Dear Dr. Ren,

Thank you for giving us the opportunity to submit a revised draft of the manuscript “Structural Equation Modeling of In silico Perturbations” for publication in the Frontiers in Genetics. We appreciate the time and effort that you and the reviewers dedicated to providing feedback on our manuscript and are grateful for the insightful comments on our manuscript. We have been able to incorporate changes to reflect most of the suggestions provided by the reviewers. We have highlighted the changes within the manuscript. We also enter the point-by-point response to the reviewers' comments and concerns in the interactive review forum. All paper numbers refer to the revised manuscript file with tracked changes.

# Reviewer 1 Comments

**Q1: Please describe the new technology or code (or new application of a known technology or code) reported in this manuscript, and its use.**

The manuscript reports implementation of an R package to process gene expression data to enable translation and testing of a given hypothesis involving perturbation of gene pathways between two systems using in silico experiments.

Thank you for your time.

**Q2: Please highlight the limitations and advantages.**

The manuscript focused on the implementation rather than carefully explaining the method that has been implemented. The manuscript is probably easy to understand by the researchers involved in this project, but it is difficult to be comprehended by an outsider. More details about the used method would be required to judge whether it is statistically valid approach, and what its limitations might be.

Thank you for pointing this out. The reviewer is correct, and we have made substantial changes in the manuscript. We include several sentences of explanation of the method in the revised manuscript. The revised texts could be found in:

1. Page XXX: detail explanation about the t-score (done)
2. Page XXX: detail explanation about the bootstrap method (done)
3. Page XXX: biological application of the method (done)

**Q3: Are there objective errors or fundamental flaws? If yes, please detail your concerns.**

The main idea to use SEM for hypothesis testing and adopt this approach to experiments involving perturbations of gene expression is sensible. However, more details are required to describe the method including all details about processing steps into the main manuscript, and I may suggest to move the implementation details to supplementary.

As suggested by the reviewer, we have moved some implementation details to supplementary material and methods section, and included the more detailed description of the SEM method and

**Q5: Please provide your detailed review report to the editor and authors (including any comments on the Q4 Check List)**

The paper is difficult to understand as many important details are not given.

*Abstract*: please explain what inputs are necessary to perform the analysis, define or explain perturbation of gene expression pathways, explain what is meant by gene activities, explain what statistical significance refers to. Some statements used in 'Contributions to the field' may be added to Abstract, but there are again unclear statements: a basic SEM model (how does it look like?), relationships among end-points (what are these points?), how to briefly explain how the functional hypothesis can be generated?

I would suggest that we include the explanation in the method and discussion sections.

*Introduction*: how are SEM models fitted? - a reference may be enough, why t-score can be used as activity metric? Implementing bootstrap random sampling is probably not that difficult. More importantly, there should be better literature survey outlined in Introduction, and also summary of contributions and advantages of the proposed method compared to other similar methods.

Steve and Lin’s revision has addressed these concerns **(any comment from other coauthors?)**

*Methods*: please add more details what has been implemented in SEMIP package or software, e.g. explain a 3-node fitting problem, it may help to add a paragraph describing what biochemical processes are considered, what type of data are assumed in the analysis, explain what is meant by system response was exemplified, the role of t-score in transferring knowledge between two stochastic systems is unclear (and this point seems to be critical for understanding the paper), how can bootstrap simulation eliminate unrelated gene signatures? Why running the bootstrap over 1000 samples is sufficient? Why not 100 or 10000? What is multicore hardware needed?

Steve’s revision, T-score elaboration, and two-class bootstrapping write up shall address these concerns. **(done)** Both bootstrap methods are non-parametric with no assumption of the population distribution, therefore sufficient large amount of simulation will provide us empirical distribution where can be consulted for statistics testing. It largely depends how much the “downstream genes target” eliminated will be impacted by the upstream regulator revealed from the SEM fitting. 100 could work but in our exercise 1000 rounds ensures a stable empirical distribution curve. In our implementation, we rely on the parallel process to conduct the bootstrap simulation, therefore a multicore hardware is recommended. As the example shown in the manuscript on KEGG pathway analysis with 28 categories, it can take up to a couple of hours to finish this step. If no multicore is detected, the application to execute a serialized solution which can take much longer time to complete. **(done)**

*Results*: line 152: sometimes ... help ... proposed new hypothesis - when does it help and when it does not? Why are the results provided in zipped file, are they so large? line 165: A Use Case of ...., line 190: ... out hypothesis ... it is unclear what is being referred to

Steve’s revision write up has addressed these concerns. **(done)**

*Discussion*: line 204: how different is your package from MplusAutomation? Are there any other similar R packages? What advantage your packages bring compared to these other software?

We agree that the MplusAutomation’s authors have done a good job in “mirroring” the commercially available software “Mplus” and implement this modeling in open-source R. It is designed to automate three major aspects of latent variable modelling, (1) create a group of models (2) run them in batches (3) allow extracting the model fitting statistics. Our SEMIPs has a similarity to MplusAutomation, where we implement SEM model in R instead of Mplus for the computational flexibility and backend automation consideration. We use the lavaan package, a highly credited/cited package exists in the research community since 2012 to implement the SEM model and extract all the statistics from the modeling output. But, our main goal in this research anchors on the biology and provide our web-lab scientists a convenient tool to explore their novel hypothesis and test the validity of their thought, and most importantly helps with hypothesis generation process. The main advantage is that our application is designed to use Rshiny to render a user’s friendly web front end. It allows web-lab scientists with limited bioinformatics skills to use the platform for this biological hypothesis testing and generation as we exemplified in the manuscript. **(done)**

*Overall*:

1. Please add details focusing on the science behind rather than the implementation.
2. It helps enormously to explain what is being modeled, what type of data are assumed, what type of hypothesis can be assumed etc.
3. Add some numerical results demonstrating the statistical validity of the developed software.
4. Please proofread the paper for some occasional English writing errors.

We appreciated the reviewer’s constructive assessment. Accordingly, throughout the manuscript, we have revised: (**based on Steve’s revision**)

Page XXX: Abstract

Page XXX: Introduction

Page XXX: Methods

Page XXX: Results

Page XXX: Discussion

Page XXX and XXX: we included details on rationale about why we chose SEM and applied T-score and bootstrapping methods **(done and need authors’ consensus)**

In addition, our colleagues helped us proofread the manuscript and the spelling and grammatical errors have been corrected. **(need Pierre’s help)**

# Reviewer 3 Comments

**Q1: Please describe the new technology or code (or new application of a known technology or code) reported in this manuscript, and its use.**

The authors developed an R Shiny application to aid in the transfer of perturbations in gene expression pathways from one system to another for determining casual inference of molecular interactions in silico. The authors used a 3-node PGR-GATA2-SOX17 gene network as a use case to evaluate the potential of using putative downstream genes of GATA2 as surrogate reporters of GATA2 activity.

**Q2: Please highlight the limitations and advantages.**

*Strength*: This manuscript presents a useful tool that can facilitate hypothesis generation and testing and allow bench scientists to perform analyses through a user-friendly interface.

*Limitations*: The content of the manuscript sometimes focus too much on procedural details and not enough on the purpose of the analysis, consideration of assumptions and interpretation of results. Schema illustrations also need improvement.

Steve’s revision has added some biological implication. The figure 1 has been updated and figure legend has been updated also. Hopefully it can address the “schema” concern. **(any comment from other coauthors?)**

**Q3: Are there objective errors or fundamental flaws? If yes, please detail your concerns.**

no comment

**Q5: Please provide your detailed review report to the editor and authors (including any comments on the Q4 Check List)**

***Major comments:***

* Line 73-75: More details about the t-score should be added here, i.e. the assumptions and observed data for the t-test.

The main motivation using a “t-score” was to achieve the cross-species projection from a model animal (mice or rats) experiment to another species or human when a perturbation was not directly applicable (Wu, S.P. et al. 2015). With a model animal (mice or rats) experiment, normally the animals are randomly assigned into two groups, where one group will receive “placebo” and/or no treatment and another group will receive the perturbation treatment. Experimental measurement will be properly collected from both groups (i.e. gene expression profile from a Microarray experiment). Significantly changed genes/probes (signatures) will be obtained from this analysis according to some thresholds followed by a statistical analysis with directionality (up/down regulation). Such a list of genes/probes are deemed collectively as the “gene expression signature” of biological responses to a particular perturbation. And these genes are referred as “signature genes”. This finding and information will be projected into another animal system (i.e. human) of interest bearing the assumption that the experimental animal of interest would respond similarly if the perturbation were applied.

In a separate experiment of interest that is done with species of interest (i.e. human), the homologous genes of those previously identified genes/probes from the experimental species will be selected, and the experimental measurement from this study (GEO accession: GSE58144, (Koot et al. 2016)) will be used. The directionality information will be used to group these genes into two separate groups. A normal t-statistics will be calculated from these two groups of measurement to represent the responses in a new species and new experiment set up of interest. Since this calculation was originated from a standard t-test statistics and the term “T-score” was coined firstly by Wu, S.P. et al (Wu, S.P. et al. 2015) and have been widely used in other research projects. Samples of interest (i.e. human) with T-score larger than 0, which share a similar signature gene expression profile from the original model animal, were classified as having gene signature activities and vice versa. **(done, need to clarify with Ty about vice versa, etc. )**

* Line 96: Regarding the two bootstrapping methods, do they have different assumptions, computational costs and/or test power? Any recommendations for when either method is preferred?

**1) Do the two bootstrap methods have different assumptions?**

Both bootstrap methods are non-parametric so there are no assumptions regarding the distribution of the data.  However, there is an assumption that the population is infinite, or sufficiently large such that that the effect of taking a sample is essentially negligible. **(done)**

**2) Do the two bootstrap methods have computational cost?**

Yes there is a computational cost for each method.  The more bootstraps performed, the longer the analysis takes but the closer the estimated parameter is to the true value.  **(done)**

**3) Do the two bootstrap methods test power?**

No.  The bootstrap methods ascertain the significance of the test. **(done)**

***Minor comments:***

* Line 70: projects -> projected

Corrected **(done)**

* Line 73: Such an information -> Such information

Corrected **(done)**

* Line 262: Figure 1 is a little confusing. The green shape is not a rectangle but was referred to as one. Varying both shape and color without appropriate annotation is confusing/distracting. The red boxes, dashed vs solid, do they have different meaning?

Already contacted Lois to assist once the final decision is achieved. Figure has been revised **(done and need authors’ consensus)**

* Supplementary Figure 1
  + - Why is SOX17 in brackets?

When the “brackets” are used, they refer to “gene expression value” instead of “projected activity”. In this model, we did use expression of SOX17, therefore, it is put in the brackets. **(done)**

* + - The top two thicker blue arrows seem to indicate the same processing step, but the text annotations are different, which is confusing and distracting.

Explained in the “revised” figure and “revised figure legend”. **(done)**

* + - Figure legend: “The resulting shrunken GATA2 gene list or reduced GATA2 [gene list] then restored by the same number of irrelevant genes are tested in the SEM model.”

Correction made in the supplemental figure. **(done)**

* Source code: Coding style in the source code could use some standardization.

Jason will communicate with the reviewer for clarification **(need Jason’s help)**