

Exploring the single-cell RNA-seq analysis landscape with the scRNA-tools database

Luke Zappia (1, 2)

Belinda Phipson (1)

Alicia Oshlack (1, 2)

1 Bioinformatics, Murdoch Children's Research Institute; 2 School of Biosciences, University of Melbourne

Abstract

As single-cell RNA-sequencing (scRNA-seq) datasets have become more widespread the number of tools designed to analyse these data has dramatically increased. Navigating the vast sea of tools now available is becoming increasingly challenging for researchers. In order to better facilitate selection of appropriate analysis tools we have been cataloguing and curating new analysis tools, as they become available, in the scRNA-tools database (www.scRNA-tools.org). Our database collects a range of information on each scRNA-seq analysis tool and categorises them according to the analysis tasks they perform. Exploration of this database gives insights into the areas of rapid development of analysis methods for scRNA-seq data. We see that many tools are developed to perform tasks specific to scRNA-seq analysis, particularly clustering and ordering of cells. We also find that the scRNA-seq community embraces an open-source approach, with most tools available under open-source licenses and preprints being extensively used as a means to describe methods. The scRNA-tools database provides a valuable resource for researchers embarking on scRNA-seq analysis and as a record of the growth of the field over time.

Keywords

Introduction

Single-cell RNA-sequencing (scRNA-seq) has rapidly gained traction as an effective tool for interrogating the transcriptome at the resolution of individual cells. Since the first protocols were published in 2009¹ the number of cells profiled in individual scRNA-seq experiments has increased exponentially, outstripping Moore's Law². This new kind of transcriptomic data brings a demand for new analysis methods. Not only does the scale of scRNA-seq datasets vastly outstrip bulk experiments but there are also a variety of challenges unique to the single-cell context³. Specifically, scRNA-seq data is extremely sparse (there is no expression measured for many genes in most cells), it can technical artefacts such as low-quality cells or differences between sequencing batches and the scientific questions of interest are often different to those asked of bulk RNA-seq datasets. For example many bulk RNA-seq datasets are generated to detect differentially expressed genes through a designed experiment while many scRNA-seq experiments aim to identify or classify cell types.

The bioinformatics community has embraced this new type of data, designing a plethora of methods for the analysis of scRNA-seq data. As such, keeping up with the current state of scRNA-seq analysis is now a significant challenge as the field is presented with a huge number of choices for approaching an analysis. Since September 2016 we have collated and categorised scRNA-seq analysis tools as they have become available. This database is being continually updated and is publicly available at www.scRNA-tools.org. In order to help researchers navigate the analysis jungle we discuss the stages of scRNA-seq analysis and their relationship to tools and categories in the scRNA-tools database. Through the analysis of this database we show trends in not only the analysis applications these methods address but how they are published, licensed and the platforms they use. Based on this database we gain insight into the state of analysis tools in this rapidly developing field.

Overview of the scRNA-tools database

The scRNA-tools database contains information on software tools specifically designed for the analysis of scRNA-seq data. For a tool to be eligible to be included in the database

it must be available for download and public use. This can be from a software package repository (such as Bioconductor⁴, CRAN or PyPI), a code sharing website such as GitHub or directly from a private website. Various details of the tools are recorded such as the programming language or platform they use, details of any related publication, links to the source code and the associated software license. Tools are also categorised according to the analysis tasks they are able to perform. Most tools are added after a preprint or publication becomes available but some have been added after being mentioned on social media or in similar collections such as Sean Davis' awesome-single-cell page (<https://github.com/seandavi/awesome-single-cell>).

Information about tools is displayed on a publicly available website at www.scRNA-tools.org. This website provides a profile for each tool, with links to publications and code repositories, as well as an index by analysis category. We also provide an interactive table that allows users to filter and sort tools to find those most relevant to their needs. A final page shows live and up-to-date version of some of the analysis presented below. Anyone wishing to contribute to the database can do so by submitting an issue to the project GitHub page (<https://github.com/Oshlack/scRNA-tools>).

When the database was first constructed there were 70 scRNA-seq analysis tools available, representing work in the field during the three years from the first published tool in November 2013 (SAMstr⁵) up to September 2016. In the year since then over 70 new tools have been added (Figure 1A). The doubling of the number of available tools in such a short time demonstrates the booming interest in scRNA-seq and its maturation from a technique requiring custom-built equipment with specialised protocols to a commercially available product.

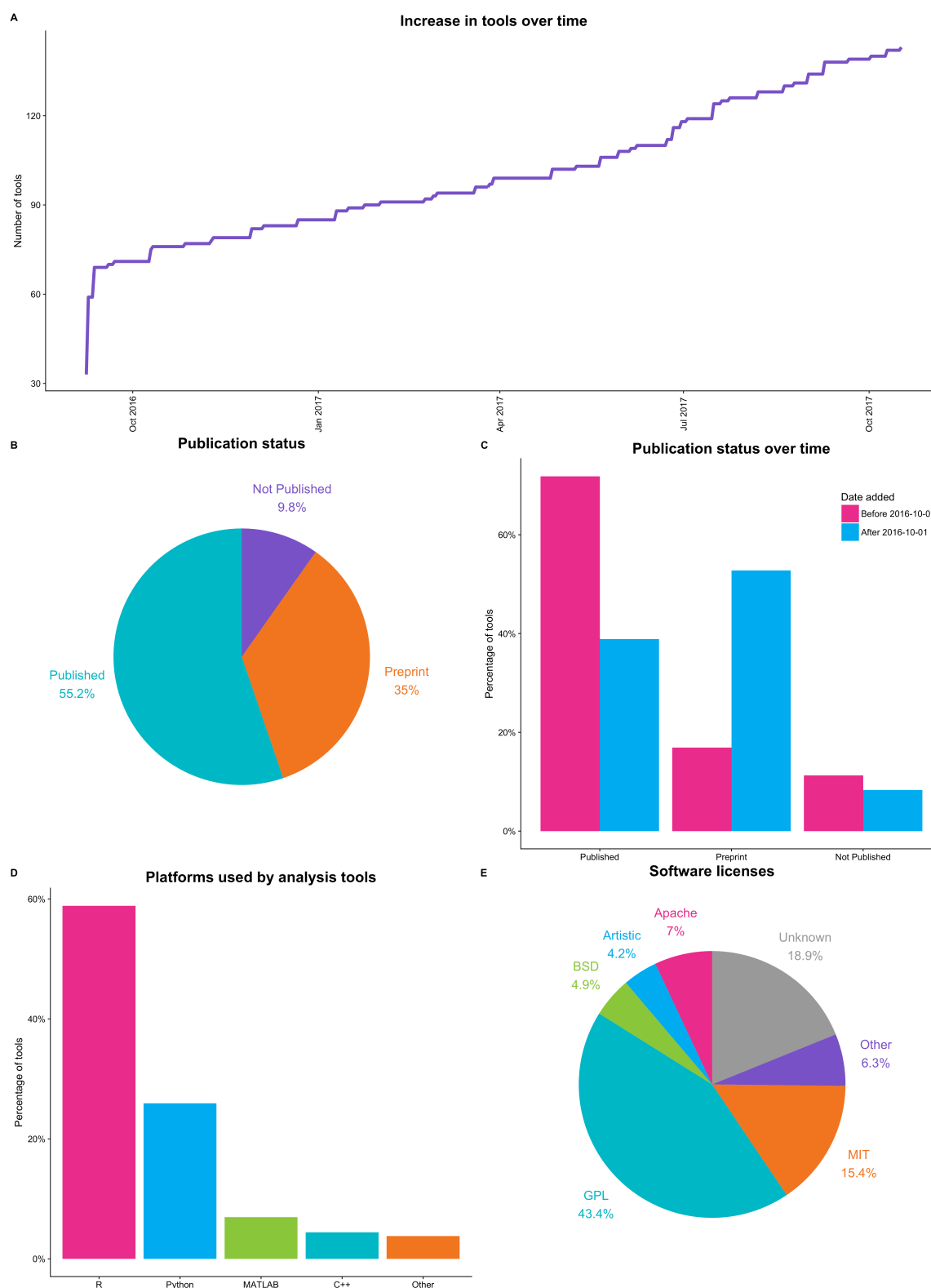


Figure 1 (A) Number of tools in the scRNA-tools database over time. Since the scRNA-seq tools database was started in September 2016 more than 70 new tools have been released. (B) Publication status of tools in the scRNA-tools database. Over half of the

tools in the full database have been published in peer-reviewed journals while another third are available as preprints. (C) When stratified by the date tools were added to the database we see that the majority of tools added before October 2016 are published, while the majority of newer tools are available as preprints. The number of unpublished tools has stayed consistent at around 10 percent. (D) The majority of tools are available using either the R or Python programming languages. (E) Most tools are released under a standard open-source software license, with variants of the GNU Public License (GPL) being the most common. However licenses could not be found for a large proportion of tools.

Publication status

Most tools have been added to the scRNA-tools database after coming to our attention in a paper describing their method and use. Of all the tools in the database about half have been published in peer-reviewed journals and another third are described in preprint articles, typically on the bioRxiv preprint server (Figure 1B). Tools can be split into those that were available when the database was created and those that have been added since. We can see that the majority of older tools have been published while more recent tools are more likely to only be available as preprints (Figure 1C). This is a good demonstration of the delay imposed by the traditional publication process. By publishing preprints and releasing software via repositories such as GitHub scRNA-seq tool developers make their methods available to the community much earlier, allowing them to be used for analysis and their methods improved prior to formal publication.

Platforms and licensing

Developers of scRNA-seq analysis tools have choices to make about what platforms they use to create their tools, how they make them available to the community and whether they share the source code. We find that the most commonly used platform for creating scRNA-seq analysis tools is the R statistical programming language, with many tools made available through the Bioconductor or CRAN repositories (Figure 1D). Python is the second most popular language, followed by MATLAB, a commercially available product, and the lower-level C++. The use of R and Python is consistent with their popularity across a range of data science fields. In particular the popularity of R reflects its history as the language of choice for the analysis of bulk RNA-seq datasets and a range of other biological data types.

The majority of tools in the scRNA-tools database have chosen to take an open-source approach, making their code available under permissive licenses (Figure 1E). We feel this reflects the general underlying sentiment and willingness of the bioinformatics

community to share and build upon the work of others. Variations of the GNU Public License (GPL) are the most common, covering almost half of tools. This license allows free use, modification and distribution of source code, but also has a "copyleft" nature which requires any derivatives to disclose their source code and use the same license. The MIT license is the second most popular which also allows use of code for any purpose but without any restrictions on distribution or licensing. The appropriate license could not be identified for almost a fifth of tools. This is problematic as fellow developers must assume that source code cannot be reused, potentially limiting the usefulness of the methods in those tools. Tool owners are strongly encouraged to clearly display their license in source code and documentation to provide certainty to the community as to how their work can be reused.

Categories of scRNA-seq analysis

As has been described in previous reviews a standard scRNA-seq analysis consists of several tasks which can be completed using various tools⁶. In the scRNA-tools database we categorise tools based on the analysis tasks they perform. Here we group these tasks into four broad phases of analysis: data acquisition, data cleaning, cell assignment and gene identification (Figure 2). The data acquisition phase (Phase 1) takes the raw nucleotide sequences from the sequencing experiment and returns a matrix describing the expression of each gene (rows) in each cell (columns). This phase consists of tasks common to bulk RNA-seq experiments, such as alignment to a reference genome or transcriptome and quantification of expression, but is often extended to handle Unique Molecular Identifiers (UMIs). Once an expression matrix has been obtained it is vital to make sure the resulting data is of high enough quality. In the data cleaning phase (Phase 2) quality control of cells is performed as well as filtering of uninformative genes. Additional tasks may be performed to normalise the data or impute missing values. Exploratory data analysis tasks are often performed in this phase, such as viewing the datasets in reduced dimensions to look for underlying structure.

The high-quality expression matrix is the focus of the next phases of analysis. In Phase 3 cells are assigned, either to discrete groups via clustering or along a continuous trajectory from one cell type to another. As high-quality reference datasets become available it will also become feasible to classify cell directly into different cell types. Once cells have been assigned attention turns to interpreting what those assignments mean.

Identifying interesting genes (Phase 4), such as those that are differentially expressed across groups, marker genes expressed in a single group or genes that change expression along a trajectory, is the typical way to do this. The biological significance of those genes can then be interpreted to give meaning to the experiment, either by investigating the genes themselves or by getting a higher level view through techniques such as gene set testing.

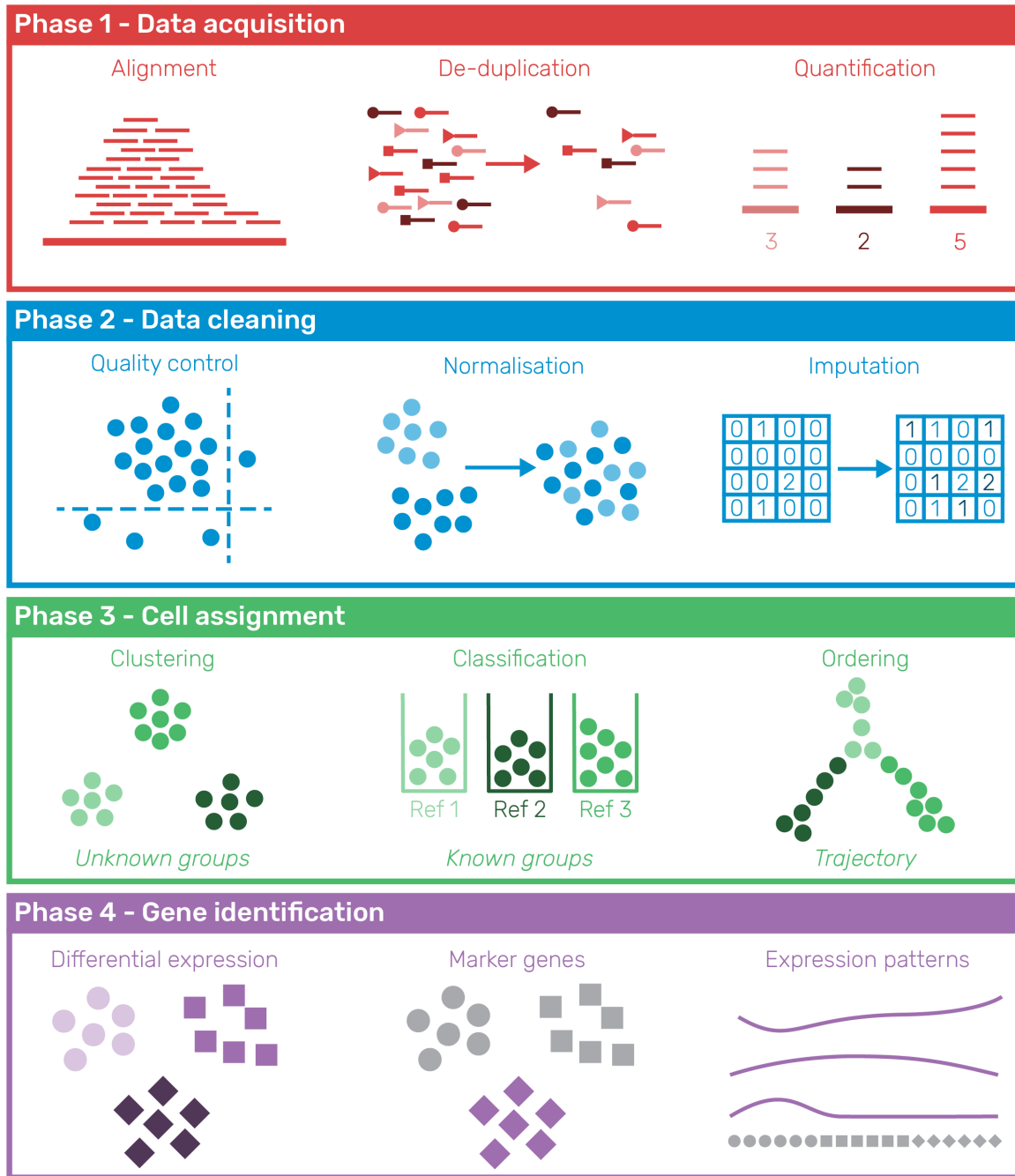


Figure 2 Phases of the scRNA-seq analysis process. In Phase 1 (data acquisition) raw sequencing reads are converted into a gene by cell expression matrix. For many protocols this requires the alignment of genes to a reference genome and the assignment and de-duplication of Unique Molecular Identifiers (UMIs). The data is then cleaned (Phase 2) to remove low-quality cells and uninformative genes, resulting in a high-quality dataset for further analysis. The data can also be normalised and missing values imputed during this phase. Phase 3 assigns cells, either in a discrete manner to known (classification) or unknown (clustering) groups or to a position on a continuous trajectory. Interesting genes (eg. differentially expressed, markers, specific patterns of expression) are then identified to explain these groups or trajectories (Phase 4).

While there are other approaches that could be taken to analyse scRNA-seq data these phases represent the most common path from raw sequencing reads to biological insight. Descriptions of the categories in the scRNA-tools database are given in Table 1, along with the associated analysis phases.

Table 1 Descriptions of categories for tools in the scRNA-tools database

Phase	Category	Description
Phase 1	Alignment	Alignment of sequencing reads to a reference
Phase 1	Assembly	Tools that perform assembly of scRNA-seq reads
Phase 1	UMIs	Processing of Unique Molecular Identifiers
Phase 1	Quantification	Quantification of expression from reads, including handling unique molecular identifiers
Phase 2	Quality Control	Removal of low-quality cells
Phase 2	Gene Filtering	Removal of lowly expressed or otherwise uninformative genes
Phase 2	Imputation	Estimation of expression where zeros have been observed
Phase 2	Normalisation	Removal of unwanted variation that may affect results
Phase 2	Cell Cycle	Assignment or correction of stages of the cell cycle, or other uses of cell cycle genes, or genes associated with similar processes
Phase 3	Classification	Assignment of cell types based on a reference dataset
Phase 3	Clustering	Unsupervised grouping of cells based on expression profiles

Phase 3	Ordering	Ordering of cells along a trajectory
Phase 3	Rare Cells	Identification of rare cell populations
Phase 3	Stem Cells	Identification of cells with stem-like characteristics
Phase 4	Differential Expression	Testing of differential expression across groups of cells
Phase 4	Expression Patterns	Detection of genes that change expression across a trajectory
Phase 4	Gene Networks	Identification of co-regulated gene networks
Phase 4	Gene Sets	Testing for over representation or other uses of annotated gene sets
Phase 4	Marker Genes	Identification or use of genes that mark cell populations
Multiple	Dimensionality Reduction	Projection of cells into a lower dimensional space
Multiple	Interactive	Tools with an interactive component or a graphical user interface
Multiple	Variable Genes	Identification or use of highly (or lowly) variable genes
Multiple	Visualisation	Functions for visualising some aspect of scRNA-seq data or analysis
Other	Allele Specific	Detection of allele-specific expression
Other	Alternative Splicing	Detection of alternative splicing
Other	Haplotypes	Use or assignment of haplotypes
Other	Immune	Assignment of receptor sequences and immune cell clonality
Other	Integration	Combining of scRNA-seq datasets or integration with other single-cell data types
Other	Modality	Identification or use of modality in gene expression
Other	Simulation	Generation of synthetic scRNA-seq datasets
Other	Transformation	Transformation between expression levels and some other measure

Other Variants Detection or use of nucleotide variants

Trends in scRNA-seq analysis tasks

Each of the tools in the database is assigned to one or more analysis categories. We investigated these categories in further detail to give insight into the trends in scRNA-seq analysis. Figure 3A shows the frequency of tools performing each of the analysis tasks. Visualisation is the most commonly included task and is important across all stages of analysis for exploring and displaying data and results. Tasks for assigning cells (ordering and clustering) are the next most common. This has been the biggest area of development in single-cell analysis with clustering tools such as Seurat¹¹, SC3¹² and BackSPIN¹³ being used to identify cell types in a sample and trajectory analysis tools (for example Monocle¹⁴, Wishbone¹⁵ and DPT¹⁶) being used to investigate how genes change across developmental processes. These areas reflect the new opportunities for analysis provided by single-cell data that are not possible with bulk RNA-seq experiments.

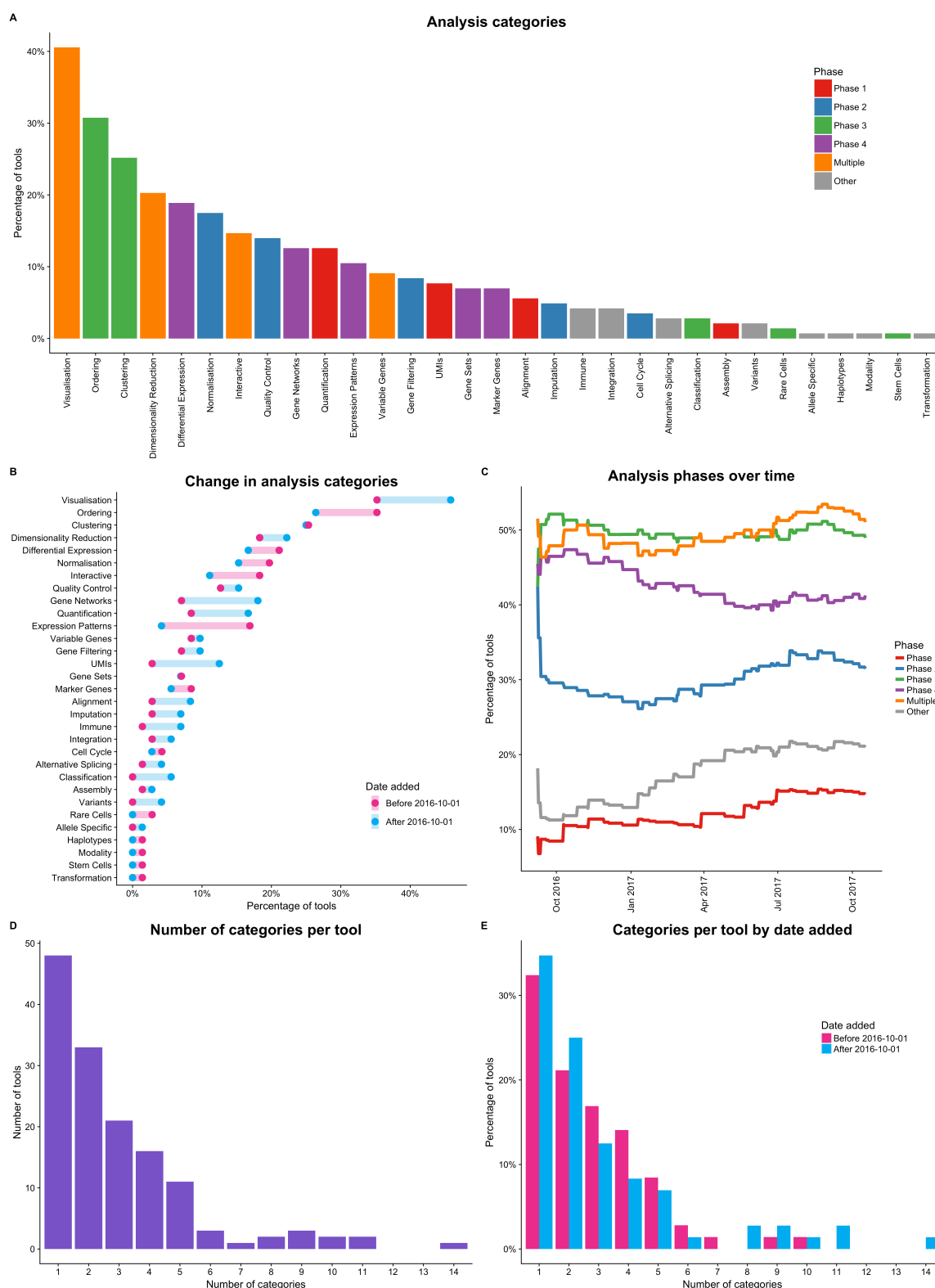


Figure 3 (A) Categories of tools in the scRNA-tools database. Each tool can be assigned to multiple categories based on the tasks it can complete. Categories associated with multiple analysis phases (visualisation, dimensionality reduction) are among the most

common, as are categories associated with the cell assignment phase (ordering, clustering). (B) Changes in analysis categories over time, comparing tools added before and after October 2016. There have been significant increases in the percentage of tools associated with visualisation, dimensionality reduction, quantification and simulation. Categories including expression patterns, pseudotime and interactivity have seen relative decreases. (C) Changes in the percentage of tools associated with analysis phases over time. The percentage of tools involved in the data acquisition and data cleaning phases have increased, as have tools designed for alternative analysis tasks. The gene identification phase has seen a relative decrease in the number of tools. (D) The number of categories associated with each tools in the scRNA-tools database. The majority of tools perform few tasks. (E) Most tools that complete many tasks are relatively recent.

Dimensionality reduction is also a common task and has applications in visualisation (via techniques such as t-SNE¹⁷), quality control and as a starting point for analysis. Testing for differential expression (DE) is perhaps the most common analysis performed on bulk RNA-seq datasets and it is also commonly applied by many scRNA-seq analysis tools, typically to identify genes that are different in one cluster of cells compared to the rest. However it should be noted that the DE testing applied by scRNA-seq tools is often not as sophisticated as the rigorous statistical frameworks of tools developed for bulk RNA-seq such as edgeR¹⁸, DESeq2²⁰ and limma²¹, often using simple statistical tests such as the likelihood ratio test. While methods designed to test DE specifically in single-cell datasets do exist (such as SCDE²², and scDD²³) it is still unclear whether they improve on methods that have been established for bulk data²⁴.

To investigate how the focus of scRNA-seq tool development has changed over time we again divided the scRNA-tools database into tools added before and after October 2016. This allowed us to see which analysis tasks are more common in recently published tools. We looked at the percentage of tools in each time period that performed tasks in the different analysis categories (Figure 3B). Some categories show little change in the proportion of tools that perform while other areas have changed significantly. Specifically, both visualisation and dimensionality reduction are more commonly addressed by recent tools. The UMIs category has also seen a big increase recently as UMI based protocols have become commonly used and tools designed to handle the extra processing steps required have been developed (UMI-tools²⁸, umis²⁹, zUMIs³⁰). Simulation is a valuable technique for developing, testing and validating scRNA-seq tools. More packages are now including their simulation functions and some tools have been developed for the specific purpose of generating realistic synthetic scRNA-seq datasets (powsimR³¹, Splatter³²). Classification of cells into known groups has also

increased as reference datasets become available and more tools are identifying or making use of co-regulated gene networks.

Some categories have seen a decrease in the proportion of tools they represent, most strikingly testing for changes expression patterns along a trajectory. This is likely related to the change in cell ordering analysis which is the focus of a lower percentage of tools added after October 2016. The ordering of cells along a trajectory was one of the first developments in scRNA-seq analysis and a decrease in the development of these tools could indicate that researchers have moved on to other techniques or that use has converged on a set of mature tools.

By grouping categories based on their associated analysis phases we see similar trends over time (Figure 3C). We see increases in the percentage of tools performing tasks in Phase 1 (quantification), Phase 2 (quality control and filtering), across multiple phases (visualisation and dimensionality reduction) and alternative analysis tasks. In contrast the percentage of tools that perform gene identification tasks (Phase 2) has decreased and the percentage assigning cells (Phase 3) has remained steady. This too may indicate a maturation of the analysis space as existing tools for performing standard scRNA-seq analyses are deemed sufficient while there is still room for development in handling data from new protocols and performing alternative analysis tasks.

Pipelines and toolboxes

While there are a considerable number of scRNA-seq tools that only perform a single analysis task, many perform at least two (Figure 3D). Some tools (dropEst³³, DrSeq²³⁴, scPipe³⁵) are preprocessing pipelines, taking raw sequencing reads and producing an expression matrix. Others, such as Scanpy³⁶, SCell³⁷, Seurat, Monocle and scater³⁸ can be thought of as analysis toolboxes, able to complete a range of complex analyses starting with a gene expression matrix. Most of the tools that complete many tasks are more recent (Figure 3E). Being able to complete multiple tasks using a single tool can simplify analysis as problems with converting between different data formats can be avoided, however it is important to remember that it is difficult for a tool with many functionalities to continue to represent the state of the art in all of them. Support for common data formats, such as the recently released SingleCellExperiment object in R³⁹, provides another way for developers to allow easy use of their tools and users to build custom workflows from specialised tools.

Alternative analyses

Some tools perform analyses that lie outside the common tasks performed on scRNA-seq data described above. Simulation is one alternative task that has already been mentioned but there is also a group of tools designed to detect biological signals in scRNA-seq data apart from changes in expression. For example alternative splicing (BRIE⁴⁰, Outtrigger⁴¹, SingleSplice⁴²), single nucleotide variants (SSrGE⁴³) and allele-specific expression (SCALE⁴⁴). Reconstruction of immune cell receptors is another area that has received considerable attention from tools such as BASIC⁴⁵, TraCeR⁴⁶ and TRAPeS⁴⁷. While tools that complete these tasks are unlikely to ever dominate scRNA-seq analysis it is likely that we will see an increase in methods for tackling specialised analyses as researchers continue to push the boundaries of what can be observed using scRNA-seq data.

Discussion and conclusions

Over the last year we have seen the number of number of software tools for analysing single-cell RNA-seq data double, with more than 130 analysis tools now available. As new tools have become available we have curated and catalogued them in the scRNA-tools database where we record the analysis tasks that they can complete, along with additional information such as any associated publications. By analysing this database we have found that tool developers have focused much of their efforts on methods for handling new problems specific to scRNA-seq data, in particular clustering cells into groups or ordering them along a trajectory. We have also seen that the scRNA-seq community is generally open and willing to share their methods which are often described in preprints prior to peer-reviewed publication and released under permissive open-source licenses for other researchers to re-use.

The next few years promise to continue to produce significant new developments in scRNA-seq analysis. New tools will continue to be produced, becoming increasingly sophisticated and aiming to address more of the questions made possible by scRNA-seq data. We anticipate that some existing tools will continue to improve and expand their functionality while others will cease to be updated and maintained. Detailed benchmarking and comparisons will show how tools perform in different situations and those that perform well, continue to be developed and provide a good user experience will become preferred for standard analyses. As single-cell capture and sequencing

technology continues to improve analysis tools will have to adapt to significantly larger datasets (in the millions of cells) which may require specialised data structures and algorithms. Methods for combining multiple scRNA-seq datasets as well as integration of scRNA-seq data with other single-cell data types, such as DNA-seq, ATAC-seq or methylation, will be another area of growth and projects such as the Human Cell Atlas⁴⁸ will provide comprehensive cell type references which will open up new avenues for analysis.

As the field expands the scRNA-tools database will continue to be updated. We hope that it provides a resource for researchers to explore when approaching scRNA-seq analyses as well as providing a record of the analysis landscape and how it changes over time.

Methods

Database

When new tools come to our attention they are added to the scRNA-tools database. DOIs and publication dates are recorded for any associated publications. As preprints may be frequently updated they are marked as a preprint instead of recording a date. The platform used to build the tool, links to code repositories, associated licenses and a short description are also recorded. Each tool is categorised according to the analysis tasks it can perform, receiving a true or false for each category based on what is described in the accompanying paper or documentation. We also record the date that each entry was added to the database and the date that it was last updated.

Website

To build the website we start with the table described above as a CSV file which is processed using an R script. The lists of packages available in the CRAN, Bioconductor and PyPI software repositories are downloaded and matched with tools in the database. For tools with peer-reviewed publications the number of citations they have received is retrieved from the Crossref database (www.crossref.org) using the rcrossref package (v0.7.0)⁴⁹. JSON files describing the complete table, tools and categories are outputted and used to populate the website.

The website consists of three main pages. The home page shows an interactive table with the ability to sort, filter and download the database. The second page shows an entry for

each tool, giving the description, details of publications, details of the software code and license and the associated software categories. Badges are added to tools to provide clearly visible details of any associated software or GitHub repositories. The final page describes the categories, providing easy access to the tools associated with them.

Analysis

The most recent version of the scRNA-tools database was used for the analysis presented in this paper. Data was manipulated in R using the dplyr package (v0.7.3)⁵⁰ and plots produced using the ggplot2 (v2.2.1)⁵¹ and cowplot (v0.8.0)⁵² packages.

Declarations

Ethics

Not applicable.

Availability of data and materials

The scRNA-tools databases is publicly accessible via the website at www.scRNA-tools.org. Suggestions for additions, updates and improvements are warmly welcomed at the associated GitHub repository (<https://github.com/Oshlack/scRNA-tools>). The code and datasets used for the analysis in this paper are available from <https://github.com/Oshlack/scRNATools-paper>.

Competing interests

The authors declare no competing interests.

Funding

Luke Zappia is supported by an Australian Government Research Training Program (RTP) Scholarship. Alicia Oshlack is supported through a National Health and Medical Research Council Career Development Fellowship APP1126157. MCRI is supported by the Victorian Government's Operational Infrastructure Support Program.

Authors' contributions

Acknowledgements

We would like to acknowledge Sean Davis' work in managing the awesome-single-cell page and producing a prototype of the script used to process the database. Daniel Wells had the idea for recording software licenses and provided licenses for the tools in the database at that time. Breon Schmidt designed a prototype of the scRNA-tools website and answered many questions about HTML and Javascript. Our thanks also to Matt Ritchie for his thoughts on early versions of the manuscript.

Additional files

References

1. Tang, F. *et al.* mRNA-Seq whole-transcriptome analysis of a single cell. *Nature methods* **6**, 377–382 (2009).
2. Svensson, V., Vento-Tormo, R. & Teichmann, S. A. Moore's Law in Single Cell Transcriptomics. (2017).
3. Stegle, O., Teichmann, S. A. & Marioni, J. C. Computational and analytical challenges in single-cell transcriptomics. *Nature Reviews Genetics* **16**, 133–145 (2015).
4. Huber, W. *et al.* Orchestrating high-throughput genomic analysis with Bioconductor. *Nature methods* **12**, 115–121 (2015).
5. Katayama, S., Töhönen, V., Linnarsson, S. & Kere, J. SAMstr: statistical test for differential expression in single-cell transcriptome with spike-in normalization. *Bioinformatics* **29**, 2943–2945 (2013).
6. Bacher, R. & Kendzierski, C. Design and computational analysis of single-cell RNA-sequencing experiments. *Genome biology* **17**, 63 (2016).
7. Wagner, A., Regev, A. & Yosef, N. Revealing the vectors of cellular identity with single-cell genomics. *Nature biotechnology* **34**, 1145–1160 (2016).
8. Miragaia, R. J., Teichmann, S. A. & Hagai, T. Single-cell insights into transcriptomic diversity in immunity. *Current Opinion in Systems Biology* **5**, 63–71 (2017).
9. Poirion, O. B., Zhu, X., Ching, T. & Garmire, L. Single-Cell Transcriptomics Bioinformatics and Computational Challenges. *Frontiers in genetics* **7**, (2016).
10. Rostom, R., Svensson, V., Teichmann, S. A. & Kar, G. Computational approaches for interpreting scRNA-seq data. *FEBS letters* (2017). doi:[10.1002/1873-3468.12684](https://doi.org/10.1002/1873-3468.12684)

11. Satija, R., Farrell, J. A., Gennert, D., Schier, A. F. & Regev, A. Spatial reconstruction of single-cell gene expression data. *Nature biotechnology* **33**, 495–502 (2015).
12. Kiselev, V. Y. *et al.* SC3: consensus clustering of single-cell RNA-seq data. *Nature methods* **14**, 483–486 (2017).
13. Zeisel, A. *et al.* Brain structure. Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. *Science* **347**, 1138–1142 (2015).
14. Trapnell, C. *et al.* The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nature biotechnology* **32**, 381–386 (2014).
15. Setty, M. *et al.* Wishbone identifies bifurcating developmental trajectories from single-cell data. *Nature biotechnology* **34**, 637–645 (2016).
16. Haghverdi, L., Büttner, M., Wolf, F. A., Büttner, F. & Theis, F. J. Diffusion pseudotime robustly reconstructs lineage branching. *Nature methods* (2016). doi:[10.1038/nmeth.3971](https://doi.org/10.1038/nmeth.3971)
17. Maaten, L. van der & Hinton, G. Visualizing Data using t-SNE. *Journal of machine learning research: JMLR* **9**, 2579–2605 (2008).
18. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140 (2010).
19. McCarthy, D. J., Chen, Y. & Smyth, G. K. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic acids research* **40**, 4288–4297 (2012).
20. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology* **15**, 550 (2014).
21. Ritchie, M. E. *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic acids research* **43**, e47 (2015).
22. Kharchenko, P. V., Silberstein, L. & Scadden, D. T. Bayesian approach to single-cell differential expression analysis. *Nature methods* **11**, 740–742 (2014).
23. Korthauer, K. D. *et al.* A statistical approach for identifying differential distributions in single-cell RNA-seq experiments. *Genome biology* **17**, 222 (2016).
24. Sonesson, C. & Robinson, M. D. Bias, Robustness And Scalability In Differential Expression Analysis Of Single-Cell RNA-Seq Data. *bioRxiv* 143289 (2017). doi:[10.1101/143289](https://doi.org/10.1101/143289)
25. Jaakkola, M. K., Seyednasrollah, F., Mehmood, A. & Elo, L. L. Comparison of methods to detect differentially expressed genes between single-cell populations. *Briefings in bioinformatics* (2016). doi:[10.1093/bib/bbw057](https://doi.org/10.1093/bib/bbw057)
26. Miao, Z. & Zhang, X. Differential expression analyses for single-cell RNA-Seq: old questions on new data. *Quantitative Biology* **4**, 243–260 (2016).
27. Dal Molin, A., Baruzzo, G. & Di Camillo, B. Single-cell RNA-sequencing: assessment of differential expression analysis methods. *Frontiers in genetics* **8**, 62 (2017).

28. Smith, T., Heger, A. & Sudbery, I. UMI-tools: modeling sequencing errors in Unique Molecular Identifiers to improve quantification accuracy. *Genome research* **27**, 491–499 (2017).
29. Svensson, V. *et al.* Power analysis of single-cell RNA-sequencing experiments. *Nature methods* (2017). doi:[10.1038/nmeth.4220](https://doi.org/10.1038/nmeth.4220)
30. Parekh, S., Ziegenhain, C., Vieth, B., Enard, W. & Hellmann, I. zUMIs: A fast and flexible pipeline to process RNA sequencing data with UMIs. *bioRxiv* 153940 (2017). doi:[10.1101/153940](https://doi.org/10.1101/153940)
31. Vieth, B., Ziegenhain, C., Parekh, S., Enard, W. & Hellmann, I. powsim: Power analysis for bulk and single cell RNA-seq experiments. *bioRxiv* 117150 (2017). doi:[10.1101/117150](https://doi.org/10.1101/117150)
32. Zappia, L., Phipson, B. & Oshlack, A. Splatter: simulation of single-cell RNA sequencing data. *Genome biology* **18**, 174 (2017).
33. Petukhov, V. *et al.* Accurate estimation of molecular counts in droplet-based single-cell RNA-seq experiments. *bioRxiv* 171496 (2017). doi:[10.1101/171496](https://doi.org/10.1101/171496)
34. Zhao, C., Hu, S., Huo, X. & Zhang, Y. Dr.seq2: A quality control and analysis pipeline for parallel single cell transcriptome and epigenome data. *PloS one* **12**, e0180583 (2017).
35. Tian, L. *et al.* scPipe: a flexible data preprocessing pipeline for single-cell RNA-sequencing data. *bioRxiv* 175927 (2017). doi:[10.1101/175927](https://doi.org/10.1101/175927)
36. Alexander Wolf, F., Angerer, P. & Theis, F. J. Scanpy for analysis of large-scale single-cell gene expression data. *bioRxiv* 174029 (2017). doi:[10.1101/174029](https://doi.org/10.1101/174029)
37. Diaz, A. *et al.* SCell: integrated analysis of single-cell RNA-seq data. *Bioinformatics* (2016). doi:[10.1093/bioinformatics/btw201](https://doi.org/10.1093/bioinformatics/btw201)
38. McCarthy, D. J., Campbell, K. R., Lun, A. T. L. & Wills, Q. F. Scater: pre-processing, quality control, normalization and visualization of single-cell RNA-seq data in R. *Bioinformatics* **33**, 1179–1186 (2017).
39. Lun, A. & Risso, D. SingleCellExperiment: S4 Classes for Single Cell Data. (2017).
40. Huang, Y. & Sanguinetti, G. BRIE: transcriptome-wide splicing quantification in single cells. *Genome biology* **18**, 123 (2017).
41. Song, Y. *et al.* Single-Cell Alternative Splicing Analysis with Expedition Reveals Splicing Dynamics during Neuron Differentiation. *Molecular cell* (2017). doi:[10.1016/j.molcel.2017.06.003](https://doi.org/10.1016/j.molcel.2017.06.003)
42. Welch, J. D., Hu, Y. & Prins, J. F. Robust detection of alternative splicing in a population of single cells. *Nucleic acids research* **44**, e73 (2016).
43. Poirion, O. B., Zhu, X., Ching, T. & Garmire, L. X. Using Single Nucleotide Variations in Cancer Single-Cell RNA-Seq Data for Subpopulation Identification and Genotype-phenotype Linkage Analysis. *bioRxiv* 095810 (2016). doi:[10.1101/095810](https://doi.org/10.1101/095810)
44. Jiang, Y., Zhang, N. R. & Li, M. SCALE: modeling allele-specific gene expression by single-cell RNA sequencing. *Genome biology* **18**, 74 (2017).

45. Canzar, S., Neu, K. E., Tang, Q., Wilson, P. C. & Khan, A. A. BASIC: BCR assembly from single cells. *Bioinformatics* **33**, 425–427 (2017).
46. Stubbington, M. J. T. *et al.* T cell fate and clonality inference from single-cell transcriptomes. *Nature methods* **13**, 329–332 (2016).
47. Afik, S. *et al.* Targeted reconstruction of T cell receptor sequence from single cell RNA-seq links CDR3 length to T cell differentiation state. *Nucleic acids research* (2017). doi:[10.1093/nar/gkx615](https://doi.org/10.1093/nar/gkx615)
48. Regev, A. *et al.* The Human Cell Atlas. *bioRxiv* 121202 (2017). doi:[10.1101/121202](https://doi.org/10.1101/121202)
49. Chamberlain, S., Boettiger, C., Hart, T. & Ram, K. *rcrossref: Client for Various 'CrossRef' APIs*. (2017).
50. Wickham, H., Francois, R., Henry, L. & Müller, K. *dplyr: A Grammar of Data Manipulation*. (2017).
51. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*. (Springer New York, 2010).
52. Wilke, C. O. *cowplot: Streamlined Plot Theme and Plot Annotations for 'ggplot2'*. (2017).