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Galaxy Integrated Omics: Web-based Standards-Compliant Workflows for Proteomics Informed by Transcriptomics*§

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With the recent advent of RNA-seq technology the proteomics community has begun to generate sample-specific protein databases for peptide and protein identification, an approach we call proteomics informed by transcriptomics (PIT). This approach has gained a lot of interest, particularly among researchers who work with nonmodel organisms or with particularly dynamic proteomes such as those observed in developmental biology and host-pathogen studies. PIT has been shown to improve coverage of known proteins, and to reveal potential novel gene products. However, many groups are impeded in their use of PIT by the complexity of the required data analysis. Necessarily, this analysis requires complex integration of a number of different software tools from at least two different communities, and because PIT has a range of biological applications a single software pipeline is not suitable for all use cases. To overcome these problems, we have created GIO, a software system that uses the well-established Galaxy platform to make PIT analysis available to the typical bench scientist via a simple web interface. Within GIO we provide workflows for four common use cases: a standard search against a reference proteome; PIT protein identification without a reference genome; PIT protein identification using a genome guide; and PIT genome annotation. These workflows comprise individual tools that can be reconfigured and rearranged within the web interface to create new workflows to support additional use cases. Molecular & Cellular Proteomics 14: 10.1074/mcp.O115.048777, 3087–3093, 2015.

Searching MS/MS spectra against a list of proteins that could be present in the sample remains the pre-eminent method for identifying proteins in liquid chromatography tandem mass spectrometry (LC-MS/MS)¹ shotgun proteomics. The relevance and quality of this protein database, as it is known, has been shown to have a significant impact on the outcome of a proteomics study (1), but even for well-studied model organisms the true set of proteins that might be expressed is debatable. For example, large scale human proteome mapping projects have recently suggested that commonly used human protein databases may include superfluous proteins while simultaneously omitting proteins for which there is evidence of expression (2, 3). For lesser studied organisms the situation is worse still, as available protein databases rely even more on computational prediction, and in some species even this is not possible as a reference genome has not yet been assembled. This is a significant problem in fields such as virology, where the disease vectors (e.g. mosquitoes, lice, ticks, birds, and bats) have poorly annotated genomes or no genome data at all. Similar challenges can be found in metaproteomics (4), where individual samples contain proteins from multiple organisms, some of which may be unidentified or not previously sequenced.

Recently, we sought to mitigate these problems by developing a methodology called proteomics informed by transcriptomics (PIT) (5) in which sample-specific protein databases are generated from transcripts that have been identified in the same sample using RNA-seq (6). These transcripts can be assembled from short reads either by mapping to a genome or entirely de novo (e.g. if no suitable genome exists). Although we showed that PIT could provide valuable new

¹ The abbreviations used are: LC-MS/MS, Liquid chromatography tandem mass spectrometry; CID, Collision induced dissociation; FDR, False discovery rate; GIO, Galaxy Integrated Omics; ORF, Open reading frame; PAG, Protein ambiguity group; PIT, Proteomics informed by transcriptomics; RNA-seq, RNA sequencing; SILAC, Stable isotope labelling of amino acids in cell culture; TSV, Tab separated values.
Galaxy Integrated Omics (GIO)

insights into the systems being studied, the complexity of the software pipeline needed to integrate the RNA-seq and proteomic MS/MS data made it difficult to configure the method for different applications and to adapt it to make use of new transcriptomic and proteomic tools. In this paper we introduce our solution to these challenges—a publicly available standards-compatible system called GIO (Galaxy Integrated Omics) that uses the popular Galaxy platform (7) to make flexible PIT workflows available via an easy to use web interface. We explain the workflows, the GIO implementation of these workflows and validate the efficacy of this software using a matched RNA-seq and LC-MS/MS dataset.

EXPERIMENTAL PROCEDURES

Software Implementation—

Use of the Galaxy Platform—Galaxy (7) is a highly customizable server-based bioinformatics platform that has already amassed a large following among the genomics community as a framework within which complex analysis of large data sets can be easily conducted in a repeatable way by non-bioinformaticians. It provides a powerful web interface through which data can be uploaded, tools executed, and workflows built, inspected, executed, and shared. Because all the necessary tools and workflows reside on a centrally managed server the need for users to download, install, and configure software is avoided. This makes Galaxy an obvious platform for PIT because PIT requires integration of a wide range of individual tools from different domains. The simple concept behind Galaxy is that any command line tool can be added to it by writing a wrapper that lists the tool’s parameters, specifies its inputs and outputs, and defines the command line syntax used to run the tool. Galaxy uses this wrapper to automatically create a user interface for each tool, substituting the tool’s command line interface with a simple web-based form. Because they define each tool’s input and output formats, wrappers also allow tools to be linked together into workflows. Wrappers allow a tool to be incorporated into Galaxy without changing the tool’s program code, regardless of the programming language in which the tool was written. Although it is possible to make tools and workflows available for administrators of Galaxy servers to download and install via a repository called Galaxy Tool Shed, configuring all the necessary components can be complicated so we chose instead to provide our workflows via a dedicated customized version of Galaxy containing all relevant tools and workflows. A demonstration of this customized Galaxy version, which we call Galaxy Integrated Omics (GIO), is freely accessible at gio.sbsc.qmul.ac.uk. We also maintain a repository of the core GIO components, together with an automated installation program, on GitHub (https://github.com/wizardfan/gio-repository) so that groups who wish to add GIO functionality to their own Galaxy server can do so.

Choice of Tools—There is a large and growing range of existing command line proteomics tools available, offering a range of functionality under various licensing arrangements (e.g., protein quantitation tools have recently been reviewed (8)). For GIO we have selected a subset of tools that covers the steps needed to construct PIT workflows. These tools were chosen based on their perception within the community and their ability to support data standards (see next section). The key transcriptomic tools were already available in Galaxy Tool Shed, as were some of the proteomics tools (wrapped by the GalaxyP project (9)). Given the difficulties of definitively benchmarking the efficacy of individual tools it is impossible to be certain that our selection is optimal, but the modular nature of Galaxy makes it straightforward to add new tools, to replace individual workflow steps in the future as better tools become available. Because the PIT workflows contain novel functionality it was necessary to implement a number of new tools, which are distinguished in GIO by names prefixed with “PIT:” These tools include a bespoke ORF finder called PIT:ORFAll, and PIT:Integrative for bringing transcriptomic and proteomic data together using our previously described method (5). Some of the PIT tools are themselves mini-workflows that combine workflow steps that are commonly used together. For example, PIT:PSM PostProcessing sequentially runs three individual tools from mzidentML-lib (10) to determine PSM level FDR scores, remove peptides below a user-defined threshold, and map peptides to proteins. Such meta-tools simplify the creation of workflows, improving their readability and reducing the chance of errors.

Data Standards—One of the prerequisites for building workflows is that individual tools must be compatible such that the output of one tool can be used as the input to another. To ensure this, GIO supports PSI data standards (10). In particular, we provide a number of tools from mzidentML-lib (11) to support the manipulation of the mzidentML (12) files. For spectral data, GIO supports the PSI standard mzML (13) format. Data in other formats can be converted to mzML using ProteoWizard’s MSConvert (14) within GIO. Because the libraries needed to convert proprietary raw binary data formats are only available for Microsoft Windows, MSConvert has been configured to run within a Windows virtual machine on the GIO server, which is accessed by GIO using Pulsar (15).

Data Processing Workflows—We have developed three primary workflows for analyzing data from PIT experiments, and a standard identification workflow for comparison purposes. Each of these workflows is available to view, edit, and execute on the GIO server (gio.sbsc.qmul.ac.uk) where detailed tutorials are also provided. Applying a workflow to a given data set is simply a case of uploading and selecting which data files to use, selecting the workflow, and clicking the execute button to run the workflow. All workflow parameters (e.g., false discovery rate, threshold) can be set by the user, and workflows can be substantially modified or created from scratch using the available tools (e.g., multiple different tools are offered for peptide spectrum matching via a tool called “Peptide spectrum matching” that wraps SearchGUI (16)).

Standard Identification Workflow—Included primarily for testing and comparison purposes, this is a standard protein identification workflow for comparison purposes. Each of these workflows is available to view, edit, and execute on the GIO server (gio.sbsc.qmul.ac.uk) where detailed tutorials are also provided. Applying a workflow to a given data set is simply a case of uploading and selecting which data files to use, selecting the workflow, and clicking the execute button to run the workflow. All workflow parameters (e.g., false discovery rate, threshold) can be set by the user, and workflows can be substantially modified or created from scratch using the available tools (e.g., multiple different tools are offered for peptide spectrum matching via a tool called “Peptide spectrum matching” that wraps SearchGUI (16)).

PIT Workflow without Reference Genome—This is a development of the standard identification workflow in which one of the inputs is a list of de novo assembled transcripts instead of a protein list. To reduce load on the public GIO server, we request that the transcript list is assembled from acquired RNA-seq data outside of GIO using a stand-alone tool such as Trinity (18) and then imported into GIO, but on a locally installed GIO installation the transcript assembly step can
be fully integrated into the workflow. The first step of the workflow (shown in Fig. 1) produces a protein database comprising the longest open reading frames (ORFs) found within all six reading frames of each transcript. This database is then used in the peptide spectrum matching step, which is followed by the same post processing as in the previous workflow. Because the ORFs identified from this process are unannotated sequences, further post processing is needed to infer what they may be. This takes place in the final part of the workflow, where all identified ORFs are BLASTed to find homologous proteins in selected species. The final result of the workflow is therefore a tabular file containing a list of ORFs for which peptide evidence has been found, together with an indication of the closest homologous protein in the selected species. This allows for the analysis of a sample from a species for which a reference genome is not available, by postidentification comparison with proteins in a closely related species. It also facilitates metaproteomics where proteins from two or more species (some of which may be unidentified) are present in the sample.

**PIT with Reference Genome**—If the species under study has a well annotated reference genome then instead of de novo transcript assembly, transcript sequences can be determined by mapping the acquired RNA-seq data to the reference genome using TopHat (20) and assembling using Cufflinks (21). This functionality is provided by a dedicated GIO workflow. In this workflow, instead of assembled transcripts, inputs are RNA-seq FASTQ files containing quality controlled forward and reverse reads. The first step in the workflow uses TopHat to map short reads to the genome of the species under study. This produces a BAM mapping file which is passed to the next step in which Cufflinks assembles the reads into transcripts. These transcripts are then used, as in the other workflows, to generate ORFs against which the acquired MS spectra are searched. Downstream processing also follows the same process as described previously for the other workflows. This workflow is shown schematically in supplemental Fig. S3 in the supplementary materials.

**Workflow Evaluation**—Workflows were evaluated by applying them to previously published (5) matched sets of RNA-seq and proteomic MS/MS data acquired from HeLa cells infected with adenovirus. This data represents a good case study because the samples contain proteins from at least two species, and the data has already been extensively analyzed using established tools so there is an opportunity to compare results. Furthermore, the human proteome is arguably the best annotated of any species, so it is possible to make a homology search so a homology search can be added at the end of this workflow to provide information as to what the identified proteins may be. This workflow is shown schematically in supplemental Fig. S2 in the supplementary materials.
A peptide level FDR threshold of 1% was applied throughout, and tolerances of 10 ppm for precursor ions and 0.8 Da for product ions. Lys and 13C6-Arg for medium) as variable modifications, essentially acquired MS/MS data against ORFs derived from Trinity benchmark against which to evaluate the other workflows. The results of this standard workflow are used below as a data using the Andromeda (22) search engine via MaxQuant.

Because the purpose of this study is protein identification, peptide spectrum matching was performed with the SILAC modifications (13C6-Lys and 13C6-Arg for heavy labeled samples and 13C6-Lys and 13C6-Arg for medium) as variable modifications, essentially treating the data as a pooled sample. Oxidation of methionine was also included as a variable modification, and carbamidomethylation of cysteine as a fixed modification. Peptide spectrum matching was performed for fully tryptic peptides, with any reasonable number of missed cleavages (according to the MSGF + algorithm), and mass tolerances of 10 ppm for precursor ions and 0.8 Da for product ions. A peptide level FDR threshold of 1% was applied throughout, and only proteins with at least two identified peptides were retained. All data used in this article is available as a Data Library within GIO.

### RESULTS

**Standard Identification Workflow**—Searching the acquired MS/MS data against a FASTA file concatenated from the complete Uniprot proteomes of human (including isoforms) and adenovirus type 5, 26,401 peptides were found by MSGF+, which mapped to 3,015 proteins (2907 human and 18 human adenovirus). These figures (provided in Table I), and the list of identified proteins that they represent, are comparable with those obtained from the original analysis (5) of the data using other tools. Table II shows more detailed information about the number of peptides searched against in each case, and the peptide identifications that were unique to each search. This shows that the majority of peptides that were only found in one search were because of the absence of the peptide from the database used for the other search. The remaining nonoverlapping peptide identifications were because of other differences in the protein database, including the presence of sample-specific peptides in the PIT database that get matched to spectra that would otherwise be matched to peptides from Uniprot, and vice versa. It should also be noted that the peptide identifications were counted after protein grouping, which also affects which peptide identifications are retained.

Overlapping at the protein level is more difficult to define, partly because proteins are longer so the chance of an exact match between a sample-specific ORF and a Uniprot protein are lower than they would be at the peptide level, but also because peptides are assigned to protein ambiguity groups (PAGs) rather than distinct proteins. A PAG contains one or more proteins that are supported by a shared set of peptide evidence, with the protein with the most evidence being designated as an anchor protein that represents the PAG. Often several members of a PAG have equal peptide evidence, e.g. they may be isoforms, so the anchor is selected using some other criteria (e.g. in alphabetical order of their ID in the case of mzIdentML-lib’s ProteoGrouper). Thus, when comparing lists of protein identifications from searches against different
databases there are several reasons why anchor proteins may not match, even if the identified proteins are essentially the same. For the purposes of this comparison, a BLAST e-value below $1 \times 10^{-30}$ (widely regarded as indicating strong homology between sequences) was taken to indicate a match between identified sequences. Using this threshold, a total of 2768 matches were found between PAGs from the standard protein identification workflow and PAGs from the PIT workflow (1955 anchor–anchor matches plus a further 813 matches between anchors and PAG submembers).

No match could be found between 247 of the PAGs from the standard workflow and the list of PAGs from the PIT workflow, i.e. 247 proteins were unique to the Uniprot search. Further analysis of these proteins was performed outside of GIO to understand why they were missed by the PIT search. Fourteen of the proteins were found to be isoforms or submembers of the overlapping PAGs. No transcript evidence could be found for the genes coding for 85 of the proteins (2.8% of the total identified by the standard workflow)—clearly PIT alone is unable to detect such proteins because the protein database is created purely from transcripts. Most of these proteins (71 out of 85), were also missing from the genome guided PIT workflow, suggesting either that the genes were not being expressed in the sample (possible because proteins can persist in a cell longer than the mRNA that was used to create them) or the RNA-seq depth was too shallow to detect them. The remaining 14 missing proteins can be attributed to Trinity’s failure to assemble a small number of transcripts. Transcript evidence could be found for the remaining 148 proteins, but the ORF finding tool had either failed to produce ORFs from these transcripts or had produced ORFs that were not sufficient matches to proteins—this suggests an area for improvement in this aspect of the PIT methodology.

A total of 158 proteins were only found in the PIT search. On further inspection, 139 of these were found to match to the Uniprot proteome but were not found by the standard search, mostly because each protein only had single peptide evidence in that search, whereas two or more peptides were found by PIT. This often occurred because of a longer ORF sequence compared with the Uniprot protein, where the extra peptide matched to the extended region, suggesting potential deficiencies in the UniProt reference proteome that was used. The remaining 19 identified ORFs did not have significant homology to any known protein so have potential for future investigation as novel translated genomic elements—these ORFs, and the evidence for them, can be found in the supplementary data.

### Table II

| Method                              | Peptides derived from database | Peptide identifications unique to this search | Unique identifications due to absence of peptide in other database |
|-------------------------------------|-------------------------------|---------------------------------------------|---------------------------------------------------------------|
| Standard (human and adenovirus)    | 11,636,910                    | 1,883                                       | 1,878 (99.7%)                                                |
| PIT–de novo assembly                | 7,843,921                     | 1,389                                       | 813 (58.5%)                                                  |
| Standard (human only)               | 11,627,809                    | 5,215                                       | 5,187 (99.4%)                                                |
| PIT–genome guided assembly          | 7,095,081                     | 846                                         | 552 (65.2%)                                                  |

**PIT Workflow for Genome Annotation**—The genome annotating workflow was successfully applied to annotate the human genome with transcripts and peptides identified from the acquired RNA-seq and proteomic MS data. An example section of the GFF3 file visualized in the Genoverse (23) genome browser is shown in supplemental Fig. S4. Obviously the GFF3 file does not provide a complete annotation of the human genome, as only genes and proteins expressed in...
the individual analyzed sample are shown but it does allow for
the rapid visualization of peptide evidence in a genomic con-
text and the localization of putative novel translated genomic
elements.

**PIT Workflow with Reference Genome**—Searching the ac-
quired MS/MS data against ORFs derived from transcripts
assembled by Cufflinks and TopHat using reference genome
hg38, 21,601 peptides were found, which mapped to 2713
PAGs, whereas the standard workflow identified 2986 PAGs
when only human proteins were used. Using the homology
search tool included in this workflow, 2676 of these ORFs
were identified as human proteins. Because this workflow
used mapping to a human reference, adenovirus proteins
were not considered.

Comparing the PIT results against those obtained using
the standard workflow, there is an overlap of 77% at the
peptide level. A visual representation of these results is
shown in Fig. 3.

Using the same PAG comparison method as in the previous
section, a total of 2435 matches were found between PAGs
from the standard protein identification workflow and PAGs
from the PIT workflow (1630 anchor–anchor matches plus a
further 805 matches between anchors and PAG submem-
bers). The reduced overlap at both peptide and PAG level,
when compared with the previous workflows, is mostly likely
because Cufflinks produced fewer transcripts than Trinity (see
Table I), resulting in a significantly smaller number of ORFs in
the search database.

No match could be found between 551 of the PAGs from
the standard workflow and the list of PAGs from the PIT
workflow. However, isoforms of two of these proteins overlap
with the PIT search. No transcript evidence could be found for
246 of these proteins. A total of 278 PAGs were only found in
the PIT search. Of these, 241 ORFs had homology to human
Uniprot proteins but were not identified by the standard
search, mostly because these proteins had single peptide
evidence in the standard search.

**DISCUSSION**

Through the creation of GIO, we have demonstrated that
Galaxy is an extremely effective platform for putting powerful
integrative data analysis workflows into the hands of re-
searchers. The PIT results obtained using GIO are very similar
to those obtained previously from the same data using a
bespoke hard-coded pipeline composed of different tools.
The principal innovation is that the data analysis can now be
reliably carried out by a typical bench biologist via a web
interface without installing a single piece of software, whereas
the original analysis required many months of programming
and command line expertise. Although we have provided four
reference workflows for PIT analysis, GIO permits huge flex-
ibility in terms of generation of bespoke workflows for differ-
ent applications. For example, users can experiment with
different parameters, different tools for certain steps (e.g.
peptide spectrum matching, post processing), or generate
their own hybrid protein databases to search against using file
manipulation tools that are already built in to Galaxy. Ad-
vanced users who install Galaxy on their own server are able
to customize workflows even further, adding their application-
specific downstream analysis tools for example.

In future, we plan to add further tools and workflows to GIO,
to bring other complex proteomics data processing tasks
within reach of the typical bench biologist. Priorities include
tools for interpreting the results of PIT experiments, e.g.
to permit fine grained comparison of mzIdentML files from dif-
ferent searches and to classify potentially novel translated
genomic elements.

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**Fig. 3. Comparison of peptides and protein groups identified by a stand-
adard protein identification workflow in which mass spectra were searched
against Uniprot, and a PIT workflow using a human reference genome, for
HeLa cells infected with adenovirus.**
Because a human reference genome was used, only human protein identifica-
tions are included in these results.
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