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# DISCLAIMER

Using YFP as a reporter of gene expression in the green alga *Chlamydomonas reinhardtii*

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**ABSTRACT**

The unicellular green alga *Chlamydomonas reinhardtii* is a valuable experimental system in plant biology for studying metal homeostasis. Analyzing transcriptional regulation with promoter-fusion constructs in *C. reinhardtii* is a powerful method for connecting metal-responsive regulation with *cis*-regulatory elements, but overcoming expression-level variability between transformants and optimizing experimental conditions can be laborious. Here, we provide detailed protocols for the high-throughput cultivation of *C. reinhardtii* and assaying Venus fluorescence as a reporter for promoter activity. We also describe procedural considerations for relating metal supply to transcriptional activity.

## 1. Introduction

Originally isolated from the jellyfish *Aequorea victoria* ***(1, 2)***, green fluorescent protein (GFP) was quickly adopted as a reporter in multiple organisms including bacteria ***(3, 4)***, plants ***(5, 6)***, and animals ***(3, 7)***. The widespread use of GFP and its variants (fluorescent proteins (FPs) engineered to have different physical and spectral properties ***(8)***) is due in large part to their ease of use. FPs derived from GFP form their chromophore autocatalically ***(3)***; detection of fluorescence *in vivo* does not require the addition of a substrate and does not necessarily require sample processing. The most common applications of FPs include localization of FP-protein fusions *in vivo* and gene expression studies of promoter-*FP* fusions. In the first case, the FP serves as a fluorescent label for proteins of interest; in the second case, the magnitude of fluorescence is a proxy for promoter activity (Figure 1).

Here, we describe the use of promoter-*YFP* fusions to profile promoter activity in response to metal availability in the green alga *Chlamydomonas reinhardtii*. YFP (Yellow Fluorescent Protein) is a general term used to refer to variants of GFP that carry amino acid substitutions that shift the protein to a yellowish emission ***(8, 9)***. This protocol specifically employs Venus, an engineered YFP resulting from several amino acid substitutions that provide increased fluorescence intensity, faster maturation, and relative tolerance to pH and chloride ions as compared to its predecessor (EYFP, enhanced yellow fluorescent protein) ***(10)***. The exploitation of FPs, specifically YFP, in *C. reinhardtii* has been facilitated in recent years by the availability of constructs and strains developed to overcome inherently poor protein expression associated with non-native genes expressed from the nuclear genome ***(11)***. The robustness of FP-based systems in *C. reinhardtii* (and thus the ability to use these reporter systems with promoters of lowly expressed genes) continues to improve ***(12-14)***.

The YFP reporter protocol described here has several attractive qualities. Individual strains are arrayed in 96-well format, and fluorescence is measured on a plate reader, enabling multiple independent transformants to be assayed simultaneously (see additional information about replicates in section 3.2). This is an important consideration for any reporter system in *C. reinhardtii*, as introduced DNA predominately recombines illegitimately into the nuclear genome, and the expression-level of the promoter

fusion varies between transformants in part because of the specific site of integration in each case ***(15-17)***. Therefore, multiple independent transformants are essential for assessing the significance of variation in promoter fusion activity between conditions or between wild-type and mutated promoter regions. Samples are assayed for YFP activity without upstream sample processing. As a result, assay time equates to the speed of a plate reader, which is typically less than 5 min per plate. Because there is no need for sample processing, fluorescence of whole cultures grown in the presence and absence of stimuli can be measured over the course of the growth curve without removing cells. By using bottom-read fluorescence, which does not require removal of the plate lid, cultures are more easily maintained free of contamination. Because of these attributes, optimizing the growth conditions and optimal point in the growth curve to measure YFP fluorescence for a subsequent mutational analysis (i.e. largest difference between WT promoter-*YFP* fluorescence from test and control conditions, see Figure 3C for an example) is straightforward and relatively simple.

Since *C. reinhardtii* is a valuable single-celled reference organism for understanding metal homeostasis in the plant lineage ***(18-20)***, we also provide details for growing cultures with controlled metal nutrition. Because many metal ions are nutrients and potential toxins, plants must fine-tune morphological, physiological, and molecular responses to meet the catalytic demand for these elements while avoiding toxicity. How the cell achieves this balance through gene regulation is an active field of investigation. For studies of metal-responsive gene regulation, *C. reinhardtii* is an advantageous experimental system for several reasons ***(21, 22)***, including: (1) metal supply can be tightly controlled without the need to resort to using metal chelators, aiding reproducibility and avoiding mis-interpretation of results due to the non-specific nature of chelators, (2) the ability to distinguish between absence of a metal and presence of the metal but with reduced accessibility (a distinction that is missing in many papers in the literature), and (3) availability of transcriptomic datasets and well characterized markers of iron, zinc and copper deficiency ***(23-25)***. To identify *cis*-acting regulatory sequences and the *trans*-acting metalloregulators in *C. reinhardtii*, we detail several considerations that should be made when correlating promoter activity with metal availability.

## 2. Materials, equipment and experimental considerations

1. *Chlamydomonas reinhardtii* str. UVM11; this strain has higher YFP expression from the nuclear genome than typical laboratory strains ***(11)***.
2. DNA for transformation: 1) pJR39GW containing the promoter fusion construct to be analyzed (referred to in the protocol as the "test promoter"), 2) pJR39GW (or other appropriate negative control), and 3) pJR39GW containing a promoter fragment that can be used as a positive control, such as the *FOX1* promoter ***(26)***, *ZRT1* promoter ***(23)*** or *CYC6* promoter ***(27)*** as a positive control for iron-, zinc- or copper-responsive *YFP* expression, respectively. pJR39GW is a Gateway-adapted version of pJR39 ***(11)*** and was constructed by replacing the *PsaD* promoter of pJR39 with a Gateway cassette. pJR39GW contains *aphVIII*, which encodes resistance to paromomycin for selection of *C. reinhardtii* transformants, *bla* for selection of *E. coli* transformants with ampicillin, and the Venus variant of YFP as the reporter protein. See (XXCITEXX Blaby and Blaby-Haas) in this series for details regarding the cloning of a promoter fragment into Gateway-adapted vectors.
3. Growth conditions for *C. reinhardtii*: Aseptic technique and clean, organized working practices should be applied at all stages. These practices are important to avoid contamination of cultures with both unwanted organisms and metals ***(28)***. *C. reinhardtii* and sterile media should be handled in a laminar flow hood with pre-sterilized culture plates, pipette tips, and pipettor (the barrel can be sprayed with 70% ethanol). Standard growth conditions are 23°C, ~80 µmol $m^{-2}$ $s^{-1}$ with a ratio of 1 warm fluorescent light bulb (3,000K) to 2 cool fluorescent light bulbs (4,100K) and rotational shaking at 180 rpm, e.g. in Innova 44 incubators (Innova, New Brunswick Scientific, Edison, NJ), for liquid cultures, or stationary in growth chambers (Percival CU-36L6 or similar) for petri dishes and 96-well plates.
4. *C. reinhardtii* "metal-free" growth medium (as described in Quinn and Merchant ***(28)***): Tris-acetate phosphate (TAP) medium ***(29)*** with high purity (>99.9% trace metals basis) chemicals (available from Sigma-Aldrich, ACROS organics or AlfaAesar; certificates of analysis are typically available before purchase of chemicals and can be used to estimate whether concentrations of contaminating metals in the prepared medium would be acceptable) and milliQ-$H_2O$: each liter of TAP contains 10mL TAP salts solution (15g $NH_4Cl$, 4g $MgSO_4 \bullet 7H_2O$, 2g $CaCl_2 \bullet 2H_2O$, final volume

of 1L milliQ-grade $H_2O$; to avoid precipitation dissolve the $CaCl_2$ in 300mL milliQ-grade $H_2O$ and the $NH_4Cl$ and $MgSO_4$ in 500mL milliQ-grade $H_2O$; then mix the two solutions together and bring up to the final volume), 8.3mL phosphate solution (18.5g $K_2HPO_4$ and KOH to pH of 7.1 (about 28mL of 20% KOH), final volume 1L), 10mL Tris-acetate solution (242g Tris, 100mL glacial acetic acid, final volume of 1L milliQ-grade $H_2O$) and 1mL each component of high-purity Kropat trace metal solutions ***(30)***. Media stock solutions (TAP salts, phosphate and Tris-acetate solutions) and Kropat trace metal solutions are stored in metal-free plastic (to prevent metal contamination of solutions in the laboratory, storage containers for metal stock solutions should not be reused to make other solutions or washed with general-use laboratory glassware). Dedicated "metal-free" plastic graduated cylinders should be used. To make appropriate metal-deficient or –free medium, reduce or leave out, respectively, the necessary metal stock solution. When making TAP, use disposable plastic serological pipette tips and make media in freshly acid-washed glassware (rinsed 3 times with 6N HCl followed by 7 rinses with milliQ-grade $H_2O$, the glassware should be used within two days or re-acid washed). Media should be made no earlier than the day before (or morning of) 96-well plates are to be filled to prevent leaching of metals from the glass/plastic into the media.

5. Select agar (Thermo Fisher 30391-023), washed three times in milliQ-grade $H_2O$, is used at 1.5% w/v for solid growth medium.
6. Agar-solidified TAP in deep-dish petri dishes (Fisher Scientific FB0875711) containing 5μg/mL paromomycin (Sigma P5057).
7. Light microscope (such as a ZEISS Primo Star; bright-field illumination is sufficient for counting *C. reinhardtii* cells).
8. Hemocytometer with cover slip (Sigma Z359629).
9. Low-adhesion microcentrifuge tubes, 2mL (USA Scientific 1420-2600), filled to the 250 – 300μL demarcation with glassbeads (particle size, 425-600μm, acid-washed; Sigma G8772), autoclaved.
10. Sterile 50mL and 15mL conical polypropylene centrifuge tubes (Thermo Scientific 339652 and 339650, respectively)
11. Centrifuge and rotors appropriate for centrifuging 50mL tubes (Thermo Scientific 339652) at 2000

x *g* and for 15mL tubes (Thermo Scientific 339650) at 1000 x *g*.

12. Vortex mixer.
13. Sterile 96-well plates: for master plates can use clear plates with clear lids (such as the Nunc™ Edge 96-Well Plate, which has a moat for humidity control, Thermo Scientific 267313); for culture plates used for fluorescence measurements use black with clear, flat bottoms and clear lids (Thermo Scientific 165305).
14. Cell culture roller drum (Fisher Scientific 14-277-2) or tube rotator.
15. Multi-channel pipette, 10 – 100µL and 30 – 300µL, and sterile pipette tips.
16. Pre-sterilized multichannel basins (Fisher Scientific 13-681-500).
17. Plate reader capable of taking measurements of $A_{750nm}$, and bottom-read fluorescence with Emission (Em): 514nm / Excitation (Ex): 530nm and Em:440nm/Ex:680nm (such as a Tecan M1000 Pro).

## 3. Methods

### 3.1. Bombardment by glass beads – see Figure 2 for a workflow schematic (please refer to Neupert, et al. *(31)* for additional details regarding transformation and Kindle *(32)* for original protocol)

1) Using PsiI (or any restriction enzyme that cuts the plasmid outside of the promoter-*YFP* fusion and *aphVIII* region) linearize the negative control, positive control and test promoter plasmids.
2) Grow a 300mL liquid culture of UVM11 under standard growth conditions to a density of $1x10^6$ cells $mL^{-1}$. Cell density is determined using a light microscope and a hemocytometer ***(33)***.
3) Transfer 50mL of culture to sterile centrifuge tubes (eg 50mL Falcon tubes).
4) Collect cells by centrifugation at 2000 x *g* for 5min, room temperature. Pour off the supernatant.
5) Gently resuspend the pellet in sterile TAP by slowly pipetting up and down with a 1mL pipette tip to a final cell density of $4x10^8$ cells $mL^{-1}$.
6) Transfer 200µL aliquots of cells to a pre-autoclaved 2mL low adhesion tube containing glass beads and DNA (500ng linearized DNA in total volume of 5µL).
7) Vortex at highest setting for 15s.

8) The beads will settle quickly to the bottom of the tube. Gently collect the cells using a 100 – 1000µL single-channel pipette and sterile pipette tip. Transfer cells to a 15mL sterile centrifuge tube pre-loaded with 5mL TAP.

9) Allow the cells to recover by incubating on a benchtop roller drum, 1 x g (~50 – 60 rpm), for 8h, in low light (<40 µmol $m^{-2}$ $s^{-1}$). The purpose of this step is to allow the cells to recover and express the resistance marker before selection, and it is important that the cells do not undergo a round of cell division to ensure that each colony is from an independent integrant.

10) Collect the cells by centrifugation at 1000 x *g* for 5min, room temperature, and pour off the supernatant.

11) Gently resuspend the pellet by slowly pipetting up and down with a 1mL pipette tip in the remaining volume of TAP left in the tube after decanting.

12) Gently spread cells with an ethanol-sterilized glass spreader or a pre-sterilized plastic spreader onto agar-solidified TAP containing 5µg/ml paromomycin.

13) Incubate in growth chamber under standard growth conditions until colonies are visible (~5 – 7 days).

**3.2. YFP assay – see Figure 3A for a workflow schematic**

1) Using a multi-channel pipette with sterile tips, fill three 96-well plates with 200µL TAP per well. These are the master plates for growing cells transformed with the 1) test promoter, 2) the negative control, and 3) the positive control.

2) Using sterile pipette tips (we find 20 – 200µL size tips to be the most appropriate) array transformants by hand into a 96-well plate (use aseptic technique when handling, open only in laminar flow hood). To keep track of inoculation, leave tip in the well of the master plate until an entire row is inoculated. Then swirl tips to ensure transfer of cells from tip to medium and throw tips away. To avoid confusion between constructs and to have plenty of transformants for robust statistics (since YFP level from each transformant will vary due to positional effects) inoculate an entire 96-well plate with 96 individual colonies per construct and label plate appropriately. Since variability due to the site of integration is substantially greater than

technical variability (due to investigator handling, aspects of the protocol or plate reader), it is advantageous to have independent transformants rather than technical replicates (repeated measurements of the same sample); for further discussion on experimental replicates, the reader is encouraged to refer to Blainey, Krzywinski, and Altman ***(34)***.

3) Place plates in growth chamber and incubate under standard growth conditions as described above for 5 days.
4) Using a multi-channel pipette and sterile tips, fill three clear-bottom, black-sided 96-well plates with 200µL TAP per well and three clear-bottom, black-sided 96-well plates with 200µL TAP minus the metal of interest or TAP with a reduced concentration of the metal of interest per well. For the rest of the protocol, TAP with minus/reduced metal content will be referred to as "inducing medium".
5) Working with a single master plate at a time, start by resuspending the cells in row A by gently pipetting up and down with a multichannel pipette using sterile tips. Without removing tips from the multi-channel pipette, transfer 10µL from row A of the master plate to row A of a fresh 96-well plate (with black sides and clear bottoms to avoid fluorescent signal bleed-through between wells) containing TAP, then add 10µL from row A of the master plate to row A of a second plate containing inducing medium. Change tips to prevent cross-contamination of wells and repeat with row B, then C and so on. Once finished inoculating from the first master plate, start with the second master plate, followed by the third master plate.
6) Incubate the 96-well plates in a growth chamber under standard growth conditions. Care should be taken to ensure that plates receive the same light intensity, thus ensuring similar growth rates between plates.
7) After 24 hours measure the absorbance (750nm), chlorophyll fluorescence (Ex: 440nm, 9nm bandwidth; Em: 680nm, 20nm bandwidth) and YFP fluorescence (Ex: 514nm, 5nm bandwidth; Em: 530nm, 5nm bandwidth) of each plate. Subsequent to reading, place the plates back in the growth chamber.
8) Repeat measurements every 24h for 7 days. For fluorescence measurements, the gain should not be changed between measurements. We routinely use a gain of 50 for chlorophyll

fluorescence and 150 for YFP (using a Tecan M1000 PRO; for other plate readers these parameters should be determined empirically). Two 96-well plates, one filled with sterile TAP medium and one filled with sterile inducing medium should be used as blanks.

9) If the YFP fluorescence signal from the cells transformed with the test promoter in inducing medium is not significantly higher than from those same transformants grown in TAP medium after 4 days, perform a second round of metal depletion. The purpose of this step is to further deplete the cells of metal ions that had been transferred during inoculation from the master plate, as the carry over may be sufficient to suppress the activity of the metal-responsive promoter fragment (see section 3.3.3 for a note on positive controls). Using a multi-channel pipette and pre-sterilized filter tips, repeat steps 4 and 5, except that instead of inoculating fresh plates from the master plates, transfer ≤ 10µL from the 96-well plate containing transformants grown in inducing medium into fresh, sterile inducing medium, and transfer the same volume from the 96-well plate containing transformants grown in TAP into fresh TAP.

10) Repeat steps 7 and 8.

### 3.3. Data Analysis

1) **Normalization**: YFP fluorescence can be normalized to absorbance and/or chlorophyll fluorescence, which are used as proxies for cell density. Which normalization to use should be determined empirically by calculating the proportionality of absorbance and chlorophyll fluorescence as a function of cell number when cells are grown in TAP and inducing medium, i.e. make standard curves. If growth in inducing medium negatively impacts chlorophyll content (for example, cells grown with suboptimal iron nutrition are chlorotic compared to growth with sufficient iron nutrition ***(35)***) then the amount of YFP per cell will be overestimated when normalized to chlorophyll fluorescence. Cell size can also change in response to metal nutrition (for example, the average cell size is larger when *C. reinhardtii* is grown in the absence of zinc compared to the presence of zinc ***(23)***), which will affect the absorbance measurement ***(36)***. If both measurements are inappropriate for normalization between the two growth conditions, an option is to take a subset

of transformants (such as the 3 transformants that appear to have the highest inducible YFP activity) and quantify the cell number using a hemocytometer and microscope. However, this measurement will reduce the volume of the corresponding wells by 20µL and should only be done as an endpoint.

2) **Plotting data**: The normalized YFP fluorescence for each transformant from a single time point can be plotted for TAP and inducing medium as in Figure 2B. Additionally, YFP fluorescence for each transformant can be plotted as a fold-change between normalized YFP fluorescence measured from TAP and inducing medium. Time course data can also be plotted, but for ease of visualization (since 96 transformants in 2 growth conditions will generate 192 curves per construct) the normalized YFP fluorescence from 2 or 3 transformants grown in TAP and inducing medium can be plotted (Figure 2C).

3) **Statistics, controls and troubleshooting**: Possible sources of false negative and false positive results include expression-level variability due to genome position effects of the YFP-construct, background fluorescence (detection of fluorescence with Ex: 514nm / Em: 530nm not due to YFP), and data normalization. Therefore, there are several ways to analyze the data to provide confident conclusions about the activity of the test promoter.

    a. Is there a statistically significant difference between the normalized YFP fluorescence of test promoter transformants grown in TAP and inducing medium?

        1. Calculate the probability associated with a Student's *t*-test. The convention in most biological research is to use a significance level of 0.05.

    b. If the answer is **yes** to (a):

        1. Is the average fold-change for the test promoter significantly different from the average fold-change for the negative control?

            i. Calculate the fold-change in normalized YFP fluorescence between inducing medium and TAP for each transformant.

            ii. Calculate the probability that the average test-promoter fold-change is different from the average negative-control fold-change with a Student's *t*-test. The convention in most biological research is to use a significance

level of 0.05.

c. If the answer is **no** to (a):

1. Is the average fold-change for the positive control significantly different from the average fold-change for the negative control?

i. Calculate the fold-change in normalized YFP fluorescence between inducing medium and TAP for each transformant.

ii. Calculate the probability that the average positive-control fold-change is different from the average negative-control fold-change with a Student's *t*-test. The convention in most biological research is to use a significance level of 0.05.

iii. The normalized YFP fluorescence from the positive control transformants should be significantly different from the negative control. If not, then there was either contaminating metal in the inducing medium leading to repression of the promoter construct or absence of induction, or technical issues with the plate reader. Differentiating between technical issues due to the growth conditions or plate reader can be tested for by repeating this protocol with a constitutively expressed YFP construct (such as pJR39 ***(11)***). The resulting transformants should have statistically significantly more YFP fluorescence when grown in TAP as compared to the negative control.

**3.6. Notes**

1) Unfortunately, not all promoters have strong enough expression levels for detectable YFP fluorescence with this system. Recently, several codon-optimized YFP constructs have been published that may aid in studying these types of promoters ***(13, 14)***.

2) YFP has a relatively long half-life ***(37)***. Therefore, if time-resolved down-regulation of promoter activity is to be studied, the reader is pointed to the luciferase reporter gene system ***(38-40)***. Luciferase has a relatively short half-life (*Renilla reniformis* luciferase expressed in *C. reinhardtii* and targeted to the cytosol has a half-life estimated to be 2 h ***(38)***). In theory, if promoter activity of

290 the reporter fusion is repressed, less time is required to observe an equivalent repression of
291 luciferase activity compared to YFP activity, however, these two reporter systems have yet to be
292 examined side-by-side in *C. reinhardtii*.
293
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303 **Figure Captions**

304 **Figure 1. Diagram of promoter-*YFP* fusions.** In panels A and B, expression of YFP is dependent on a
305 promoter fragment that contains a binding site for a transcriptional inducer. When transferred to inducing
306 medium the transcription factor binds to the promoter fragment, transcription occurs, and YFP
307 accumulates in the cytosol. If the binding site for an inducer is deleted, YFP will no longer be expressed in
308 the inducing medium. In panels C and D, expression of YFP is dependent on a promoter fragment that
309 contains a binding site for a transcriptional repressor. In non-repressing medium, the transcription factor
310 no longer binds to the promoter fragment, transcription is allowed to occur, and YFP accumulates in the
311 cytosol. If the binding site for a repressor is deleted, YFP will be expressed in both media. In the protocol,
312 the medium that leads to YFP expression is referred to as the “inducing medium” without an assumption
313 of the regulatory mechanism. Abbreviations: RNAP, RNA polymerase; YFP, yellow fluorescent protein.

314

315 **Figure 2. An experimental workflow of protocol step 3.1.** Circled numbers correspond to steps in the
316 protocol for UVM11 transformation by glass bead bombardment. Three plasmids – the first contains a

promoter-less *YFP* construct (negative control), the second contains the promoter of interest upstream of *YFP* (test promoter) and the third contains a promoter-*YFP* fusion that is metal-responsive (positive control) – are linearized with a restriction enzyme. Prior to transformation in step 6, this DNA should be added to the pre-autoclaved glass beads. Since there are three *YFP* constructs, steps 6 – 13 are carried out on three separate aliquots of cells.

**Figure 3. An experimental workflow of protocol 3.2**. A, Circled numbers correspond to steps in protocol section 3.2. Since there are three *YFP* constructs, step 1 involves three petri dishes of transformants (corresponding to the negative control, test promoter and positive control) and three 96-well master plates filled with TAP. Step 3 requires a total of 6 black-sided, clear-bottom plates; inducing medium is abbreviated as IM. B, YFP fluorescence from 96 UVM11 cultures each transformed with the same WT promoter fragment (WT) and 96 UVM11 cultures each transformed with the same promoter fragment that contains a 50 bp deletion (mutant) abolishing activity. The 192 transformants were grown in 96-well plates filled with TAP (TAP) and 96-well plates filled with inducing medium (IM) for 6.5 days. In this example, YFP fluorescence is normalized to chlorophyll fluorescence. C, plot of YFP fluorescence normalized to chlorophyll fluorescence for two individual transformants (tf-1 and tf-2) containing the WT promoter fragment grown in TAP (TAP) and inducing medium (IM).

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