Worden and Begley Lab Protocol

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Quantifying Thiamin Cellular Quotas in Algae

We use the described methods for determining the internal cellular quota of thiamin and its different phosphorylation states in marine algae, in the below case for the haptophyte *Emiliania huxleyi*. The chemical assay used – the thiochrome assay - was developed in the 1930s but has been modified over the decades to include instrumentation with improved detection (Backstrom et al. 1995, Reddick et al. 2001). The assay is based on cyclization/oxidation of thiamin to thiochrome under basic conditions in the presence of an oxidizing agent such as potassium ferricyanide. Thiochrome is intensely fluorescent and easy to detect and quantify with high sensitivity in an HPLC assay. This method has previously been used to determine cellular quotas of thiamin and its different phosphorylation states in algae from brackish waters (Pinto et al. 2002, Sylvander et al. 2013).

Culture Preparation and Growth

Important note: regularly check the axenicity of the cultures using the DNA stain 4,6-diamidino-2-phenylindole (DAPI) and epifluorescence microscopy. Use dedicated media bottles for each thiamin concentration to avoid carry-over of thiamin between media batches.

1. Maintain cultures of CCMP2090 *Emiliania huxleyi* in mid-exponential growth for ≥10 generations using semi-continuous batch culture techniques. Grow cultures using artificial seawater based medium L1-Si (Guillard 1975).

2. Transfer cells daily (replete vitamin amendment conditions) or every second day (limited vitamin amendment conditions) to not exceed 1 x 10^6 cells mL^{-1}. Quantify the cell concentrations on a flow cytometer (e.g., Accuri C6 cytometer BD Biosciences, USA).

3. Select the vitamin amendment conditions for the experiment and set up triplicate cultures for each treatment. For luxury treatment, supply thiamin at 300 nmol L^{-1} (standard f/2 medium concentration). For replete conditions supply thiamin and HMP at 10 nmol L^{-1}. For limiting conditions supply thiamin at 500 pmol L^{-1} and HMP at 100 pmol L^{-1}.

4. Maintain cells in mid-exponential growth for ≥10 generations using semi-continuous batch culture techniques. Quantify the cell concentrations on a flow cytometer.

Cell Washing and Concentration

Important note: Work on ice and have the centrifuge pre-cooled to 4°C. Resuspend the cells gently between washing steps.

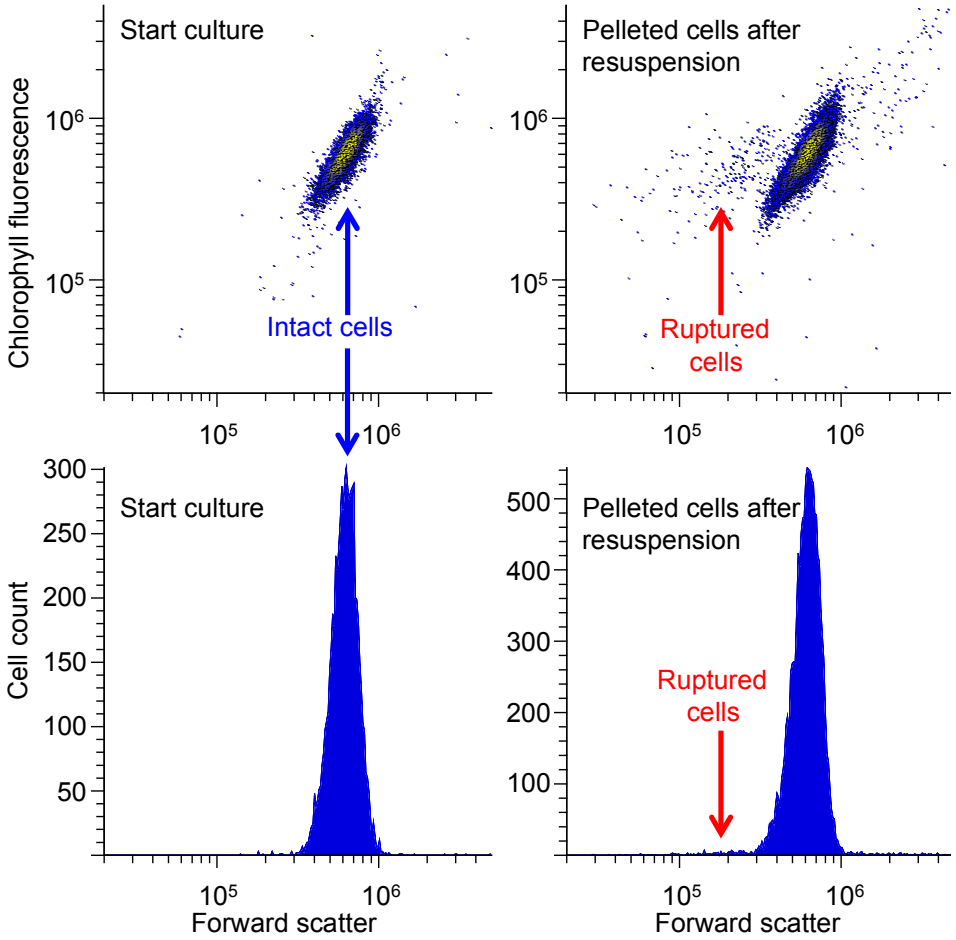
1. Remove vitamin amendments from media by using centrifugation wash steps with thiamin-free medium. Centrifuge 100 mL of culture at 4000 x *g* for 10 min at 4°C. Remove the supernatant, add 50 mL of thiamin-free medium to wash cells and repeat (wash, centrifugation etc.) three times.

2. After the last wash step, resuspend the pellet in 1.5 mL of thiamin-free medium and quantify the cell concentration on a flow cytometer.

3. Generate a final cell pellet. Centrifuge at 10,000 x *g* for 30 min at 4^{o}C. Collect the supernatant. Remove as much of the supernatant as possible and retain in a separate tube. Flash-freeze the pellet in liquid nitrogen prior to storage at -80^{o}C.

4. Combine the removed supernatant aliquots and quantify the cell concentration in the supernatant on a flow cytometer. Subtract these numbers (in the supernatant) from the total cell numbers determined prior to the final centrifugation step. This will give you the final cell count in the cell pellet.

5. Monitor the chlorophyll fluorescence and FALS distribution on the flow cytometric histograms to make sure cells have remained intact during the washing and centrifugation steps. See Figure below for representative cytograms that depict an intact cell population from a start culture, versus a cell population that has been resuspended from a pellet. A low (<1%) number of ruptured cells is evident – and these cells and released thiamin would be removed with removal of the final supernatant (after final spin) – so it is unlikely the influence quotas.

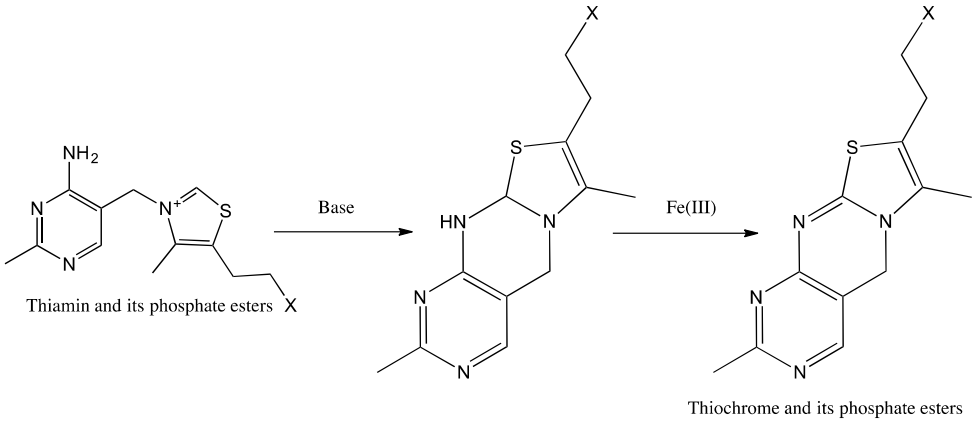


Determination of cellular thiamin quota using the thiochrome assay

Pellet extraction

Important note: The thiochrome assay is based on the facile and efficient cyclization/oxidation of thiamin to thiochrome under basic conditions in the presence of an oxidizing agent such as potassium ferricyanide (see schematic below).

1. Suspend pellet in 100 µL of 7% $HClO_4$ and sonicate for 2 min
2. Add 50 µL 4 M CH_3CO_2K and 50 µL 30 mg mL^{-1} $K_3[Fe(CN)_6]$ in 7 M NaOH. Pipette to mix. Leave 1 min.
3. Immediately neutralize the reaction with 6 M aqueous HCl.
4. Centrifuge the sample and collect the supernatant for further analysis.



Reverse phase HPLC

1. Purify the deionized water that is use for reagents with active charcoal and filter.
2. Use a reverse phase HPLC (Agilent 1200 series) with fluorescence detection. Set the detector at an excitation of 365 nm and emission of 444 nm.
3. Prepare a Supelcosil SPLC-18-DB (25 cm x 10 mm, 5 µm) column with a gradient of the following compounds. A) H_20, B) K_2HPO_4 (pH 6.6), C) CH_3OH. Set the gradient to 0 min, 100% B; 5 min, 100% B; 14 min, 7% A/70% B/23% C; 25 min, 25% A/75% C; 28 min to 34 min, 100% B.
4. Construct a calibration curve with known concentrations of thiamin pyrophosphate (TPP), thiamin monophosphate (TMP), and free thiamin (ThF). Integrate the fluorescence signal peak area.

Quantification of thiamin quota

1. Quantify ThF, TMP and TPP concentrations in the samples by plotting the fluorescence signal peak area on the HPLC chromatogram against the respective standard curve.

References

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