IMPaLA tutorial

http://impala.molgen.mpg.de/

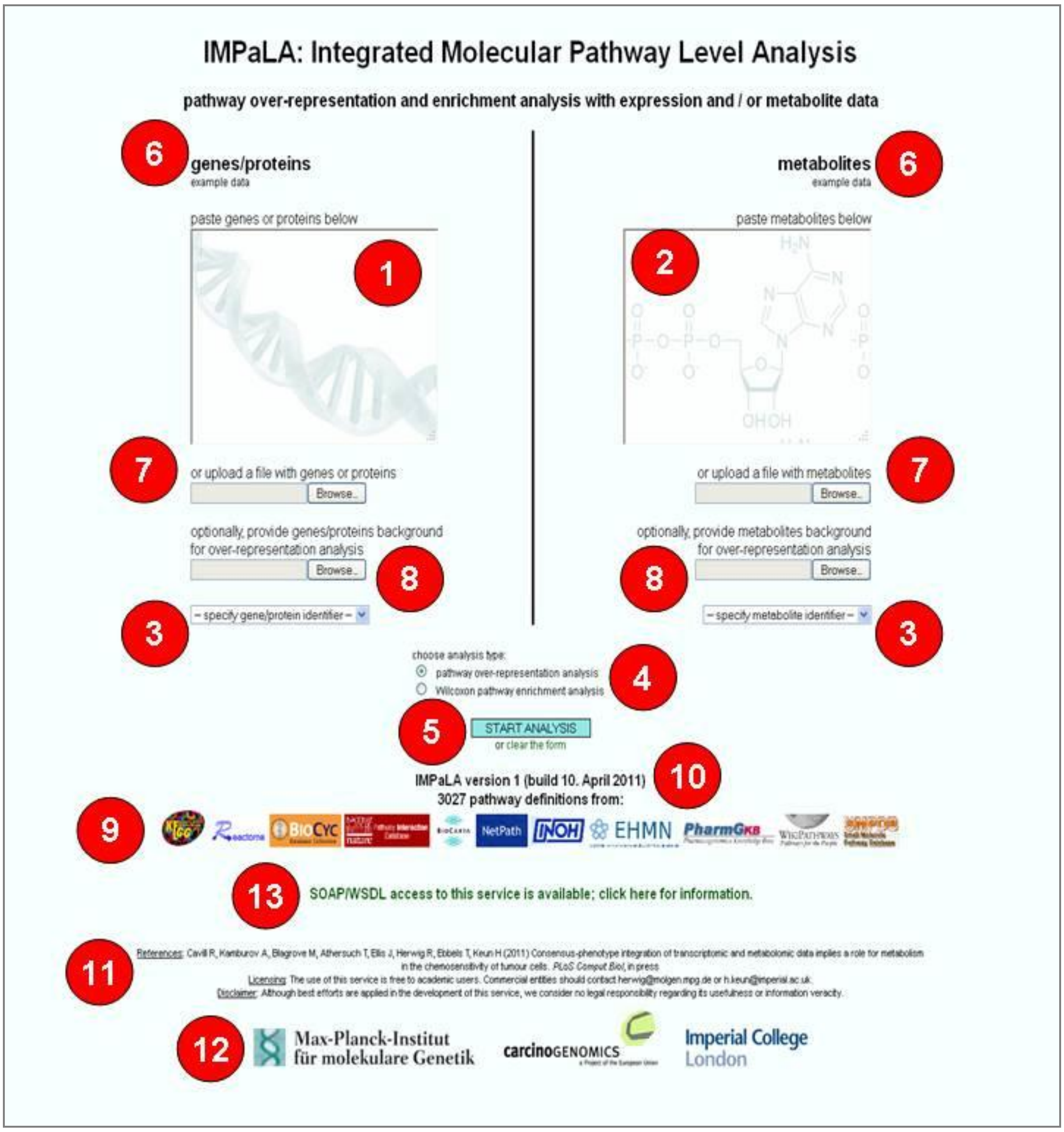
1. Introduction

IMPaLA is a web tool, developed for integrated pathway analysis of metabolomics data alongside gene expression or protein abundance data. It works through extending over-representation and enrichment analyses to multiple dataypes. The methodology was first presented in Cavill, R. et al. (2011) Consensus-Phenotype Integration of Transcriptomic and Metabolomic Data Implies a Role for Metabolism in the Chemosensitivity of Tumour Cells. *PLoS Computational Biology*, *7*(3), 12.

IMPaLA was developed to provide a straightforward publicly available platform for performing these analyses. It provides pathway over-representation and enrichment analysis functionality with user-specified lists of genes/proteins and/or metabolites, generally termed physical entities.

2. IMPaLA screenshots

2.1 IMPALA home screen.



IMPaLA: Integrated Molecular Pathway Level Analysis

pathway over-representation and enrichment analysis with expression and / or metabolite data

genes/proteins
example data

paste genes or proteins below

or upload a file with genes or proteins

optionally, provide genes/proteins background for over-representation analysis

metabolites
example data

paste metabolites below

or upload a file with metabolites

optionally, provide metabolites background for over-representation analysis

choose analysis type:

● pathway over-representation analysis

○ Wilcoxon pathway enrichment analysis

IMPaLA version 1 (build 10. April 2011)
3027 pathway definitions from:

SOAP/WSDL access to this service is available; click here for information.

References: Cavill R, Kamburov A, Blagrove M, Athersuch T, Ellis J, Herwig R, Ebbels T, Keun H (2011) Consensus-phenotype integration of transcriptomic and metabolomic data implies a role for metabolism in the chemosensitivity of tumour cells. *PLoS Comput Biol*, in press

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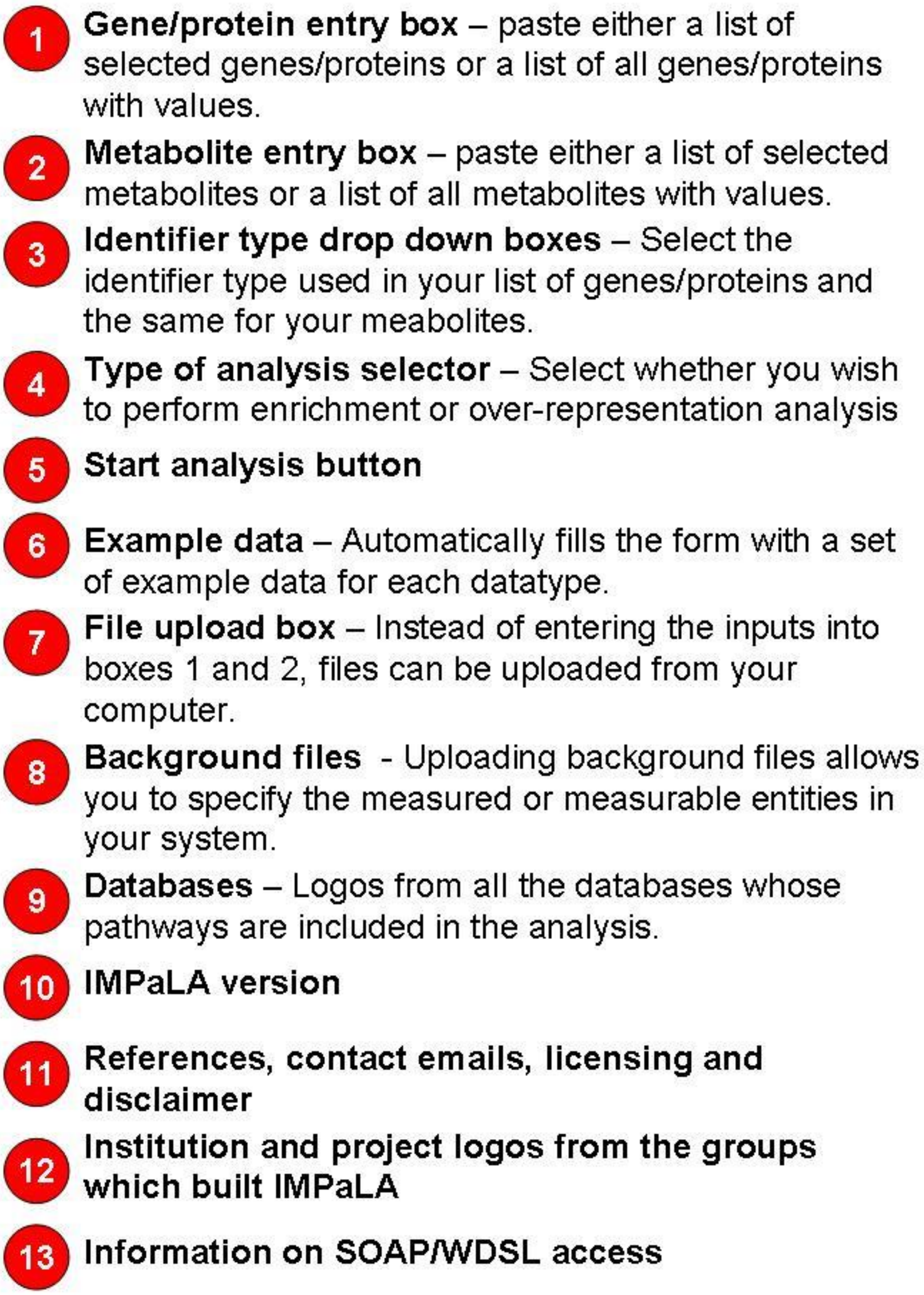
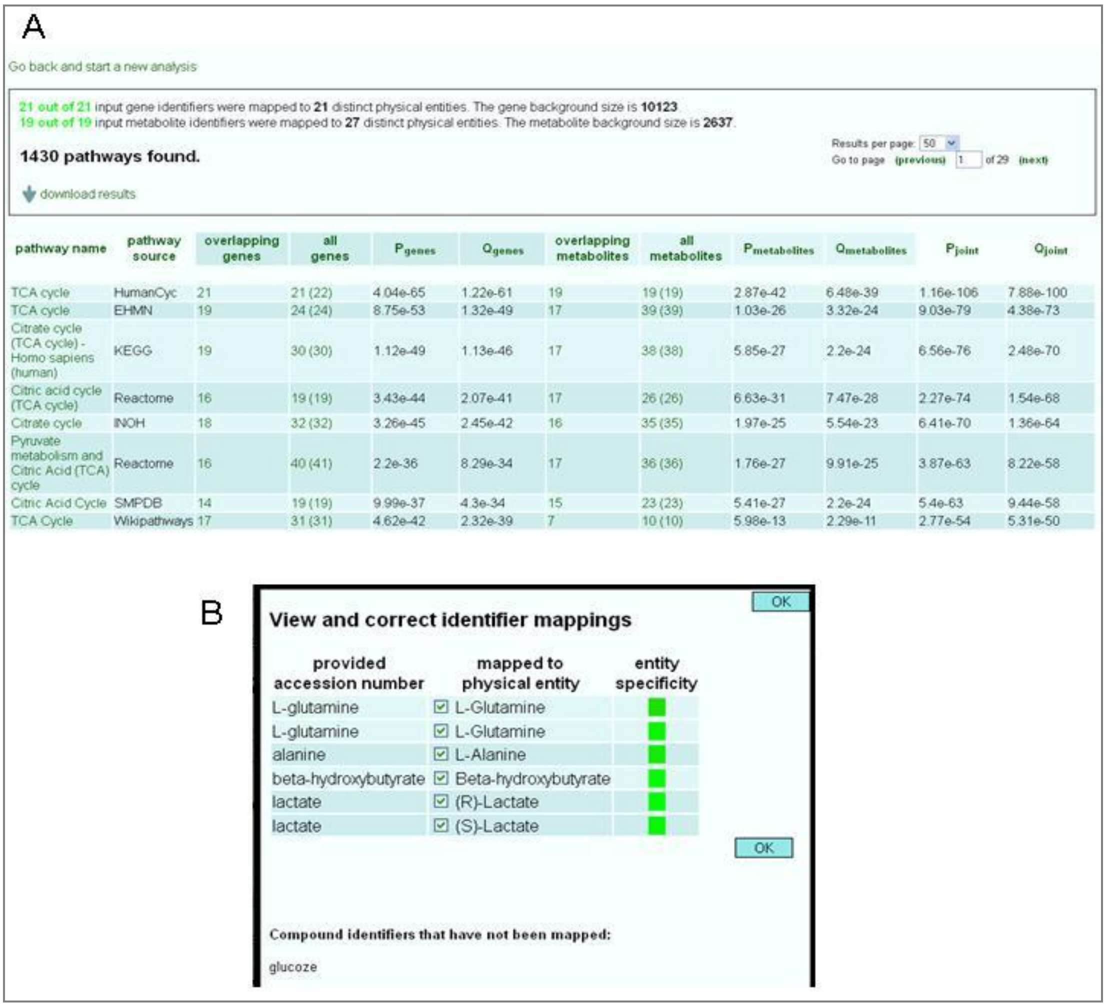
Figure 1 - Screenshot from IMPaLA with important features numbered (see next page for key)



1. **Gene/protein entry box** – paste either a list of selected genes/proteins or a list of all genes/proteins with values.
2. **Metabolite entry box** – paste either a list of selected metabolites or a list of all metabolites with values.
3. **Identifier type drop down boxes** – Select the identifier type used in your list of genes/proteins and the same for your meabolites.
4. **Type of analysis selector** – Select whether you wish to perform enrichment or over-representation analysis
5. **Start analysis button**
6. **Example data** – Automatically fills the form with a set of example data for each datatype.
7. **File upload box** – Instead of entering the inputs into boxes 1 and 2, files can be uploaded from your computer.
8. **Background files** - Uploading background files allows you to specify the measured or measurable entities in your system.
9. **Databases** – Logos from all the databases whose pathways are included in the analysis.
10. **IMPaLA version**
11. **References, contact emails, licensing and disclaimer**
12. **Institution and project logos from the groups which built IMPaLA**
13. **Information on SOAP/WDSL access**

Figure 2 – Key to figure 1.

2.2 IMPaLA output screen



Go back and start a new analysis

21 out of 21 input gene identifiers were mapped to **21** distinct physical entities. The gene background size is **10123**.
19 out of 19 input metabolite identifiers were mapped to **27** distinct physical entities. The metabolite background size is **2637**.

1430 pathways found.

Results per page: 50
Go to page (previous) 1 of 29 (next)

download results

pathway name	pathway source	overlapping genes	all genes	P_{genes}	Q_{genes}	overlapping metabolites	all metabolites	$P_{metabolites}$	$Q_{metabolites}$	P_{joint}	Q_{joint}
TCA cycle	HumanCyc	21	21 (22)	4.04e-65	1.22e-61	19	19 (19)	2.87e-42	6.48e-39	1.16e-106	7.88e-100
TCA cycle	EHMN	19	24 (24)	8.75e-53	1.32e-49	17	39 (39)	1.03e-26	3.32e-24	9.03e-79	4.38e-73
Citrate cycle (TCA cycle) - Homo sapiens (human)	KEGG	19	30 (30)	1.12e-49	1.13e-46	17	38 (38)	5.85e-27	2.2e-24	6.56e-76	2.48e-70
Citric acid cycle (TCA cycle)	Reactome	16	19 (19)	3.43e-44	2.07e-41	17	26 (26)	6.63e-31	7.47e-28	2.27e-74	1.54e-68
Citrate cycle	INOH	18	32 (32)	3.26e-45	2.45e-42	16	35 (35)	1.97e-25	5.54e-23	6.41e-70	1.36e-64
Pyruvate metabolism and Citric Acid (TCA) cycle	Reactome	16	40 (41)	2.2e-36	8.29e-34	17	36 (36)	1.76e-27	9.91e-25	3.87e-63	8.22e-58
Citric Acid Cycle	SMPDB	14	19 (19)	9.99e-37	4.3e-34	15	23 (23)	5.41e-27	2.2e-24	5.4e-63	9.44e-58
TCA Cycle	Wikipathways	17	31 (31)	4.62e-42	2.32e-39	7	10 (10)	5.98e-13	2.29e-11	2.77e-54	5.31e-50

View and correct identifier mappings

provided accession number	mapped to physical entity	entity specificity
L-glutamine	☑ L-Glutamine	🟩
L-glutamine	☑ L-Glutamine	🟩
alanine	☑ L-Alanine	🟩
beta-hydroxybutyrate	☑ Beta-hydroxybutyrate	🟩
lactate	☑ (R)-Lactate	🟩
lactate	☑ (S)-Lactate	🟩

Compound identifiers that have not been mapped:

glucoze

Figure 3 – IMPaLA outputs: A Full output table (using example data link figure 1, item 6) **B** Checking and correcting identifier mappings when metabolite names were entered.

3. Using IMPaLA with lists of selected genes/proteins and/or metabolites

3.1 Step-by-Step input

1. Create a list/s of genes/proteins and/or metabolites from your data. These lists should typically contain genes with significantly altered expression or metabolites with significantly altered concentration
2. Copy these lists into boxes 1 and 2 as shown in figure 1.
3. Select the Identifier types which you used in the lists from the drop-down boxes marked 3 in figure 1.
4. Select **pathway over-representation analysis** from 4 in figure 1.
5. Click the **START ANALYSIS** button shown as 5 in figure 1.
6. Wait for the results table to appear, this should take 10-15 seconds depending on the input size, internet connection, and on how heavily used the tool is at that moment.

3.2 Step-by-step Understanding the output

1. **Check the mappings for your identifiers**; at the top of the ouptut screen (figure 3A), the mapping performance is shown. The color of the text "**X out of Y** input gene/metabolite identifiers..." scales from green to red according to the mapping performance (green=many of the provided identifiers have been mapped to physical entities, i.e. genes/proteins or metabolites, available in pathways). If you click on the text "**X out of Y** input gene/metabolite identifiers", then the popup dialog shown in figure 3B appears.
2. **View and correct the mapping (Figure 3B)**; if incorrect mappings are made, then you can deselect them here. If mappings are missed, or identifiers have not been mapped due to entry errors (see "glucoze" at the bottom of figure 3B), then you can use the link at the top of the results page to "go back and start a new analysis" and correct your input. The entity specificity column (color scaling from green, specific to red, non-specific) helps you spot e.g. currency metabolites that are not pathway-specific (such as ATP) and it might be a good idea to exclude them. If you mouse-over the colored area, you will get information about the number of pathways the according entity is present in.
3. **Reading the results table**; for each pathway found, the following columns are in the results table:
   a. **Pathway name –**
      Pathway name; clicking on the name links to the pathway's original source, containing a detailed pathway diagram if supported by the source database
   b. **Pathway source** – Name of the database in which the pathway is found.
   c. **Gene columns** – If genes/proteins were entered into the input then the next 4 columns are about the results of the analysis with just the genes/proteins.
      i. **Overlapping genes** – Gives the number of your entered genes which are found in that pathway.
      ii. **All genes** – Gives the total number of genes in the pathway, with the number found in the background list given in brackets.
      iii. $\mathbf{P_{genes}}$ – A P-value for the pathway based only on the genes.
      iv. $\mathbf{Q_{genes}}$ – The False Discovery Rate which results from correcting the P-values for multiple testing using the method set out by Benjamini and Hochberg.
   d. **Metabolite columns –** If metabolites were entered into the input then they have an identical set of 4 columns to the genes, showing the results of the analysis with just the metabolites.
   e. **Joint columns –** If both genes/proteins and metabolites were input, then joint P-values and Q-values are calculated for each pathway. Evidence for pathway de-regulation from the provided genes and metabolites is treated independently, thus the according probabilities (P-values) are integrated with Fisher's method to obtain the joint P-value. By default the table is sorted on these columns.

4. **Sorting the table by other columns;** Click any column title to sort the table by the values in that column.

Downloading the data; Just above the results table is a “download results” link, which downloads a .csv file (comma separated value file) that can be read by Excel or other programs.

4. Using IMPaLA with continuous values for all genes/proteins/metabolites

With continuous values for all the genes/proteins/metabolites, IMPaLA can perform Wilcoxon pathway enrichment analysis. The continuous values entered could be correlation coefficients to a phenotype of interest, fold changes of expression between two groups, results from t-tests or any of the many other values which emerge from analysis. The input data should contain all measured entities, with either one or two values per entity (one value: e.g. expression difference; two values: e.g. mean expression in two phenotypes).
Important – To get meaningful results the list of values must contain **both** positive and negative values. If you are using a continuous measure that is all positive (or all negative) then centre the data first, by subtracting the mean of each value from all values..

Top Tip! Since you will be entering an identifier plus value for each item in you have measured, you will have a much longer input than with the over-representation analysis, prepare your inputs as files and use the file upload facility (figure 1, number 7) to send your data to the IMPaLA server.

To run the analysis, follow the step-by-step instructions in section 3.1 except in step 4 select “Wilcoxon pathway enrichment analysis”.

Background lists in Wilcoxon enrichment analysis

Unlike over-representation analysis, there is no need to upload a background list for the Wilcoxon analysis because the Wilcoxon test applied here is essentially different from the hypergeometric test..

Top Tip! When preparing data for Wilcoxon analysis, make sure each element is only listed once. If an element features in the list repeatedly a warning message will appear, and the mean value of all those supplied per identifier will be used.

5. Case Study

Using data freely downloaded from the NCI60 website (http://dtp.nci.nih.gov/ mtargets/download.html) with baseline transcriptomics measurements of all genes (affymetrix U133 chip) and accompanying values for the GI50 (dose level at which the growth of the cell line is inhibited by 50%) for doxorubicin, I performed a wilcoxon enrichment analysis of those pathways which are enriched in cell lines which are particularly resistant or sensitive to doxorubicin.

5.1 Step-by-Step

- I combined all the data into a single excel sheet, by pasting the doxorucin GI50 for each cell line above the column of gene expression readings for the cell line. (see the first sheet in preparing_doxorubicin_data_for_wilcoxon_analysis.xlsx) and calculated the correlation between each probeset and the doxorubicin GI50 values.
- Next I copied and pasted just the gene name identifier and the correlation columns to a separate sheet (using the paste special command to paste the values from the correlation calculations rather than the formulae).
- In order to summarise all the probesets which pertain to each gene name into a single value I used a pivot table. In excel click insert> pivot table, using the data from this second sheet as input. I then placed "gene" in the row labels section of the pivot table, and "correlation to doxorubicin" in the values section. By default the values will use the sum of all correlations, but for this purpose we want the average value, right clicking on the top of the column in the pivot table, give the "summarise by" option in the menu, where "average" can be selected.
- I then copied and pasted this data into dialog box in impala, selecting wilcoxon analysis and gene symbols as the input type, and obtained the results shown in "enrichment_results_doxorubicin.csv".

6. Frequently Asked Questions

- *I want to do over-representation analysis – how do I make my list of selected items?*
    - The list of selected items for over-representation analysis is usually the result of a statistical test on your data. For instance those items which show statistically significant differential expression in a t-test between two phenotypes, or whose values are significantly correlated with a phenotypic value of interest.
      Top Tip! It might be useful to check the validity of your analysis by making a minimum of 10-20 random lists of items, where each list is the same length as the list you entered with your real data – check that the pathways which come out are different each time. If you are always getting the same pathways at the top of your analysis, then you have some bias in your analysis. To deal with this bias, try using summary values for each gene/protein/metabolite before selecting the list and using background lists which contain all the items you have measured.

- *Which analysis type should I use with my data?*
    - Which analysis type to use depends on what data you have and the questions you want to ask, however for most biological problems both types of analysis will be applicable. Over-representation analysis will be much faster through the website as you will have to

upload a much smaller amount of data. However, the disadvantage with over-representation is that once an item passes your statistically signifcant threshold and is included in the list, it will be treated with identical weight to all the other items in the list. Therefore, it is important to use multiple-testing correction when selecting your threshold, so that your list does not contain too many false-positives.

- *The identifiers I have for my genes/proteins/metabolites are not in your list of identifiers, how can I use your tool?*
    - IMPaLA supports all popular identifier types for genes and metabolites, but there are many identifier types out there. There are many free tools available for changing between different identifier types. Bridgedb (www.bridgedb.org) and BioMart (http://www.biomart.org), and the metabolite ID conversion module of MSEA (http://www.msea.ca) are useful tools for doing this. Please also let us know the identifier types which you use if they are missing, and we may be able to add them into the next version.

- *I used IMPaLA a few months ago, and now with the same data I get a slightly different answer – what's going on?*
    - IMPaLA updates its pathways knowledge base every three months, based on the latest versions of its source databases. If a source database changes the pathway definition, then the results from the analysis will change. Always note the version number and build number of IMPaLA when you do the analysis, then it will be clear when IMPaLA has changed since your last visit. Also note, we are always working to extend the databases used by IMPaLA to increase its pathway coverage, so with new versions, new pathways may also appear in your analysis.

- *Why should I use a background list for over-representation analysis?*
    - A background list should contain all the elements which you have measured, or in cases where you have only identified the differentially expressed elements, a list of what would typically be measurable by your technique in that sample type. Background lists are particularly important when you have only measured a small percentage of the elements of that type in the source databases. For instance, if I just enter 4 amino acids; alanine, proline, glutamine and lactate, then I get a p-value of 3.64e-08 for the Amino acid and oligopeptide SLC transporters from Reactome. However, if all I measured was the amino acids, then my background is much smaller than all the metabolites in the database, so I get a much less significant p-value of 0.293.

- *I have lots of scenarios which I want to test, do you provide a programming interface to IMPaLA?*
    - Yes, there is a well documented programming interface available, see the link about SOAP/WDSL access shown at number 13 in figure 1.

- *How do you cope with ambiguity in metabolite names? If I enter Lactate, will it map to R-lactate or S-lactate?*
    - Lactate will map to both R- and S- forms, see figure 3B. To specify just one form of a chiral metabolite enter the name for that speficic form. To check which entities your names have mapped to, click on the “X out of Y input identifiers” text at the top of the results screen (figure 3A) and you will get the dialog seen in figure 3B. If the mappings are not what you desire, then you can use the link at the top of the screen to return to the input screen and re-enter your amended indentifiers.

- *What are the forumulas used to make the calculations?*
    - For over-representation analysis the formula is:

$$\forall i \in pathways, \; p_i = \sum_{j=k_i}^{\min(K,N_i)} \frac{\binom{N_i}{j}\binom{M-N_i}{K-j}}{\binom{M}{K}}$$

Where Ni is the effective size of pathway i, M is the overall number of genes or metabolites available to be picked, K is the number of genes or metabolites picked and ki is the number of genes or metabolites picked from the pathway.

For Wilcoxon analysis x_j, y_j denote the expression level of the j-th gene in the pathway. The differences $d_j = x_j - y_j$ are calculated for each pathway and ranked. The ranks of differences with negative signs, R_{neg}, and those with positive signs, R_{pos}, were summed. The test statistic is based on, $R = \min\{R_{\text{pos}}, R_{\text{neg}}\}$. The Z-score is defined as $z = |R - E(R)| / \sqrt{Var(R)}$ where $E(R) = n_i(n_i + 1)/4$ and $Var(R) = [n_i(n_i + 1)(2n_i + 1)]/24$, respectively

- *I have a great pathway database – can it be included in IMPaLA?*
    - Let us know about it, and we will consider it for integration.

If you have further questions, do not hesitate to contact us at kamburov@molgen.mpg.de and rachel.cavill@maastrichtuniversity.nl.