**Guest editor comments:**

*1) Please consider proof reading of the manuscript by a metabolomics expert in order to improve weak points in descriptions and terminology (as suggested by referee 1)****.***

TODO: Rainer. Nochmal drüber lesen am Ende.

“Mögliche Änderungen im Text…”

*2) Please provide some additional results (preferably from metabolomics studies) and comparison with the performance of other software tools.*

In order to emphasize the increasing importance of cross-omics studies, which particularly involve the profiling of the metabolome, we cited two pioneering studies in this field, which have recently been conducted by Gruden *et al.* and Armiour *et al.*, respectively. Furthermore, Figure 2 is now based on real data from as yet unpublished collaboration work, which is briefly described in the revised manuscript.

TODO: Lars. Comparison with other visualization tools for metabolomics data

“Furthermore, modern mass spectrometry platforms are able to detect and measure the relative intensity of thousands of metabolites with high accuracy. In recent years, increasing attention has been drawn to the integration of metabolomics with molecular profiling on the transcriptional and protein level [PMID: 22253695, 22936829].”

TODO Rainer: Möglicherweise ist hier auch noch möglich über die Wichtigkeit von kombinierten Analysen in der Zukunft zu sprechen insbesondere mit dem Blick auf Proteomics. Dass solche Analysen bald ausstehen werden und wir deshalb schon entwickeln wäre ja auch ein guter Grund. Die Software erst anzupassen, wenn die Daten schon da sind, wäre ja nun wirklich dämlich. Aber man kann ja nicht schon jetzt die Experimente beschreiben.

“Mögliche Änderungen im Text…”

*3) The way that data is imported could be described more eloquently.*

TODO: Lars. Über den Import werde ich noch etwas mehr schreiben. Habe da auch noch paar Bugs gefixt.

“Mögliche Änderungen im Text…”

*4) Comments from referee 2 on targeted metabolomics data should be given consideration.*

See revision notes for reviewer 2.

**Reviewer 1 comments:**

*1) Also the implementation of the metabolomics part is very simple: only differentially expressed metabolites are considered. First of all, this terminology is strange: metabolites are not espressed but formed.*

We agree with Reviewer 1 that the algorithm used for pathway enrichment analysis is simple in the sense that only differentially formed metabolites are considered. However, please note that InCroMAP now also allows for importing enrichment results from other tools. Since InCroMAP version 1.7, you can, for instance, import metabolite set enrichments from the MSEA software (<http://www.msea.ca>) which offers more sophisticated algorithms (e.g., single sample profiling or quantitative enrichment analysis) accounting for metabolite concentrations. Please note that we do not intend to replicate these features in our software. Nevertheless, we set a high value on ensuring the compatibility to related tools in order to overcome potential limitations of our software.

Due to the fact that metabolomics datasets are often limited to specific metabolites which could be identified in the profiled samples, we added an option to limit the background set (universe) to the quantified metabolites. Besides avoiding a potential bias arising from the unequal coverage of the tested pathways this change may also increase the sensitivity of the applied statistics.

* TODO: Import von MSEA implementieren (optional auch IMPaLA, MBRole, MPEA)

“Umsetzung im Text…”

TODO: Lars. Tatsächlich verwenden wir “expressed” nur wenn da metabolites, genes and proteins steht. Ich füge halt noch „formed“ hinzu. Idiotisch aber o.k.

LARS: Ich werde mich drum kümmern, dass Universe implementiert wird und dass wir auch andere Ergebnisse importieren können.

*2) Secondly, which kind of metabolomics is considered here? The authors talk about pathways but if one measures in blood, then the concept of pathway is very difficult.*

TODO: Rainer. Veränderungen in Pathways sollten sich auch in Blut/Urin wiederspiegeln. Hier wäre möglicherweise ein Beispiel gut.

“Umsetzung im Text…”

*3) The authors are clearly not from the field of metabolomics (they do not mention the proper and much used tools for data (pre)m processing in metabolomics. They do not refer to existing tools or software for metabolite enrichment analysis.*

TODO: Vergleich mit existierenden Tools machen.   
TODO: Rainer/Lars. Was gibt es noch, was für die Vorprozessierung der Daten in Metabolomics verwendet wird? Wir haben R und MayDay (für Transcriptomics/Proteomics bisher) zitiert. In den Sinn käme mir noch kommerzielle Statistiksoftware.

Mit was werden sonst die Rohdaten prozessiert (mal die Software der Gerätehersteller ausgeschlossen)? Hier fällt mir noch XCMS (von Ralf Tautenhahn am Scripps) ein, welches in R läuft und wohl auch relativ bekannt ist.

“Umsetzung im Text…”

**Reviewer 2 comments:**

**Major Revisions**

*1) The multi-level integration is clearly an advantage of the proposed tool. But this could be demonstrated by a powerful application to show which new biological insights can be provided by integrating more than two levels. Since the first software version was published in 2012, are there experimental studies using this tool? This should be cited. Also unpublished collaboration work could be shortly explained.*

*Since the main purpose of the manuscript is the integration of metabolomics data, an example application from the viewpoint of metabolomics would be important.*

In the EU-funded project MARCAR (<http://www.imi-marcar.eu/>), we perform extensive cross-omics profiling based on liver samples from rodent in-vivo studies. Most notably, we recently submitted a manuscript entitled „Metabolic programs orchestrated by the activated Ha-ras and β-catenin oncoproteins in mouse liver tumors” to Int. J. Cancer. In this study the InCroMAP software was extensively applied to the pathway-based, integrated analysis of transcriptomics, proteomics and metabolomics data obtained from mouse liver tumors differing in their mutation status. Since the manuscript has not been published yet, we can not cite it. Nevertheless, as proposed by Reviewer 2 this work is now shortly explained in the revised version of the manuscript. Additionally, we updated Figure 2, which is now based on real data from the above-mentioned study. However, since we would like to remain the focus on the presentation of the novel features of the InCroMAP software (e.g., enhanced support for metabolomics data), an in-depth discussion of the gained biological insights is beyond the scope of this article.

TODO: Nach Rücksprache mit Michael Schwarz 🡪 Figure 2 updaten

“Umsetzung im Text“

*2) How does the proposed tool outperform existing tools?*

*The authors avoid a detailed comparison with existing tools like MassTRIX and Paintomics by using the argument that those tools consider only two omics levels. Even though the proposed tool has the advantage of integrating several omics levels, typical practical applications consider only two levels, e.g., metabolomics and transcriptomics. A more detailed comparison of the proposed software tool with existing tools would therefore be necessary.*

TODO: Tabelle, die die Fähigkeiten vergleicht. Da natürlich insbesondere auf Interaktive Analyse eingehen. (Java standalone tool). Des Weiteren auf gradielle Farbdarstellung im Vergleich zu MassTRIX. Nachteil wäre die Annotation, aber ich denke hier kann man durchaus argumentieren, dass man dies ja professionellen Tools wie z.B. OpenMS/XCMS überlassen kann. Tools die für einen Vergleich in Frage kommen: (MetaMapp (O. Fiehn), MassTRIX (Philippe), Paintomics , Cytoscape)

“Umsetzung im Text“

Ich würde zusätzlich noch folgende Punkte hervorheben:

* Metabolic Overview Feature 🡪 geordnete Darstellung der Änderungen im zellulären Metabolismus (besser strukturiert als Tabelle/Barplot mit „enriched pathways“)
* Direkte Anbindung an KEGG und Nutzung von aktuellen Daten
* Integrierte Enrichment-Analyse und Visualisierung von mehr als zwei Typen von Omics-Datensätzen, die anhand derselben biologischen Proben generiert wurden

Können ja mal ne Tabelle reinsetzen und dann immer mal wieder paar Häkchen hinzufügen.

*3) The authors emphasize their strong effort in accepting and merging different kinds of gene or metabolite identifiers. But it remains unclear which of those tasks are new compared to similar software.*

TODO: Johannes: bezüglich Gene Identifier, was ist neu, bzw. was sollte man da sagen

Es gibt da schon ein paar nette Features, aber diese sind eben nicht neu seit Version 1.6:

* Automatische Erkennung von Probeset IDs (z.B. Affymetrix, Agilent, etc.) oder Gene IDs (z.B. Gene Symbols, EntrezGene, etc.) im Import-Dialog
* Mapping von Probesets auf Gensymbole (z.B. anhand von Affymetrix IDs)
* Annotation von miRNAs mit experimentell bestimmten oder vorhergesagten Target-mRNAs

TODO: Lars: bezüglich Metabolite Identifier? Mega neues gibt es da wohl nicht, ich habe nur versucht möglichst sorgfältig zu sein beim mergen der Datenbanken und immer wieder kontrolliert. Es gab ja auch eine Studie die Überlappungen gesucht hat und dann auch Strukturen verglichen hat. Ich gucke mal ob ich da was finde. Zusätzlich versuche ich noch was wegen InChIKeys zu finden.

“Umsetzung im Text“

*4) To identify pathways affected by an experiment, a "special pathway enrichment" algorithms is used, but a detailed description of how metabolomics data are included in the algorithm is missing as well as a comparison to pathway enrichment of other tools. Also, what does it mean "pathway enrichment is limited to targeted metabolomics data"? Is the visualisation in general limited to targeted data?*

We thank Reviewer 2 for this critical remark. To the best of our knowledge only one other software (IMPaLA) exists which is capable of the integrating both transcriptomics and metabolomics data for the purpose of pathway enrichment analysis. In short, IMPaLA calculates the enrichment p-values independently for each platform using either a hypergeometric test or Wilcoxon enrichment analysis. Then, as the experiments are considered independent, the joint p-value is computed based on the product of the p-values calculated for the individual platforms. On the contrary, InCroMAP performs only one statistical test in which all genes and metabolites showing significant changes are combined to one list. Analogously, the gene and metabolite universe are pooled in the hypergeometric test statistic. As suggested by Reviewer 2, the conceptual differences between the InCroMAP and IMPaLA algorithm for cross-platform enrichments are now explained comprehensively in the revised version of the manuscript.

“Umsetzung im Text: siehe Punkt 1 von Reviewer 3“

TODO: Lars: Natürlich auch untargeted daten möglich, aber Problem der Signifikanz bei Enrichment wegen many-to-many Mappings beschreiben. Ebenso werden keine möglichen Metabolitannotationen generiert. Tools zitieren und vergleich anstreben. Zeigen, dass analyse mit many-2-many mappings noch problematisch ist.

TODO: Rainer. Gibt es noch andere Tools, die du kennst, die zur Analyse verwendet wird. Womit macht ihr z.B. eure Heatmap plots?

“Umsetzung im Text“

*5) The presented tool requires input data in form of significant values (p-value/ log fold change). This means the complete data pre-processing remains by the user. A big user friendly improvement could be done by integrating some parts of data processing or even accepting raw data as input, as partly done by other tools.*

We thank Reviewer 2 for this suggestion. However, we would like to point out that the use of tabular processed data corresponding to fold changes and/or p-values is one of the central design concepts underlying the InCroMAP software. All core developers agreed on this concept as firstly, the central aim of InCroMAP is not the replication of features (e.g., for the pre-processing of raw data) offered by existing tools and secondly, as the ease-of-use is ensured by a largely uniform input format which is suitable for the import of heterogeneous omics datasets. Thus, beyond elementary convenience functions (e.g., for the detection and conversion of IDs) no support in data preprocessing is provided.

“Umsetzung im Text: Im Text (Methodenteil) wird auf diese Einschränkung der Software klar hingewiesen. Ich würde daher im Text gar nichts ändern.”

**Minor Revisions**

*1) What is the meaning of the acronym InCroMAP? I could not find.*

Kurze Antwort und möglicherweise im Paper erwähnen?

“Umsetzung im Text“

*2) The title "Straightforward interpretation… " might be misleading since there is no data interpretation in the manuscript.*

TODO: Alle. Änderung des Titels? Vorschläge?

Integrated enrichment analysis and pathway-centered visualization of metabolomics, proteomics, transcriptomics and genomics data by using the InCroMAP software

“Umsetzung im Text“

*3) Figure 2B shows a pathway, but metabolite data (colored circles) seems not to be included in the visualization.*

Metabolitdaten waren nur im ersten Bild vorhanden (2A) vorhanden. Das hat möglicherweise verwirrt. Dieses Bild könnte geändert werden. Was meint ihr?

TODO: Johannes. Nach Genehmigung von Michael Schwarz, Signaling-Pathway aus Figure 2B durch metabolischen Pathway überlagert mit Daten aus Maustumorstudie ersetzen

“Umsetzung im Text“

**Reviewer 3 comments:**

**Major Revisions**

*1) I´m missing more detailed explanations on the computation, which is important in the field of such tools. E.g. good background set for genes, proteins and metabolites are needed for calculation of enrichment and p-values. Furthermore, the authors do not explain in detail how combined enrichment works and how values are calculated.*

We fully agree with Reviewer 3 that a more detailed description of the algorithm used for the pathway enrichment is required. Thus, we added a comprehensive explanation of the adopted methods to the methods section (see subsection 2.2). Furthermore, as suggested by Reviewer 2 we also explain how the algorithm implemented in InCroMAP differs from the approaches used in related software.

„The InCroMAP software allows for the calculation of single-platform and cross-platform enrichments, based on omics experiments from individual and multiple platforms, respectively. In the former case, a hypergeometric test is applied to assess the significance of the overrepresentation of predefined gene sets (e.g., KEGG pathways) within a list of deregulated genes derived from a certain experiment based on fold-change and/or p-value cutoffs. The same statistical approach is used to determine pathways enriched with differentially formed metabolites. By default the union of all genes/metabolites in KEGG is considered as the universe. In order to enhance the support for high-throughput techniques which do not permit molecular profiling on a global level, the universe may optionally be limited to the genes/metabolites covered by the given experimental data. For the integrated enrichment analysis across multiple platforms, InCroMAP uses a straightforward extension of the single-platform method. Specifically, a hypergeometric test is used, where the sample corresponds to the union of the molecules which were changed in terms of abundance on any of the platforms and the universe is given by the union of the platform-specific background sets. To our knowledge the only alternative method for calculating an integrated enrichment based on both transcriptomics and metabolomics data is implemented in the tool IMPaLA (PMID: 21893519). In contrast to our software, IMPaLA calculates the enrichment p-values independently for each platform using either a hypergeometric test or Wilcoxon enrichment analysis. As the experiments are considered independent, the joint p-value is then computed based on the product of the p-values calculated for the individual platforms.

*2) I would also like to know how they implemented the metabolite database for mapping between the different identifiers. This is important, because a lot of people are struggling with harmonizing different metabolomics databases*

TODO: Lars. Nochmal genau beschreiben, wie die Integration gelaufen ist.

“Umsetzung im Text“

**Minor Revisions**

*1) Page4 line 2-3. Sentence "A main goal..." can be improved.*

In response to the suggestion made by Reviewer 3 the corresponding sentence was revised accordingly.

“The application was specifically designed to provide a high ease of use for experimental biologists.“