evolution rate and faster CO\textsubscript{2} fixation rate.
5.1_Optimization of Lipid Extraction Protocols for *N. oceanica*

Jonah, Yuan

December 2016

**Outlines:**
1. Background
2. Progress
3. Challenges
4. Future Potential
Brief Introduction:

- **Overall GCEP Goal**: screen and categorize mutant strains of Nannochloropsis oceanica to look for a) increased lipid productivity and b) uncompromised (ability) growth at high CO\textsubscript{2} levels
  - **Rational**: Algae placed near powerplants, where flue gas fed directly into growth apparatus to mitigate CO\textsubscript{2} release while producing bioproducts
  - **Desire**: Mutant strains able to produce more lipids (biofuel) and sequester/utilize more carbon

- Targeted and Random Mutants generated via Christoph Benning’s group at MSU
  - Mutant screening procedures developed by fellow GCEP members
Downstream Lipidome Analysis:

• Major goal of project is to assess biofuel potential in a variety of mutant strains
  • Triacylglycerides (TAGs) are the main component used in lipid-based biodiesel

• **Thus, we sought to develop a lipid-extraction protocol for our lab to use in this investigation**

• Extracting lipids is useful for numerous downstream analyses:
  
  • **Problem:** Poor literature available, not specific enough, and myriad of possible procedures
  
  • **Solution:** Procedure optimization based on materials, organism, and cost of extraction
Literature Review:

- Surveyed 15+ literature articles (2009-present) in top journals (Bioresource Tech., Science, Algal Research) for extraction protocols

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</thead>
<tbody>
<tr>
<td>1. Culture Used</td>
<td>Nannochloropsis sp. and Chlorella sp. (Used in methanol, acetonitrile)</td>
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<td>Nannochloropsis sp. and Chlorella sp. (Used in methanol, acetonitrile)</td>
</tr>
<tr>
<td>2. Lysis and Pelleting</td>
<td>10 μL sample added into 100 μL of 100 mM phosphate buffer (pH 7.4)</td>
<td>10 μL sample added into 100 μL of 100 mM phosphate buffer (pH 7.4)</td>
<td>10 μL sample added into 100 μL of 100 mM phosphate buffer (pH 7.4)</td>
<td>10 μL sample added into 100 μL of 100 mM phosphate buffer (pH 7.4)</td>
<td>10 μL sample added into 100 μL of 100 mM phosphate buffer (pH 7.4)</td>
<td>10 μL sample added into 100 μL of 100 mM phosphate buffer (pH 7.4)</td>
<td>10 μL sample added into 100 μL of 100 mM phosphate buffer (pH 7.4)</td>
</tr>
<tr>
<td>3. Separation Process</td>
<td>1. Repeated pellets in 1 mL of 0.1M sodium acetate + 0.1M sodium phosphate buffer (pH 7.4)</td>
<td>1. Repeated pellets in 1 mL of 0.1M sodium acetate + 0.1M sodium phosphate buffer (pH 7.4)</td>
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</tr>
<tr>
<td>4. Decantation</td>
<td>1. Cells pellets were centrifuged for 5 min at 11000 x g</td>
<td>1. Cells pellets were centrifuged for 5 min at 11000 x g</td>
<td>1. Cells pellets were centrifuged for 5 min at 11000 x g</td>
<td>1. Cells pellets were centrifuged for 5 min at 11000 x g</td>
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<td>1. Cells pellets were centrifuged for 5 min at 11000 x g</td>
<td>1. Cells pellets were centrifuged for 5 min at 11000 x g</td>
</tr>
<tr>
<td>5. Collection</td>
<td>1. Cells pellets were washed by the following procedures: 3 x 5 min of centrifugation at 1000 x g</td>
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<td>1. Cells pellets were washed by the following procedures: 3 x 5 min of centrifugation at 1000 x g</td>
</tr>
<tr>
<td>6. Quantification</td>
<td>1. GC-MS - 75 mg pellet sample was separated with 1 ml of 95% MeOH (v/v)</td>
<td>1. GC-MS - 75 mg pellet sample was separated with 1 ml of 95% MeOH (v/v)</td>
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<td>1. GC-MS - 75 mg pellet sample was separated with 1 ml of 95% MeOH (v/v)</td>
</tr>
</tbody>
</table>
Main Components of Extraction:

• **Liquid Extraction**: Bligh and Dyer (1959) and Folich (1957) most used procedure with methanol:chloroform (2:1 v/v) as solvent mixture
  - Chose this method due to widespread success and use
  - 9th most cited paper of all time (45,131 citations)\(^1\)
  - Governing principle is polarity of solvents and standard phase extraction

• Mode of Biomass Processing:
  - Source: Lyophilized, *wet*, or oven dried
  - Method: Crushed, liquid N\(_2\) frozen, *mortar and pestle*

• Drying:
  - Centrivamp, inert gas purge (Ar, N\(_2\)), or air dry

Initial Considerations:

• Liu et al. (2013) describe a method for *N. oceanica* lipid analysis that was adopted in first round testing based on Folich’s (1957) method\(^2\)
  - Used 10-20 mL pelleted wet biomass, methanol:chloroform:formic acid (2:1:0.1 v/v/v), KCl phase separator

• **Result:** too much variability in lipid extracts, due to low starting biomass
  - Biomass weight: ~ 20 mg
  - Dry Lipids: ~ range of 2 mg
  - Too much room for gravimetric error

• After consultation with Hongxiang, upped biomass to 100mg per reaction and decided to dry biomass and grind with mortar and pestle
  - Jun Cheng’s “method”\(^3\)

---

Initial Results:
- *N. oceanica* has ridged cell wall comprised of algaenan so perhaps sonication is necessary?
  - **Observation:** sonication prior to extraction results in no yield increase

**Lipid Wash:** further inspection of literature revealed washing lipids with low salt buffer (another phase extraction) prior to drying can increase downstream purity
  - Removes protein, acid, and residual debris

- Kodner et al. (2009) purified and categorized algaenan but first removed lipids with Folich’s method – disrupt/permeate the membrane?
  - Other have done similar carbohydrate assays
Current Method:
• Final SOP is now on version 10 and was subsequently modified
• Follows general scheme; two day procedure, ~4 hrs total, scalable

Day 1)

**Biomass**

- Spin down and wash to remove residual salts
- Dry Overnight

Day 2)

- Grind dry biomass for 3-5 min in precooled mortar

**MeOH:**CHCl\(_3\):FA (2:1:0.1 v/v/v)

- Vortex and Incubate
- **Extraction**
- **Salt Buffer and Spin**
- **Phase Separation**
- **Organic Separation and wash**
- **Washed Lipids & Residual**
- Transfer and Drying
- Argon purge to dry
- Storage in dark at -20 C under Ar

Mass Samples
Extraction Accuracy Between Technical Replicates:

- Method was tested for potential through error between technical replicates in batch processing
  - Observed very reproducible replicates; low standard error of about 0.5%

<table>
<thead>
<tr>
<th>Characteristics of the Noc WT culture for lipid extraction</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD750 (Neo 2)</td>
<td>0.555</td>
</tr>
<tr>
<td>Volume</td>
<td>100 ml</td>
</tr>
<tr>
<td>Chl. a Concentration</td>
<td>3.22 μg/ml</td>
</tr>
<tr>
<td>Dry Biomass (avg.)</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Dry Lipid (avg.)</td>
<td>0.0268 g</td>
</tr>
<tr>
<td>Lipid content % Dry Biomass</td>
<td>26.79%</td>
</tr>
<tr>
<td>Chl. a content % Dry Biomass</td>
<td>0.31%</td>
</tr>
<tr>
<td>Chl. a content % Dry Lipid</td>
<td>1.15%</td>
</tr>
</tbody>
</table>
Initial Application to *N. Oceanica* in Absence of Nitrogen:

- Previous studies have shown that *Nannochloropsis* *sp.* can accumulate significant amount of total lipid content under nitrogen stress\(^4\):
  - The organism diverts efforts to energy storage (lipid biosynthesis) as opposed to replication and protein synthesis – in anticipation for return of nitrogen

<table>
<thead>
<tr>
<th>Sample</th>
<th>OD 750</th>
<th>Nile Red/OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR 1</td>
<td>1.066</td>
<td>876.6417</td>
</tr>
<tr>
<td>NR 2</td>
<td>1.038</td>
<td>955.2023</td>
</tr>
<tr>
<td>ND 1</td>
<td>1.008</td>
<td>4523.313</td>
</tr>
<tr>
<td>ND 2</td>
<td>0.971</td>
<td>4304.84</td>
</tr>
</tbody>
</table>

- High Nile Red/OD reflected in elevated total lipids observed in the nitrogen deplete culture
Preliminary Analysis of Targeted Mutants:

- Scaled-up three “robust” lipid producers as categorized previously to analyze total lipid yields.
- In interest of time, cultures harvested too young (early log phase), so no substantial differences in total lipid yields observed.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lipid Yield/Dry Biomass %</th>
<th>OD 750</th>
<th>Nile Red/OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>20.90</td>
<td>0.71</td>
<td>582</td>
</tr>
<tr>
<td>CISi</td>
<td>21.99</td>
<td>0.539</td>
<td>812</td>
</tr>
<tr>
<td>G2</td>
<td>19.93</td>
<td>0.557</td>
<td>540</td>
</tr>
<tr>
<td>G+C1</td>
<td>21.10</td>
<td>0.545</td>
<td>627</td>
</tr>
</tbody>
</table>

Growth: 16:8 hr. diurnal, 2% CO2, 40 uE, 22 C

- Nile red/OD for cultures reflects relative lipid yields – good for primary screening.
Conclusions:

• **Overall:** method is reproducible, easy, and reliable – could tweak if needed in future

• **Note:** as Laurens et al. (2012) note, the wide array of extraction methods impact the results collected on similar organisms – thus it is hard to compare results to other research groups
  
  • However, useful for internal comparisons (exactness of growth conditions and standards is imperative)
  
  • Compare total lipid yields of mutants at a plethora of conditions (elevated CO₂)

• **Goal:** bridge to more exact profiling, namely GC-MS
  
  • What types of fats do our transgenic algae produce?
  
  • What is the TAG composition/profile? (Biofuel!!)
  
  • Does this composition change with stress and CO₂ composition?
  
  • Better understanding of lipid biosynthesis pathways in *Nannochloropsis*
  
  • Joe Dixon – collaboration?

• *Please see me if you’d like a demo or to learn more!*

5.2 Optimization and standardization of lipid quantification using Nile Red

Yunbing, KT, Yuan, Hoa
November 2016
Motivation

• A rapid, accurate method are mandatory to screen for high lipid producing algal strains
• Primary problems with existing Nile Red method
• Dye aggregates might be the main factor contributes to the problems in existing Nile Red method
• A more reliable method is proposed
Ideal quantification assay criteria

1. High signal to noise ratio (low background, high signal)
2. Small variations among identical samples and blank.
3. Stable signal and small decay over period of time (kinetics)
4. Linear correlation between Nile Red signal and lipid content
5. Linear correlation between Nile Red signal and cell culture density
6. Wide linearity that satisfies daily uses.
7. High throughput
Primary problems with existing Nile Red method

1. Variations in blank
   In this method, huge variations are always observed when measuring aliquots from identical blank samples. Samples were measured for 10min, 15min, 30min.
   The middle horizontal line indicates where the average for culture samples.

2. High signal from background albeit high signal from samples
   In the method proposed by BioTek company, the variations in blank is acceptable. However, other issues exist: background is 1/3 to 1/2 of real sample signal. An example of the raw data from BioTek is

   | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 |
---|----|----|----|----|----|----|----|----|----|----|----|----|
| 3287 | 2652 | 2788 | 2610 | 2089 | 2782 | 2356 | 1474 | 1411 | 1014 | 847 | 680 |
| 3382 | 2865 | 2897 | 2581 | 2327 | 2076 | 1880 | 1589 | 1476 | 1064 | 953 | 667 |
| 3178 | 2900 | 3291 | 2468 | 2035 | 1987 | 1942 | 1512 | 1563 | 953 | 872 | 691 |
| 3125 | 2755 | 2838 | 2521 | 2346 | 1957 | 1723 | 1489 | 1206 | 1065 | 862 | 766 |

Figure 4. Fluorescence of Nile red stained C. vulgaris cell dilutions.
Where the high background, high variation come from?

Left: flow cytometry revealed the presence of two types of noncellular events:
• cell debris
• aggregated dye precipitate

Right: Microscope confirmed the presence of dye aggregates.
Optimized method proposed in Cirulis et al., 2012

- **Stock**: 1mg/mL Nile Red in Acetone, -20°C dark
- **Working solution**: 4 μg/mL of Nile Red in 2% acetone, 1:1 mix with sample, dark incubation for 30 min
- **Final concentration**: 2ug/mL in 1% acetone
- **Excellent uniformity of blank solution** (cooking oil is used as a positive control)
Correlation between
NR signal (4ug/mL) and lipid content in milk

Correlation curve between the residual fat in non-fat milk and Nile red (4 μg/mL of Nile Red 4% acetone) shows a good correlation.

The rational of using milk as a choice of our standard reagent (positive control) is based on:
1. Milk fat is primarily triglycerides (98%)
2. Amphiphilic nature of fat composition in milk. Readily dissolvable in reagents such as culture medium (unlike cooking oil or other TAGs).
3. Shares the same solvent as samples and blank.
4. Can make standard curve by using whole milk with known fat content.
5. Nile red tested with BSA yielded minimal reading.
Correlation between NR signal (4µg/mL) and culture OD

A good correlation in general.

However the highest linear correlation is only observed within 0-0.4OD culture (insert)

The saturation curve suggests that the Nile Red is saturated when cell reaches higher density. Therefore, higher Nile Red concentrations are explored.

Secondly, based on this data set recorded every 30 minutes over a 90 minutes period, the signal is quite stable. It does not decay while might increase a little, possibly due to 1) increased dye penetration into the cell; 2) prior repeated laser excitation during kinetics record.

The correlation figure from Biotek publication is listed here as a reference.
Correlation between NR signal (8µg/mL) and culture OD

- A set of 8 µg/mL of Nile Red in 4% acetone solutions were tested
- All show a good correlation in a wider range (OD: 0-0.8).
- Among these, the one prediluted in 200µg/mL acetone showed the best correlation and smallest blank variation.
Saturation curve of Nile Red (8ug/mL) Fluorescence using whole fat milk

Nile Red reaction is tested for saturation using whole milk containing 25% fat using 8ug/mL Nile Red in 4% acetone. Final concentration is 4ug/mL in 2% acetone.

A serial milk dilution is prepared ranging from 0.05g/mL to 0.00004 g/mL in A+ medium. Data is plotted and fits the sigmoidal curve.

Fat (ug) = milk concentration (g/mL) \times 15.625\% \times 200\mu L / 400\mu L \times 100\mu L \times 10^3
= 7.8125 \times 10^3 \times \text{milk concentration (g/mL)}
Linear correlation between milk fat and Nile Red falls between 0-100ug/0-7000 (a.u.)

Best linear correlation between milk fat and Nile Red falls between 0-8ug/0-900 (a.u.) with $R^2 = 0.996$.

A good correlation ($R^2 = 0.973$) is also seen in the range of 0-50ug fat/0-6000 (a.u.) which totally satisfies our needs when using Nanno culture for Nile Red measurement. Typically when staining nanno culture using Nile Red, the fluorescence signal is in the range below 6000 au. For dense culture (OD>1.0) or nile red signal over 7000, dilution is needed to ensure assay linearity.
Correlation between NR signal (8μg/mL) and culture OD

With this selected concentration and specific preparation approach, 8 μg/mL of Nile Red in 4% acetone shows a good correlation in a much wider range (0-1.4), and better slope.
Kinetics of Nile Red (8ug/mL) Fluorescence

Nile Red reaction kinetics was measured over a period of 70 minutes.

Protocol:

0-5 min: same samples measured repeatedly every 1 minute for 5 minutes
6-11min: 2nd batch of same samples measured repeatedly every 1 minute for 5 minutes
12-17min: 3rd batch of same samples measured repeatedly every 1 minute for 5 minutes
18-33min: 4th batch of same samples measured repeatedly every 5 minute for 15 minutes
34-64 min: 5th batch of same samples measured repeatedly every 10 minute for 30 minutes.

Conclusion

1. All readings are generally at the right position (blank very low, and milk very high)
2. For milk or culture, NR signal reaches maximum immediately (consistent with results in Biotek publication).
3. Some fluctuations observed during initial 20 minute incubation. After 30 minutes, the signal tends to become stable and does not appear to decay substantially.
Protocol

• Nile red stock solution is made in 200ug/mL in pure acetone, in glass bottle, foil covered and store in 4C. Replenish every 3 months.

• Dilute 200ug/mL stock solution to 8ug/mL in dH$_2$O. (100uL+2.4mL, use fresh dH$_2$O every time).

• Make 0.01g/mL non-fat milk in A+.

• Wait for 30 minutes letting NR reach equilibrium before start mixing NR and samples (200uL sample +200uL NR). Vortex sample before add.

• Vortex again after mix. Incubate reaction in dark for 30min.

• Vortex again then add 100uL reaction into each well (one sample=3 triplicated well) and read.
Conclusions

• With newly developed method, the issues with blank reading (big variation and high background) was solved.

• Newly developed method showed good correlation between Nile Red fluorescence intensity and cell culture OD, as well as milk fat content. The correlation showed both good $R^2$ and a wide OD range.

• For dense culture (OD>1.0) or Nile Red signal over 7000, dilution is needed to ensure reading falls within assay linearity range.

• Nile Red fluorescence kinetics was measured and is consistent with Biotek publication. Sample signal reaches maximum within a few hundred seconds. Some fluctuations seen initially but stabilizes after 30 minutes. Signal does not decay significantly over period time.

• Overall, Nile Red is a promising lipid dye as long as optimized method is established.
Overall

Two different approaches were taken for lipid quantification. One is the traditional lipid extraction followed by weighing. The other is the more recent, micro-scale Nile red based fluorometry method.

Overall, the gravimetric method is quite reliable. However it doesn’t work very well for quantifying small amount of algal culture (<= 10 ml). GC-FID or GC-MS is necessary for more accurate quantification of total lipid or TAG content.

The Nile Red based method is optimized and standardized, and is suitable for rapid and high throughput screening. Further correlation between results generated using Nile red method and gravimetric method needs to be established.