Dear Dr. Nguyen,

We thank the editor(s) and reviewers for their thoughtful and constructive comments and recommendations; we are pleased that our manuscript was found to be of interest. We have addressed all the reviewers' concerns by modifying the text and the video, and providing clarifications. We believe our manuscript has been improved greatly by making these changes based on the reviewers' comments. All the changes in the manuscript have been tracked. Below we provide a detailed point-by-point response to address the reviewers' comments.

Editorial and production comments:
Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: *We have proofread the manuscript and corrected any spelling or grammar issues.*

2. Please revise the following lines to avoid previously published work: 32-38, 74-78, 138-142, 154-157, 194-197, 198-205, 252-271, 335-348, 380-384

Response: *We have revised the text in the specified lines.*

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Response: *Frontiers articles are published under the Creative Commons Attribution License and can be reproduced contingent on crediting the source. The link to the Frontier Copyright Policy is: https://zendesk.frontiersin.org/hc/en-us/articles/201904552 The figure legends have been revised to provide the citations of the sources accordingly.*

4. If the figures are not reused from a previous publication, please provide new figure legends as they are from previously published work: https://www.frontiersin.org/articles/10.3389/fbioe.2014.00068/full

Response: *The figures are reused from a previous publication and have been cited in the figures legends in the revised version.*

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names

before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

Response: *We have removed all commercial language from the manuscript and used generic terms in the revised version. All commercial products are referenced in the Table of Materials.*

6. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Response: *We have added more steps to our protocol in the revised version. We have added a reference to gram staining protocol step 1.8 in the revised manuscript.*

7. 1.2: What are the growth conditions?

Response: *We have added the growth conditions to step 1.2. The growth conditions are to grow the cells in fresh Tris-Acitate-Phosphate (TAP) media with final concentrations of 400μg/mL timentin, 50μg/mL ampicillin, and 100μg/mL kanamycin to mid-log phase.*

8. 1.7: Please expand on the standard assay plates. What are they?

Response: *We have explained what are the standard assay plates in the revised manuscript. The standard assay plates are carbon sources, nitrogen sources, phosphorus and sulfur sources plates, and peptide nitrogen sources.*

9. 1.9: How is the gram staining done?

Response: *We have added a reference to the gram staining protocol step 1.8 in the revised manuscript.*

10. Please include all user input commands (File | Save | etc. or run xxx -t etc) so that the steps 2-4 are explicitly detailed. We need these details so that others can faithfully replicate the protocol.

Response: *We have added all the user input commands to steps 2-4 in the revised manuscript..*

Changes to be made by the Author(s) regarding the video:
1. Please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol.

Response: *The narration was revised as requested, and we added more detailed descriptions for the protocol steps 2 and 4 as described in the manuscript, with screen recording representing the different steps of the computational data analysis.*

2. Please reduce the frame rate to 30 frames/second.

Response: *All video parts were retaken at 30 frames/second.*

3. Please stabilize the video and avoid the fast zooms.

Response: *The videos were taken using a fixed tripod, and zoomed shots were taken separately to avoid zooming while filming.*

4. Add some transitions to the footage, it will smooth out the video and make it easier to follow

Response: *We added the titles for each part of the protocol as described in the manuscript, in addition to the previously present parts (results and conclusion).*

5. 2:17: Please use the Greek symbol mu for the microgram abbreviation. Please capitalize the L in the mL abbreviation.

Response: *The Greek symbol mu was used as requested for the antibiotics' concentrations.*

6. 2:39: Please avoid commercialism in the video. Remove the Biolog feature. Use generic terms whenever possible.

Response: *We removed the Biolog feature in the video, and made sure the logo doesn't show on the instrument.*

7. 3:03: Please do not feature the Omnilog system. Use generic terms whenever possible.

Response: *We removed the Omnilog system and replaced it with Phenotype Microarray assay system (when possible), except for one section in the data analysis part where the software used includes the Omnilog mention in the R-package software.*

8. Add a white background behind the graphics and charts

Response: *White background was added behind graphics and charts as requested.*

Please upload a revised high-resolution video here:
https://www.dropbox.com/request/HJGfhygJyxuTIMInMc9T?oref=e

Response: *The revised video is uploaded.*

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The authors use commercially available Biolog plates to metabolically phenotype a model microalgae (C. reinhardtii). They use the resulting substrate utilization patterns as evidence for the presence/absence of metabolic pathways via a computational pipeline. After their verification, metabolic pathway information was used to update an existing metabolic model. This new model was then benchmarked against the previous model by addition of data from another method (GC-TOF) and via a final model test by running shadow cost analysis for light and dark reactions for both models.

Major Concerns:
The authors developed a satisfactory pipeline for addressing gaps within metabolic models and I have few minor comments that need to be addressed before acceptance of the manuscript.
Bacterial growth. The authors do not control for bacterial growth in cultures of C. reinhardtii during the primary cultivation step. Three types of antibiotics are added after microalgae have been grown to a high enough density for subsequent Biolog incubation. How have the authors ensured that this antibiotic treatment truly inhibited all bacterial growth (as claimed in line 104-105) and that their signal is not an artefact from bacterial metabolism? I am not fundamentally questioning the validity of their data but would like clarification on this and also like the authors to suggest the addition of antibiotics at the initial incubation step (line 96).

Response: *We have added descriptions of antibiotic addition at the incubation step as recommended by the reviewer. We now also state in the protocol, step 1.8, that gram staining was performed before and after the assays to monitor bacterial contamination.*

Light conditions. The authors acknowledge (and state repeatedly) that the OmniLog system does not allow for the illumination of microalgae samples and therefore all metabolism occurs under prolonged darkness (up to 7 days). This shortcoming is discussed by the authors but only by stating what other strains also show heterotrophic growth. What I a missing in this discussion is a section on how the absence could affect their model interpretation.

Response: *We have expanded the discussion on how the absence of light could affect the model interpretation in the revised manuscript (lines 422-432).*

Minor Concerns:
Line 96: Please provide additional details on how C. reinhardtii was grown (i.e. light conditions, temperature, and duration to 'mid-log' phase).

Response: *We have added the growth conditions in the protocol step 1.2 in the revised manuscript.*

Line 111: Please provide a volume for these cell numbers

Response: *We have provided the volume for the cell number in the revised manuscript; the volume is in mL.*

Line 120: Why was bacterial contamination checked here and not before growth? Further, why was C. reinhardtii not grown with antibiotics added (step 1.5 in the protocol describes the addition of antibiotics after the cells were already grown)

Response: *Cultures were determined to be axenic before inoculation and grown with antibiotics. We have added descriptions of antibiotic addition at the incubation step. We now also state in the protocol at step 1.8 to perform gram staining before and after the assays to monitor bacterial contamination.*

Line 203: 'Figure 3 shows that the majority of the data were nearly identical as they fall on the 45-degree line'. Please perform a linear regression and display the resulting R2 value onto the figure and state it in the text.

Response: *We have performed a linear regression and displayed the resulting R^2 value of 0.9 onto the figure and stated it in the text in the revised manuscript (lines 691-692).*

Line 256-257: The author state that the addition of new metabolites did not introduce additional root gaps in previously published (and now augmented) metabolic models. Why did your phenotyping method not close some of these root gaps?

Response: *In this revised version, we have stated that it should be noted that the phenotyping method used in this protocol does not close root gaps, because the original root gap metabolites lack transport or production mechanisms, which were not addressed in the phenotyping assays.*

Line 276: Respiration is not growth. Please delete growth

Response: *We thank the reviewer for pointing this out; we have deleted "**growth"** in the revised manuscript (line 663).*

Line 301: Please indicate which test values (i.e. pathways/compounds) deviate away from the supposed linear relationship. Please indicate how this deviation was measured (standard deviation/error?).

Response: *Values of compounds, a linear regression was performed and the obtaoned coefficient of determination (R^2) of 0.9 is stated in the text of the revised manuscript (lines 691-692) and indicated in the revised figure 3.*

Table 1: Please change the layout/size of this table so the PSI-blast values/ annotations are visible together with the EC numbers on the same page.
Table of materials: Same comment as above, ensure readability by placing table contents onto same page

Response: *We have changed the layout of the tables in the revised version.*

Reviewer #2:
Manuscript Summary:
This manuscript aims to detail a protocol for using Biolog data for improving genome-scale models of metabolism, particularly for the organism Chlamydamonas reinhardtii. The paper is generally fine until section 3 dealing with identification and genes associated with new metabolites. From that point on, it is confusing and not detailed enough.
Response:

Major Concerns:
Statement 3.1 doesn't make sense. Compounds don't have EC numbers, enzymes do. The biolog data does not identify enzymes.

Response: *We have corrected this error and have edited this sentence in the revised manuscript. We now state that we search KEGG (http://www.genome.jp/kegg/) and MetaCyc (http://metacyc.org/) to identify the Enzyme Commission.*

In section 3.3, the authors need to detail what should happen if no candidate genes are found for the EC. Also, they need to expand on the term "other organisms" does it refer to other algae, specific family of bacteria, or all sequenced genomes. The statement is vague.

Response: *The reviewer is referring to protocol step 3.3. We have clarified this to describe a search for homologs in other organisms, starting with species closest to C. reinhardtii, to identify the relevant protein for the query reaction.*

If no corresponding protein can be identified in C. reinhardtii, the reaction can be added without an associated enzyme.

For section 3.4, E value of 0.05 is large. The number of possible genes that could be identified could be large. How should they be further pared?

Use of Pfam and Interpro should be detailed.

Response*: We agree with the reviewer that, in general, an E value of 0.05 is too relax; however, in our experience, we did not find the problem of having too many hits. Further, we like to draw attention that this step is followed by additional QC steps to evaluate the obtained candidate proteins for having the correct enzymatic activity associated with them. Namely, the obtained hit will be evaluated by Interpro and PFam scans. Last, the Pfam and Interpro tools described here are web-based tools, and their use does not require any informatics skills or detailed explanation.*

In section 4, there are multiple versions of COBRA toolbox available. It should be noted; particularly since due to the pay nature of matlab, many researchers are switching to cobrapy.

Response: *We have mentioned the availability of a version of COBRA toolbox that runs in python (COBRApy) in the revised version and does not require Matlab.*

Section 4.1.2, there is a hierarchy to transport of metabolites. The way the sentence is written it seems as though one can directly transport metabolites from external environment to the mitochondria and other compartments surrounded by cytosol. This is not proper modeling.

Response: *We have added more details and command line in step 4.1.3 in the revised version.*

Line 178 and 179, states "two columns correspond to reactions; flux and reduced cost, and one column corresponds to metabolites' shadow prices." It would be lot less confusing to say 3 columns. This only serves to confuse a novice user.

Response: *We have added more details and command line in step 4.1.4 in the revised manuscript.*

4.1.5 is too simplified. A much more detailed comparison of the models can be conducted beyond just comparing shadow prices.

Response: *We have added more details and command line to step 4.1.6 in the revised manuscript.*

Why wait until representative results to refer to figures? The figures would drastically help explain concepts earlier in the manuscript.

Response: *We thank the reviewer for his/her suggestion. We have arranged the manuscript's material based on the journal's template; the revised accompanying video does integrate results and analyses and should be helpful in this regard.*

Also it is very confusing to say in this study we ...., when what has been described is a protocol.

Response: *We have modified it to "in this methods description".*

The authors need to explain why plates PM01 and PM03 where chosen.

Response: *We used PM01 and PM03 plates only as examples of our results. Any phenotype microarray plates could be used for the analysis.*

On line 198, what pipeline are the authors referring to?

Response: *The pipeline of refining genome-scale metabolic network using PM data. Line 465 in the revised manuscript.*

They found 149 metabolites that overlap between biology and old model. However, later they mention 128, where did this later number come from?

Response: *149 metabolites were overlapped between the 662 PM metabolites and the 1068 iRC1080 metabolites, while only 128 metabolites were identified from literature.*

In conclusion, the paper as written is confusing for both experimentalist and theoretical scientists. There is not enough detail on protocols after section 3.

Response: *We have added more details, steps, and command lines in steps 3 and 4 in the revised manuscript.*

Minor Concerns:
1. In the abstract: systems-level is a complex word and needs a hyphen.

Response: *We have added a hyphen to* ***"systems-level"*** *word in the revised version.*

2. line 50, don't need to add "or pathways".

Response: *We have deleted* ***"or pathway"*** *in the revised manuscript.*

3. line 53, This sentence seems dated. It references a paper from 6 years ago. There have been more algal models developed since then. Perhaps the authors should enumerate the number of current models available and contrast it to the

number of algal genomes that have been sequences. This vague sentence does a disservice to the paper.

Response: *We have added a reference to the number of sequenced algal species and have stated in the revised version that "Although approximately 160 microalgal species have been sequenced5, there are, to our knowledge, only 44 algal metabolic models available."*

4. line 62, run on sentence. Change to (entailing ~~-~~250 sources). It has 1,706

Response: *We have edited the sentence in the revised manuscript.*

5. The entire section 3 is missing relevant references.

Response: *We have added the references to section 3 in the revised manuscript.*

6. line 154, genetic instead of genic

Response: *We have corrected the word to genetic in the revised manuscript.*