CLK refactor and test of robustness

Tests of Robustness in Peer Review

A Thesis
Submitted to the Faculty
of
Drexel University
by
Jeremy Leipzig
In partial fulfillment of the
Requirements for the degree
of
Doctor of Philosophy
August 2021

Acknowledgments

I am deeply indebted to my wife Mary Ellen McCarty for her support and sacrifices during this long journey.

I wish to thank my patient and calm advisor, Jane Greenberg, the thesis committee - Karthik Ram, Gail Rosen, Xiaohua Tony Hu, Erjia Yan - and Chamoei Chen, who suggested I write the pipeline paper.

My work in this dissertation originated from my exposure to the brilliant statistician Charles Berry, who introduced me to reproducible research in 2004. I am also grateful to Frederic Bushman, who brought me in touch with Dr. Berry, and to my master's advisor Steffen Heber, who recommended me to Dr. Bushman.
I am thankful to CHOP for funding a portion of the tuition and providing much of the experiential fodder for this unusual research topic. Many of the reproducibility frustrations of working in a large research organization were made more bearable by my past colleagues - Deanne Taylor, Zhe Zhang, Perry Evans, Pichai Raman, and Eric Lim.

Throughout this dissertation, I received valuable contributions from Sandeep Amberkar, Stephen Anderson, Israel Barrantes, Juan Caballero, Barry Digby, Harish K, Ammar Naqvi, Daniel Nüst, Wade Rogers, Yi Xing, and Karl Sebby.

Declaration

This dissertation is the result of my own work, except where explicit reference is made to the work of others. It has not been submitted for another qualification to this or any other university. This dissertation does not exceed the word limit for the respective Degree Committee.

Contents

Abstract

Purpose: The purpose of this dissertation is to investigate the feasibility of using tests of robustness in peer review. This study involved selecting three high-impact papers which featured open data and utilized bioinformatic analyses but provided no source code, and refactoring these to allow external survey participants to swap tools, parameters, and data subsets in order to evaluate the robustness and underlying validity of these analyses. This approach has been enabled by technical advances that have taken place in recent years - scientific computing infrastructure has matured to support the distribution of reproducible computational analyses. These advances, along with cultural shifts encompassing open data and open code initiatives, promise to address technical stumbling blocks that have contributed to the "reproducibility crisis". To take full advantage of these developments toward improving scientific quality, a logical next step is to integrate reproducible analysis into the peer review process. Seven existing major case study types - reproduction, replication, refactor, robustness test, survey, census, and case narrative - have been invaluable toward establishing reproducibility as a serious and independent area of research. Of particular interest are refactors, in which an existing analysis with abstract methods is reimplemented by a third party, and robustness tests, which involve the manipulation of tools, parameters, and data to assess the scientific validity of an analysis. This thesis describes efforts to test the feasibility of robustness testing in the context of in silico peer review. The contributions described are complemented with extensive source code.

Design and Methods: A multi-method approach was employed for this study consisting of user surveys and tests of robustness - hands-on self-directed software development exercises. Three high-impact genomics publications with open data, but no source code, were selected, refactored, and distributed to active study participants who acted as quasi-external reviewers. The process of the refactor was used to evaluate the limitations of reproducibility using conventional tools and to study how best to present analyses for peer review, and the tests of robustness were employed under the hypothesis this practice would help to evaluate the underlying validity of an analysis. Three different approaches were taken in these tests of robustness - a faithful reproduction of the original manuscript into a framework that could be manipulated by participants, a workflow-library approach in which participants were encouraged to employ modern "off-the-shelf" pre-built pipelines to triangulate tests, and an advisor-led approach in which senior experts suggested alternate tools to be implemented and I generated a report for their evaluation.

Findings: The refactors and tests of robustness produced numerous discoveries both in terms of the underlying scientific content and, more importantly, into the strengths and weakness of the three robustness approaches (faithful/workflow-library/advisor-led) and pain points in the analytic stack which may be addressed with appropriate software and metadata. The principal findings are that the faithful approach may often discourage aggressive robustness testing because of the inertia imposed by the existing framework, the workflow-library approach is efficient but can prove inconclusive, and the advisor-led approach may be most practical for journals but requires a higher level of communication to be effective. The vast majority of time in all these refactors was spent on sample metadata management, particularly organizing sample groups of biological and technical replicates to produce the numerous and varied tool input manifests.

Practical Implications: Reproducibility-enabled in silico peer review is substantially more time-consuming than traditional manuscript peer review, and will require economic, cultural, and technical change to bring to reality. The work presented here could contribute to developing new models to minimize the increased effort of this type of peer review while incentivizing reproducibility.

Value: This study provides practical guidance toward designing the future of reproducibility-enabled in silico peer review, which is a logical extension of the computational reproducibility afforded by technical advances in dependency management, containerization, pipeline frameworks, and notebooks.

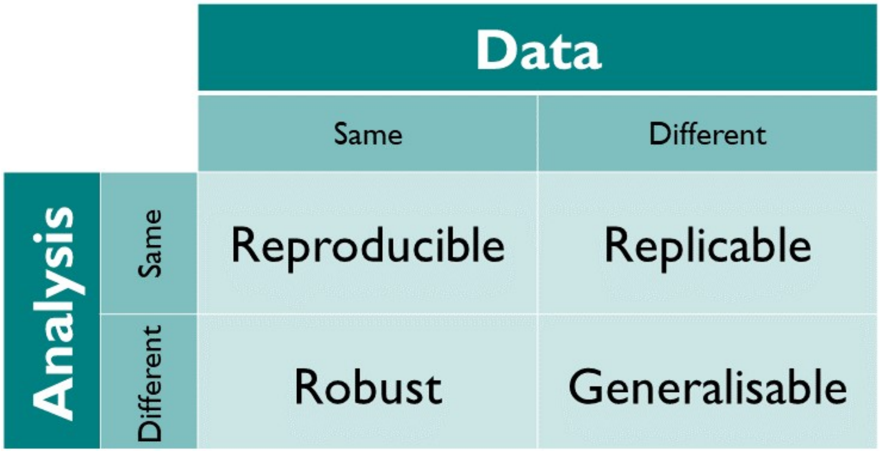
1 Introduction

In recent years, various fields - namely the biomedical sciences, psychology, and neuroscience, but also newer areas such as artificial intelligence - have decried a "reproducibility crisis" \cite{Baker2016-ri} in the form of unreproducible analyses or unreplicable results. The primary origins of these problems are selective reporting and insufficient detail provided in methods, which can include missing code, data, versions, or parameters. These details encompass all steps of the scientific endeavor, from laboratory protocols, data collection, data processing, analysis, and manuscript editing.

Reproducible research is not yet a formally established area of study or curricula in information science, despite increasing attention within an array of scientific fields and as a primary focus of several researchers, institutions, and journals. Only one dissertation exists with a title bearing the phrase "reproducible research" or "reproducible computational research" \cite{Pham2014-ad}. The field of information science, straddling the fence between computer science and its applied domains, is uniquely positioned to accommodate reproducible computational research as a topic of study in its own right. This proposal intends to serve as a progenitor for this new subdomain, by exploring metadata as a syntactic glue to bind layers of the "analytic stack".

1.0.1 Reproducible Research

Reproducible Research is an umbrella term that encompasses many forms of scientific quality - from generalizability of underlying scientific truth, an exact recreation of an experiment with or without communicating intent, to the open sharing of analysis for reuse. Specific to computational facets of scientific research, Reproducible Computational Research (RCR)\cite{Donoho2010-xp} encompasses all aspects of in silico analyses, from the propagation of raw data collected from the experimental lab, field, or instrumentation, through intermediate data structures, computational hardware, to open code and statistical analysis, and finally publication. Reproducible research points to several underlying concepts of scientific validity – terms that should be unpacked to be understood. Stodden et al. \cite{Stodden2013-ce} devised a five-level hierarchy of research, classifying it as – reviewable, replicable, confirmable, auditable, and open or reproducible. Whitaker \cite{Whitaker2016-gl} describes an analysis as "reproducible" in the narrow sense that a user can produce identical results provided the data and code from the original, and "generalisable" if it produces similar results when both data is swapped out for similar data ("replicability"), and if underlying code is swapped out with comparable replacements ("robustness") (Figure 1).



		Data	
		Same	Different
Analysis	Same	Reproducible	Replicable
	Different	Robust	Generalisable

Figure 1: Whitaker's matrix of reproducibility \cite{The_ Turing_ Way_ Community2019-fn}

While these terms may confuse those new to reproducibility, a review by Barba disentangled the terminology while providing a historical context of the field \cite{Barba2018-qv}. One major conflicted use of terms (reproducible/replicable) has since then been harmonized \cite{noauthor_undated-or}. A wider perspective places reproducibility as a first-order benefit of applying FAIR principles: Findability, Accessibility, Interoperability, and Reusability \cite{Wilkinson2016-qr}.

Reproducible computational research (the "reproducible" in Whitaker's table) is attainable with current technology with a few caveats - namely external resources and manual steps. There is some debate whether implementing reproducibility is "still a challenge" \cite{FitzJohn2014-wk} or "not hard" \cite{Edzer_Pebesma2016-gl}. The tools to achieve highly portable and automated analyses such as Conda, Docker, cloud computing, pipeline frameworks, notebooks, and script provenance tools are readily available, even if some are quite new.

RCR is necessary but not sufficient to achieve the other three types of reproducibility (replicability, robustness, and generalizability). It is impossible to evaluate the replication (swapping in new data) or robustness (swapping in new tools) of a complex computational workflow if it is not first reproducible. Those types of reproducibility rely on some level of scientific validity (i.e. truth) to realize. Methods to measure replicability, robustness, and generalizability are also indirectly measuring the strength of scientific hypotheses rather than best practices in reproducible research. As mentioned above, a binary condemnation of "Not replicable" might infer a scientific result is not valid, when the infraction might be relatively minor - for example, a missing but ultimately derivable parameter. Conversely, a highly transparent and automated analysis can still suffer from design problems and small sample sizes that lead to artefactual results. . The Begley and Ellis Amgen study cited poor design, poor statistics, and perhaps most commonly, selective reporting, rather than record-keeping or lack of lab notebooks as the primary factor in a failure to validate \cite{Begley2012-mt}. This contrasts somewhat with the Ioannidis microarray review discussed above \cite{Ioannidis2009-at}, which identified most reproducibility problems as being due to a lack of detailed methods, missing controls, and other failures in protocol.

Munafò and Smith contend that the four types of reproducibility cited by Whitakerare not sufficient to verify scientific validity because they do not eliminate common confounders. They posit that *triangulation*, defined as applying entirely different approaches (each with differing biases) and multiple lines of evidence to the same problem, are a more appropriate use of resources.\cite{noauthor_undated-xz}

Millman and colleagues in "Is tagging of therapist-patient interactions reliable?" \cite{K_Jarrod_Millman_ ql} classify reproducibility into four categories.

- Computational reproducibility and transparency
- Scientific reproducibility and transparency
- Computational correctness
- Statistical reproducibility

These divisions correspond to the divides between reproducibility/replicability and between wet-lab and *in silico* analysis.

Secondary Attributes of Reproducibility Building on those primary types are nine secondary attributes of manuscript reproducibility, perhaps best described by Stodden -

replicable, reproducible, repeatable, confirmable, generalizable, reviewable, auditable, verifiable, and validatable. Many of these attributes revolve around the free and unrestricted availability of data. The role of open data and the open data movement, as a tenet or a prerequisite to reproducibility, is a lengthy topic by itself but also affects the standards and means of measuring reproducibility. The data-sharing movement - including Open Access and FAIR \cite{Wilkinson2016-qr}– has developed somewhat tangentially to the reproducibility movement, although these share many of the same values of reuse, evaluation, and scientific validity. In biomedical science, open data is largely driven by the need to reach a critical mass of patient data to derive statistical power, especially in rare disease. Sample sizes in the thousands are often necessary to utilize machine learning techniques. "Deep learning" requires even more. In data science, open data is often associated with larger social and political issues of government transparency.

The reproducibility movement can be presented as an "open analysis" (as opposed to "open data") movement, although this characterization oversimplifies both the challenges of creating reproducible workflows in terms of portability, transparency and other characteristics in the same way "open data" glosses over some of the finer points of tidiness and metadata.

Rokem, Marwick, and Steneva \cite{K_Jarrod_Millman_Kellie_Ottoboni_Naomi_A_P_Stark_and_P
ql} classify the three facets of reproducibility as

- Automation and provenance tracking - includes the single button press criteria
- Availability of software and data
- Open reporting of results

Tertiary Attributes of Reproducibility Finally, there are quality attributes that are associated with reproducibility in a tangential fashion, most often connected with software engineering, and information and library science.

Attributes from software engineering The computational reproducibility community is closely aligned with computer science and software development communities, which have developed various software engineering habits, development methodologies, best practices, and standards that may embody these high-level qualitative characteristics, engendering "checklist"-style rubrics. These include version control, provenance tracking (tracing the origins of data and intermediates), documentation, pipeline frameworks, and continuous integration \cite{Sandve2013-yv,Noble2009-ad}. Most of the concepts here have been directly borrowed from research in industrial software engineering settings \cite{Losavio2004-tg,Technology2003-gl} and many have formal software quality ISO9126 entries.

- Automation - Are there checkpoints that require human judgment to proceed, either because a machine learning or other automated routine has not yet been developed, or because there are steps that involve web applications, desktop software, or other interactive tools which disrupt flow?

- Swappability - Refers to how readily can alternative tools and analytical steps be replaced with substitutes. This is incredibly important for testing whether a result is an artifact created by a particular tool or model. In software quality terms this is referred to as ISO 9126 Maintainability Sub-characteristic Replaceability

- Modularity - can individual tools, steps, and reports be extracted and used by others. Maintainability Sub-characteristic Modularity

- Discoverability - how readily is this project going to be found by others, even those in a different field but whose workflow resembles the project

- Readability - is source code well enough documented to be inspected and understood by programmers familiar with the tool but unfamiliar with the underlying implementation

- Abstracted - does the project use conventional standard frameworks, such as pipeline frameworks - either CWL-based or a DSL\cite{Leipzig2017-hv}.

- Portability - can the software dependencies of an analysis be seamlessly installed on a different server? Are they specified in a server-agnostic dependency manager such as Conda? Are Docker \cite{Schulz2016-or} or Singularity containers \cite{Kurtzer2017-wb} used to isolate individual processes? Is the infrastructure "cloud-ready"?

- Uncoupledness - is the software designed as a loosely coupled service-oriented architecture that leverages application programming interfaces (APIs), preferably in a stateless or RESTful web interface. A popular framework for such services is Swagger \cite{Haupt2017-ud}. For more highly linked data, a SPARQL endpoint may be preferable.\cite{Gonzalez2014-dq}

- Scalability - can a process be configured to use multiple cores, multiple nodes, batch submission systems, or more sophisticated big data shared memory frameworks such as Spark.

- Loggability - does the workflow record steps during progression?

- Monitoring - can the workflow be monitored in real-time?

- Tested - have tests been developed? Are there test coverage statistics?

- Debuggability - can bugs in the project be easily identified? is continuous integration used?

- Updatability - can the project be updated with new resources?

- Extensibility - can one easily build on an analysis to suit a different experimental design?

- Robustness - can the software work in a variety of contexts?

- Gracefulness - can the software handle exceptions and report meaningful errors?

- Defensiveness - does the software detect error states early on?

- Reentrancy and memoization - can a workflow be restarted where it left off if interrupted? Can a downstream target deliverable be produced from intermediates?

Attributes from information science Another facet of RCR research is provided by researchers in information and library science.

- Semantic encoding - semantic data contains markup, metadata, that defines the meaning of data for computation, discovery, reuse, and attribution. Metadata is essential for the wet-lab technical, computational, scientific, and bibliographic layers of a research project.

- Metadata - are data dictionaries used? Are the Dublin Core elements complete?

- Linkedness - Are standards for linked metadata used to provide means of unambiguous identification with uniform resource identifiers? Are resource description frameworks leveraged to enable the relationship between entities to be defined using standard ontologies? Research objects \cite{Bechhofer2010-lr} provide a framework for tying together the data, code, workflows, and publications related to a project using a standard ontology.

- Provenance - are the origins of data properly recorded? \cite{Herschel2017-yc}

- Sustainability - are sustainable and permanent data identifiers used that won't decay? \cite{Gomez-Perez2013-th,Zhao2012-ou}. Are external databases or files cached such that the version used is available?

Attributes from statistics and data science

- Tidiness - is the raw input data munged into a format by which conforms to tidy data standards as described by Hadley Wickham \cite{Wickham2014-xj}. Tidy data tends to be "tall", rather than "wide", with value classifier variables as row values rather than as column headings, and only one observation or measurement, all of the same data type, appearing in each row.

- Subsetability - can a project's data be easily subset or randomly sampled for cross-validation?

- Literacy - the statistical code is interspersed with contextual text that describes the intent of each block

- Prospective stability - the model, operation, or algorithm can map new data points without affecting existing results

To the author's knowledge, there is no word or phrase that encompasses all of the attributes above. One possible term would be "broad-sense reproducibility" (BSR), which I use to describe "narrow-sense RCR" (aka "hit return reproducibility" - the ability to execute a packaged analysis with little effort) with the added goals of discovery, reuse, and transparency in line with Findable, Accessible, Interoperable, and Reusable (FAIR) principles \cite{Wilkinson2016-qr}. A recent effort to develop metrics for FAIR has emphasized a rubric that is "clear", "realistic", "discriminating", "measurable", and "universal" \cite{Wilkinson2017-hf}

Reproducibility Crisis The scientific community's challenge with irreproducibility in research has been extensively documented \cite{Baker2016-ri}. Two events in the life sciences stand as watershed moments in this crisis – the publication of manipulated and falsified predictive cancer therapeutic signatures by a biomedical researcher at Duke and subsequent forensic investigation by Keith Baggerly and David Coombes \cite{Baggerly2010-qy}, and a review by scientists at Amgen who could replicate the results of only 6 out of 53 cancer studies \cite{Begley2012-mt}. These events involved different aspects of research practice - poor data structures and missing protocols, respectively. Together with related studies \cite{Ioannidis2009-at}, they underscore recurring reproducibility problems due to a lack of detailed methods, missing controls, and other protocol failures including inappropriate statistical tests and or misinterpretation of results also play a recurring role in irreproducibility \cite{Motulsky2014-vg}. Regardless of intent, these activities fall under the umbrella term of "questionable research practices". It bears speculation whether these types of incidents are more likely to occur in novel statistical or computational approaches compared to conventional ones. Subsequent surveys of researchers \cite{Baker2016-ri} have identified selective reporting, while theory papers \cite{Ioannidis2005-se} have emphasized the insidious combination of underpowered designs and publication bias, essentially a multiple testing problem on a global scale. We contend that metadata has been undervalued in the role it can play in addressing all of these issues and shifting the narrative from the current crisis to new opportunities \cite{Fanelli2018-ek}.

In the wake of this newfound interest in reproducibility, both the variety and volume of related case studies increased after 2015 (Figure 2). Likert-style surveys and high-level publication-based censuses (see Figure 3) in which authors tabulate data or code availability are most prevalent. Additionally, low-level reproductions, in which code is executed, replications in which new data is collected and used, tests of robustness in which new tools or methods are used, and refactors to best practices are also becoming more popular. While the life sciences have generated more than half of these case studies, areas of the social and physical sciences are increasingly the subjects of important reproduction and replication efforts.

The majority of studies into reproducible research have focused on the first row of Whitaker's grid, and replication in particular. The reasons for this bias are most perhaps rooted in how science has typically been conducted in those areas that have experienced the most public reproducibility crises - psychology and the life sciences - where generating new data to test existing hypotheses is more common than modeling existing datasets, as is more common in the physical sciences.

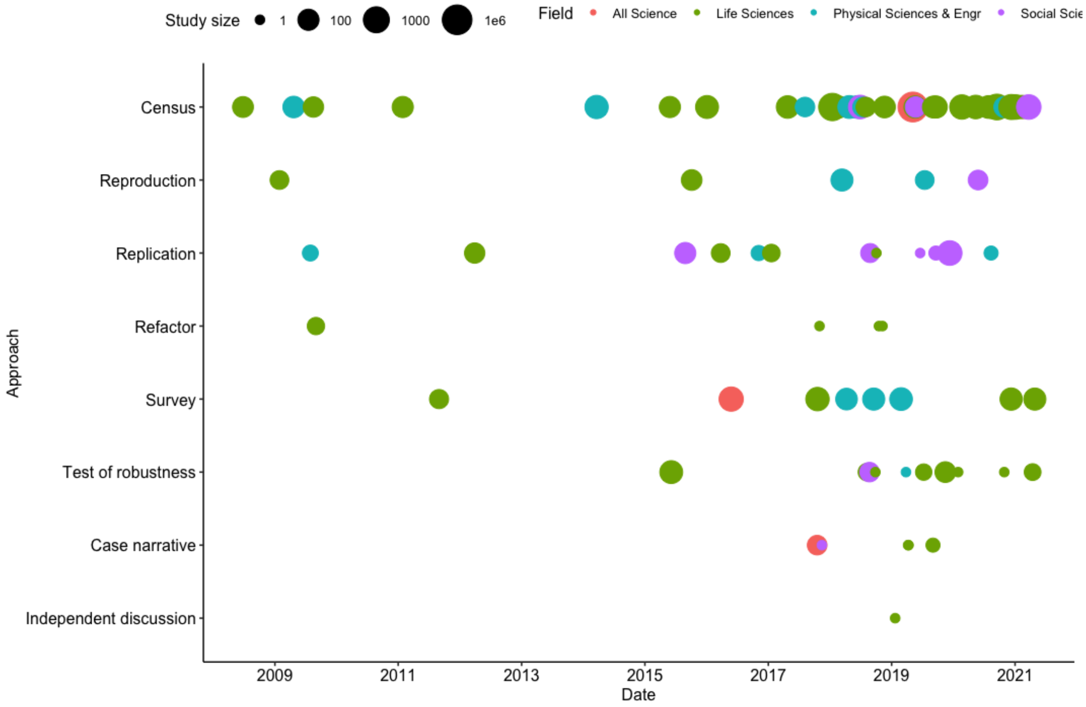


Figure 1: Case studies

Big Data, Big Science, and Open Data The inability of third parties to reproduce results is not new to science \cite{Lehrer2010-pm} but the scale of scientific endeavor and the level of data and method reuse suggest replication failures may damage the sustainability of certain disciplines, hence the term "reproducibility crisis." The problem of irreproducibility is compounded by the rise of "big data," in which very large, new, and often unique, disparate or unformatted sources of data have been made accessible for analysis by third parties, and "big science," in which terabyte-scale data sets are generated and analyzed by multi-institutional collaborative research projects on specialized and possibly unique infrastructure. Big data and big science have increased the demand for high-performance computing, specialized tools, and complex statistics, with attention to the growing popularity and application of machine learning and deep learning (ML/DL) techniques to these data sources. Such techniques typically train models on specific data subsets, and the models, as the end product of these methods, are often "black boxes," i.e., their internal predictors are not explainable (unlike older techniques such as regression) though they provide a good fit for the test data. Properly evaluating and reproducing studies that rely on such algorithms presents new challenges not previously encountered with inferential statistics \cite{Warden2018-ak,Bouthillier2019-yq}. Computational reproducibility is typically focused on the last analytic steps of what is often a labor-intensive scientific process that often originates from wet-lab protocols, fieldwork, or instrumentation and these last in silico steps present some of the more difficult problems both from technical and behavioral standpoints, because of the amount of entropy introduced by the sheer number of decisions made by an analyst. This "decision entropy" is a possible contributor to many problems in replications, Hoffmann et al state "there are concerns that this multiplicity of analysis strategies plays an important role in the non-replicability of research findings"\cite{Hoffmann2020-sb}. Ironically, these choices are also being utilized to evaluate the quality of science, which is the point of this dissertation.

The ability of third parties to reproduce studies relies on access to the raw data and methods employed by authors. Much to the exasperation of scientists, statisticians, and scientific software developers, the rise of "open data" has not been matched by "open analysis" as evidenced by several case studies \cite{Obels2019-sy,Rauh_undated-ej,Stodden2018-ls,Stagge2019-fv}.

Missing data and code can obstruct the peer review process, where proper review requires the authors to put forth the effort necessary to share a reproducible analysis. Software development practices, such as documentation and testing, are not a standard requirement of the doctoral curriculum, the peer-review process, or the funding structure – and as a result, the scientific community suffers from diminished reuse and reproducibility \cite{Nust2018-wx}. Sandve et al. \cite{Sandve2013-yv} identified the most common sources of these oversights in "Ten Simple Rules for Reproducible Computational Research" – lack of workflow frameworks, missing platform and software dependencies, manual data manipulation or forays into web-based steps, lack of versioning, lack of intermediates and plot data, and lack of literate programming or context can derail a reproducible analysis.

An issue distinct from the availability of source code and raw data is the lack of metadata to support reproducible research. We have observed many of the findings from case studies in reproducibility point to missing methods details in an analysis, which can include software-specific elements such as software versions and parameters \cite{Collberg2014-cj}, but also steps along the entire scientific process including data collection and selection strategies, data processing provenance including hardware, statistical methods and linking these elements to publication. We find the key concept connecting all of these issues is metadata.

An ensemble of dependency management and containerization tools already exist to accomplish narrow-sense reproducibility \cite{Piccolo2016-kd} – the ability to execute a packaged analysis with little effort from a third party. But context to allow for robustness and replicability, "broad-sense reproducibility," is limited without endorsement and integration of necessary metadata standards that support discovery, execution, and evaluation. Despite the growing availability of open-source tools, training, and better executable notebooks, reproducibility is still challenging \cite{FitzJohn_undated-bq}. The following sections address these issues by first defining metadata, defining an "analytic stack" to abstract the steps of an in silico analysis, and then identifying and categorizing standards both established and in development to foster reproducibility.

Overall, the review above documenting aspects of the reproducibility crisis underscores the need to investigate this topic from multiple perspectives to ameliorate further crises. The research presented below, first examining existing case studies, then implementing tests of robustness to explore their viability as a natural extension of peer review, seeks to address the reproducibility crisis in terms of critical and exploratory evaluation.

Existing Methods in Reproducible Research Case Studies The questions posited above will be examined using a mixed-methods approach consisting of a *refactor*, followed by a survey and a brief user testing exercise called a test of robustness. A *refactor* is a type of case study used in reproducible research in which a study that is presumably valid but poorly reproducible is brought up to higher standards. A refactor is one of several available approaches that have been used in this area. To survey the existing efforts of formal measurement, I collected 40 case studies on reproducible research. This is published on Awesome Reproducible Research, a crowdsourced curated list of reproducible research case studies, projects, tutorials, and media.

Case Study Methodologies

2 Reproducibility case studies 2005-2019

The term "case studies" is used in a general sense to describe any study of reproducibility. A *reproduction* is an attempt to arrive at comparable results with identical data using computational methods described in a paper. A *refactor*, as described above, involves refactoring existing code into frameworks and other reproducibility best practices while preserving the original data. A *replication* involves generating new data and applying existing methods to achieve comparable results. A *robustness test* applies various statistical models or parameters to a given data set to study their effect on results. A *census* is a high-level tabulation conducted by a third party. A *survey* is a questionnaire sent to practitioners. A *case narrative* is an in-depth first-person account. A *theoretical case study* measures global reproducibility using non-empirical evidence.

Ioannidis 2005 \cite{Ioannidis2005-se}	Science	Theoretical	(all studies)
Glasziou et al 2008 \cite{Glasziou2008-of}	Medicine	Census	80 studies
Baggerly & Coombes 2009 \cite{Baggerly2010-gt}	Cancer biology	Refactor	8 studies
Hothorn et al. 2009 \cite{Hothorn2009-sx}	Biostatistics	Census	56 studies
Ioannidis et al 2009 \cite{Ioannidis2009-at}	Genetics	Reproduction	18 studies
Anda et al 2009 \cite{Anda2009-lr}	Software engineering	Replication	4 companies
Vandewalle et al 2009 \cite{Vandewalle2009-pe}	Signal processing	Census	134 papers
Prinz 2011 \cite{Prinz2011-ej}	Biomedical sciences	Survey	23 PIs
Horthorn & Leisch 2011 \cite{Hothorn2011-pb}	Bioinformatics	Census	100 studies
Begley & Ellis 2012 \cite{Begley2012-mt}	Cancer biology	Replication	53 studies

Collberg et al 2014 \cite{Collberg2014-cj}
Collberg & Proebsting 2016 \cite{Collberg2016-we}
	Computer science	Census	613 papers

OSC 2015 \cite{Open_Science_Collaboration2015-rm}	Psychology	Replication	100 studies
Bandrowski et al 2015 \cite{Bandrowski2015-qu}	Biomedical sciences	Census	100 papers
Patel et al 2015 \cite{Patel2015-zu}	Epidemiology	Robustness test	417 variables
Névéol et al 2016 \cite{Neveol2016-ou}	NLP	Replication	3 studies
Reproducibility Project 2017 \cite{Nosek2017-jk}	Cancer biology	Replication	9 studies
Vasilevsky et al 2017 \cite{Vasilevsky2017-vd}	Biomedical sciences	Census	318 journals
Kitzes et al 2017 \cite{Kitzes2017-qx}	Science	Case narrative	31 PIs
Barone et al 2017 \cite{Barone2017-ac}	Biological sciences	Survey	704 PIs
Kim & Dumas 2017 \cite{Kim2017-rz}	Bioinformatics	Refactor	1 study
Camerer 2017 \cite{Camerer2016-tr}	Economics	Replication	18 studies
Olorisade 2017 \cite{Olorisade2017-wt}	Machine learning	Census	30 studies

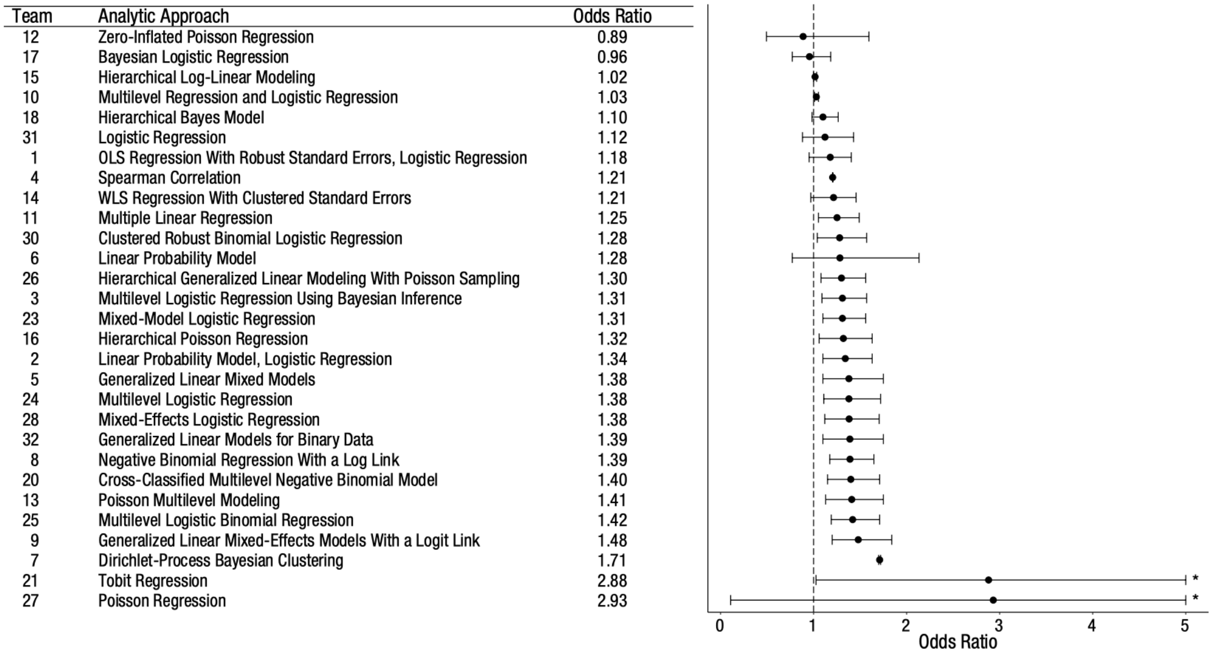
Case studies in reproducible research. This dissertation proposal posits three refactors (existing public examples in blue) to be completed by the candidate, and robustness tests (existing examples in green) as an exercise for the reviewers.

Surveys and censuses have provided a valuable understanding of the scope and nature of the reproducibility crisis. However, reproductions, refactors, and tests of robustness are most appropriate for in-depth investigations into reproducible computational research of the in silico variety.

Systematic Runnability Tests Several groups have begun to formalize the development of systematic in silico reproducibility tests, distinct from individual case studies or larger replication efforts such as the Open Science Framework Reproducibility Project. Software-based testing challenges such as CODECHECK \cite{Eglen2019-qm} focus on runnability, whereby a "code checker" certifies the code submitted with a paper executes on cloud-based infrastructure and produces outputs roughly identical to those in the paper, allowing for some leeway in terms of visible figures. ReScience is a peer-reviewed journal that targets computational replications \cite{Rougier2017-ys}. Rigorous reproducibility standards for submissions to NeurIPS have also been institutionalized \cite{Pineau2020-bs}. Despite the new opportunities afforded by reproducibility standards, none of these tests typically involve the manipulation of tools or parameters. In the following sections more involved techniques are discussed, refactoring and tests of robustness.

Refactoring for reproducibility A reproduction can merely be as perfunctory as running someone else's code on their data, with no regard to other qualities of the code itself. A reproduction by its very nature assumes the paper is easily reproducible. A refactor involves an attempt to improve the broad-sense reproducibility of a study to a level higher than its initial state. Broad sense reproducibility is defined as the ability to execute a packaged analysis with little effort (narrow-sense) with the added goals of discovery, reuse, and transparency in line with Findable, Accessible, Interoperable, and Reusable (FAIR) principles \cite{Wilkinson2016-qr}. A recent effort to develop metrics for FAIR has emphasized a rubric that is "clear", "realistic", "discriminating", "measurable", and "universal" \cite{Wilkinson2017-hf}

A refactor is an appropriate initial approach for this dissertation for three reasons. First, A refactor enables an evaluation of the latest tools including workflows, data-as-a-dependency, literate programming, and metadata standards. Second, A refactor enables the modularization and parameterization of tools and steps in a workflow to easily enable tests of robustness, in which a similar tool is swapped in or the parameter landscape is explored to examine the robustness of the p-value or result being posited. A recent paper by Vaquero from the laboratory of Barash explores the refactoring aspect by swapping out both tool versions and data in a replication of an existing RNA splicing study \cite{Vaquero-Garcia2018-ax}. Finally, analyses, even more than data processing, involve numerous choices about appropriate statistical tests, dealing with outliers and missing data, normalization, correction for multiple comparisons, null and full models. An interesting study by Silberzahn et al examined the various routes a statistical analysis could take by distributing a fixed data set of soccer official interactions and player variables to 29 teams, each charged with determining whether soccer referees are more likely to give red cards to dark-skin-toned players than to light-skin-toned players \cite{Silberzahn2015-yx}. Differences in statistical tests, treatment of covariates, and underlying model distributions created wide ranges of odds ratios, underscoring how nuances of statistical analysis can affect results and the importance of reproducibility when evaluating a study.



Team	Analytic Approach	Odds Ratio
12	Zero-Inflated Poisson Regression	0.89
17	Bayesian Logistic Regression	0.96
15	Hierarchical Log-Linear Modeling	1.02
10	Multilevel Regression and Logistic Regression	1.03
18	Hierarchical Bayes Model	1.10
31	Logistic Regression	1.12
1	OLS Regression With Robust Standard Errors, Logistic Regression	1.18
4	Spearman Correlation	1.21
14	WLS Regression With Clustered Standard Errors	1.21
11	Multiple Linear Regression	1.25
30	Clustered Robust Binomial Logistic Regression	1.28
6	Linear Probability Model	1.28
26	Hierarchical Generalized Linear Modeling With Poisson Sampling	1.30
3	Multilevel Logistic Regression Using Bayesian Inference	1.31
23	Mixed-Model Logistic Regression	1.31
16	Hierarchical Poisson Regression	1.32
2	Linear Probability Model, Logistic Regression	1.34
5	Generalized Linear Mixed Models	1.38
24	Multilevel Logistic Regression	1.38
28	Mixed-Effects Logistic Regression	1.38
32	Generalized Linear Models for Binary Data	1.39
8	Negative Binomial Regression With a Log Link	1.39
20	Cross-Classified Multilevel Negative Binomial Model	1.40
13	Poisson Multilevel Modeling	1.41
25	Multilevel Logistic Binomial Regression	1.42
9	Generalized Linear Mixed-Effects Models With a Logit Link	1.48
7	Dirichlet-Process Bayesian Clustering	1.71
21	Tobit Regression	2.88
27	Poisson Regression	2.93

Fig. 2. Point estimates (in order of magnitude) and 95% confidence intervals for the effect of soccer players' skin tone on the number of red cards awarded by referees. Reported results, along with the analytic approach taken, are shown for each of the 29 analytic teams. The teams are ordered so that the smallest reported effect size is at the top and the largest is at the bottom. The asterisks indicate upper bounds that have been truncated to increase the interpretability of the plot; the actual upper bounds of the confidence intervals were 11.47 for Team 21 and 78.66 for Team 27. OLS = ordinary least squares; WLS = weighted least squares.

Results from the Silberzahn study on soccer referee bias demonstrating the value of robustness tests on statistical approaches

Existing refactors in the literature A conference poster by the GATK group at the Broad Institute outlined attempts to perform a refactor of an existing exome study while applying WDL-compliant workflows, Docker containers, and Jupyter notebooks. This was very much in the spirit of this dissertation proposal, but because the underlying study involved protected data, the Broad group was forced to generate synthetic variants to mimic the original data set. While an admirable task, reverse engineering these called and filtered variants introduces considerable doubt into the validity of the reproduction, as a major component of exome studies involves dealing with the vagaries of variant and genotype calling, which is exactly what GATK is designed to do. Synthetically generating noisy data to imitate real noisy data only to arrive at the same intermediates falls short of true reproduction.

Defining Robustness Here we define the robustness of an analysis as its ability to maintain core findings while withstanding perturbations introduced by tool and parameter changes. This is converse to the understanding of tool or model robustness - being able to be applied to a wide variety of experimental designs without returning spurious or biased results. The robustness of an analysis should reflect both the experimental design and the strength of the underlying theory. The former is commonly known as "test validity" or more specifically "construct validity" - the degree to which a test measures what it claims to be measuring. \cite{OLeary-Kelly1998-ll}. The latter may reflect generalizability or "external validity", more generally "experimental validity", or scientific truth.

Understanding the history of omics method development To understand what makes it possible to perform tests of robustness, aside from justifying the practice as an evaluation tool, it is first necessary to explain why there are often many competing similar software tools that perform roughly the same tasks in the sciences.

- Different conceptual models - models can be derived from a physical or biological concept, for instance, many differential expression tools assume that there is a floor and a limit to the number of transcripts a cell transcribes in a given time, and that should inform normalization of libraries derived from biological replicates. Other models may not be informed of the underlying biology but will strive for the best possible fit.

- Different statistical models - tools developed using different mathematical or stat models of the underlying phenomena will exhibit different error profiles in the face of experimental noise. One semi-recent example is the adoption of the negative binomial model to more accurately portray between-sample read count distributions ("dispersions") of next-generation sequence expression data.

- Different experimental scenarios - tools developed to address a specific question are often adopted by other areas without formal vetting - for example, fusion detection in cancer being used to detect readthrough events \cite{Haas2019-rq}.

- Different test sets - tools are developed to model-specific data sets available to developers will be fitted to those sets. This is often the root of complaints about tool description papers, as opposed to formal benchmarking papers, as a tool fitted or optimized to a test set will appear superior to untuned "straw men" competitors \cite{Buchka2021-fa,Peters2018-av}.

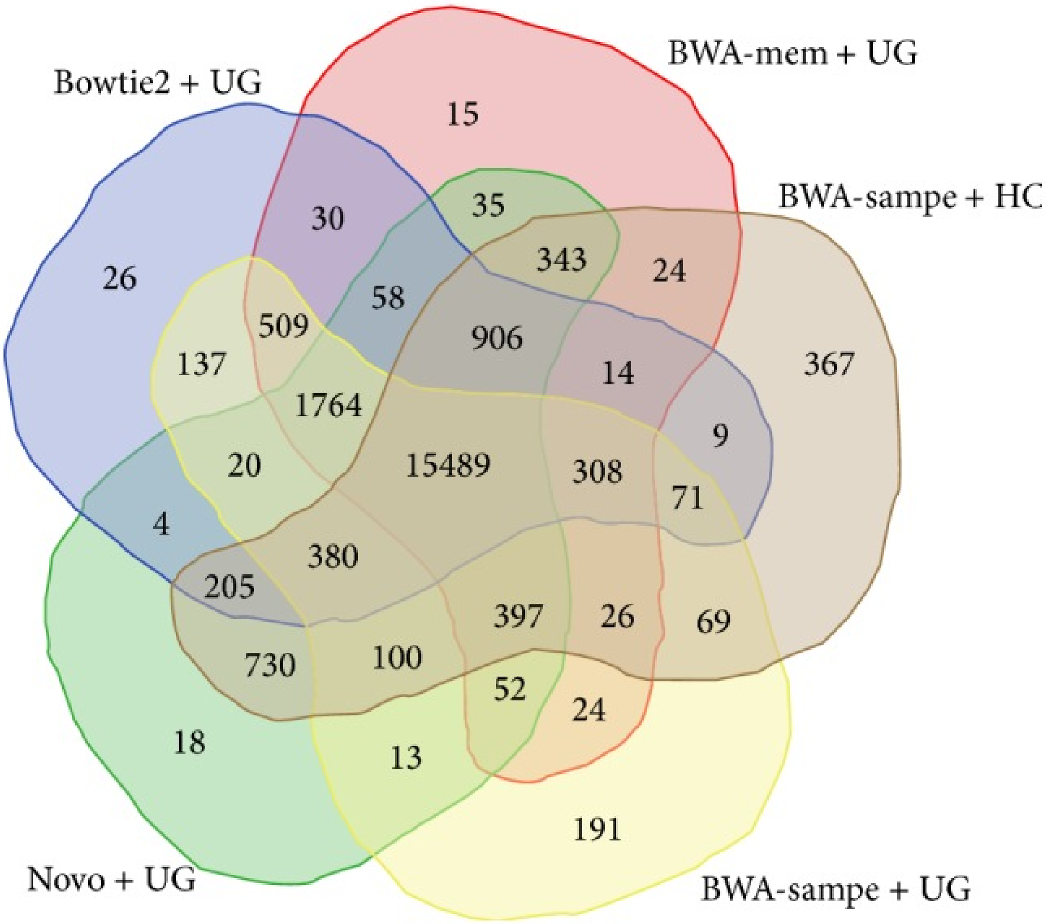
- Improved computational resources have enabled the implementation of computational approaches that would have been previously impractical. Both the transcript aligner STAR and the de novo assembler Velvet were developed during a time when single-node random access memory became available at gigabyte scale.

- Improved software implementations - this can be driven by the influx of developers from computer science or other disciplines and may take the shape of more modular, tested, or faster implementations.

- Improved algorithmic performance - Algorithms derived from other areas of mathematics, such as Burrows-Wheeler algorithm for compression, have provided indisputable improvement to omics tools, allowing either greater performance or more sensitivity with equal performance.
- Improved bias management - tools may improve as more data is collected about scientific instruments and biases are understood. In microarrays, MAS5 normalization has been largely replaced by RMA \cite{Lim2007-jb} and its successors, which utilize cohort normalization.
- Utilization of machine learning or deep learning approaches

A recent publication text-mining over 1000 single-cell tool papers revealed many of the trends above, with trends toward data integration, greater performance, and specialization \cite{Zappia2021-op}.

Degrees of Freedom and Questionable Research Practices To vet robustness testing as an evaluation tool, we must define the possible outcomes. The main theory behind robustness is that results are prone to inflations due to "researcher degrees of freedom", \cite{Bakker2020-qr} The flexibility afforded by computational choices enables both intentional and unintentional biases in the form of questionable research practices (QRPs). The most common types of QRPs are p-hacking, in which analyses are manipulated to achieve significance, and hypothesizing after results are known, in which the hypothesis driving an experiment is developed to explain a set of results as a confirmation, rather than a priori. Certain analytical approaches may invite these problems. Gene set enrichment analysis (GSEA) or Gene Ontology enrichment, for instance, are especially prone to QRPs \cite{John2012-lb} \cite{Timmons2015-yg,Wijesooriya2021-jc} because they so often generate results that can be explained post-hoc.



This Venn diagram from Cornish & Guda illustrates the lack of consensus among various alignment-variant caller combinations. In theory, each of these combinations could produce substantially different findings. \cite{Cornish2015-qo}

Exploring Scenarios of Robustness Testing Though there is no formal determination for whether a test of robustness has "passed" or "failed", criteria should be developed from the results and discussions in the manuscript. The test of robustness may prove simply inconclusive if there are limitations such as lack of computing resources, software bugs, time, data accessibility, that prevent it from being executed. Assuming a determination can be made by reviewers, multiple interpretations can be derived from that finding, and still, multiple conclusions and courses of action can be drawn from those interpretations.

- Possible interpretations given analysis fails the test of robustness
  - The original tool or model is the "correct" model and alternatives introduce error
  - Authors engaged in one or more QRPs
  - Authors implementation contained software bugs

    - Test of robustness itself contained software bugs
    - The analysis is fundamentally flawed

- Possible interpretations given analysis passes the test of robustness
    - Test of robustness was too limited
    - Both original tool and test models suffer from low test validity
    - Analysis is robust

Given the extensive time it takes to perform a test of robustness - a conservative estimate extrapolated from traditional peer review is 10 hours per reviewer and the experiences in this thesis suggests 20 to 100 hours may be more realistic - the peer review infrastructure should be revamped to yield downstream benefits from this work. An imagined endpoint that attempts to leverage yields from robustness testing is discussed further in the recommendations section.

Opportunities for robustness testing While a refactor is the most appropriate approach to test the state-of-the-art, the process of refactoring and modularization of steps should lend components of these analyses to be easily swapped out (i.e. test of robustness). This is a fortuitous opportunity as there is a renewed interest in systematic benchmarking of bioinformatic tools \cite{Mangul2019-cy} and statistical methods \cite{McIntyre2017-wr}, both of which greatly benefit from reproducible setups, especially modularized and parameterized workflows.

Benchmarking vs robustness testing Benchmarking can be viewed as the other side of the coin from robustness testing. A benchmarking exercise involves the evaluation of a tool or model using a gold standard truth set. In competitive machine learning circles such as Kaggle competitions, this gold standard would typically be a holdout test set sequestered from entrants. A common gold standard in bioinformatics is Genome in a Bottle, a collection of "High-confidence" variant calls and regions used in variant calling competitions. A benchmark without loss criteria or subjective judging is called a "bake-off", and typically involves groups with pipelines rather than individual tools. As bioinformatics is one of the few disciplines where every paper draws scrutiny of both the underlying theory and the tools used to study it, every paper is an opportunity to engage simultaneously in benchmarking and robustness testing.

An interesting and novel part of this dissertation will involve a "user testing" component of robustness. Reviewers will be charged with swapping out a tool or statistical test from each analysis with an equivalent replacement and reporting the results.

Bioinformatic analyses lend themselves to tests of robustness because they often suggest several analysis choices of equal suitability. As discussed below, these analytical choices stem from different assumptions about the underlying biology, mathematical models at hand, computing power, and other criteria which change over a long period of time. An example of this phenomena (one not used in this dissertation) is the decomposition or manifold learning of single cell data. Single-cell studies study individual cells or clonal populations, rather than bulk heterogeneous populations. A variety of genomic, transcriptomic (scRNA-Seq), and proteomic methods can be used to distinguish these populations. Most single-cell studies attempt to cluster and visually distinguish cell subtypes using dimensionality reduction (DR). The go-to technique for this since 2008 has been T-distributed Stochastic Neighbor Embedding (t-SNE) \cite{Van_der_Maaten2008-bm}. However, t-SNE has itself been criticized for low reproducibility in terms of achieving a given cluster for the same data, or when adding new data to an existing set ("prospective stability"), or in preserving local or global cluster distances. A technique called UMAP reportedly produced more similar clusters using various subsamples of a data set than other tools, including t-SNE \cite{Becht2018-df}. As a first order test of robustness, it would be desirable to implement UMAP on the raw data of a paper that uses t-SNE.

The above sections described existing case studies into reproducibility and presented robustness testing, in which tools, parameters, and data subsets are swapped out of existing analyses, as a natural extension of existing approaches that has been enabled by reproducible research and a practice that could be applied to peer review. These case studies informed the methods chosen for this dissertation. The next section presents my primary research questions guiding my work to implement robustness testing on existing published manuscripts with the intent to study both the requirements of packaging analyses for this exercise and evaluate the feasibility of this practice as a potential element of peer review.

2.0.1 Primary Research Questions

This dissertation addresses the primary question of whether reproducibility-enabled peer review, as implemented with tests of robustness designed to evaluate the strength of analyses, is feasible and practical given the state-of-the-art tools and standards. Secondly, this thesis is designed to reveal what is required for reviewers to conduct a test of robustness in terms of both software organization and computational environment. Finally, the dissertation seeks to identify next steps to improve this process should it become a standard practice.

2.0.2 Study Design

Methods This study employed a mixed-method methodology consisting of both user surveys and respondent-produced or respondent-directed analyses. A sample of three high-impact research papers were selected, each with open data and utilizing bioinformatic tools. These analysis, described only in the the methods sections, were then refactored into pipeline frameworks in order to provide a basis tests of robustness to be performed. Three different approaches were used. The first involved a complete reproduction of the paper for participants to manipulate, the second involved providing scaffolding for the use of pre-existing workflow libraries, and the third involved implementing expert suggestions in the form of tool swaps.

3 Sample:

Three papers in genomics were selected based on criteria including data availability, significance, and critical reception. None of the papers featured open source code so refactors were based on descriptions in the methods section. Three different approaches were taken in these tests of robustness - a faithful reproduction of the original manuscript into a framework that could be manipulated by participants, a workflow-library approach in which participants were encouraged to employ modern "off-the-shelf" pre-built pipelines to triangulate tests, and an advisor-led approach in which senior experts suggested alternate tools to be implemented and I generated a report for their evaluation.

Participants were contacted about this opportunity via several means - through Twitter, Slack groups, message boards, and direct personal contacts. IRB approval was obtained before conducting the survey and test of robustness.

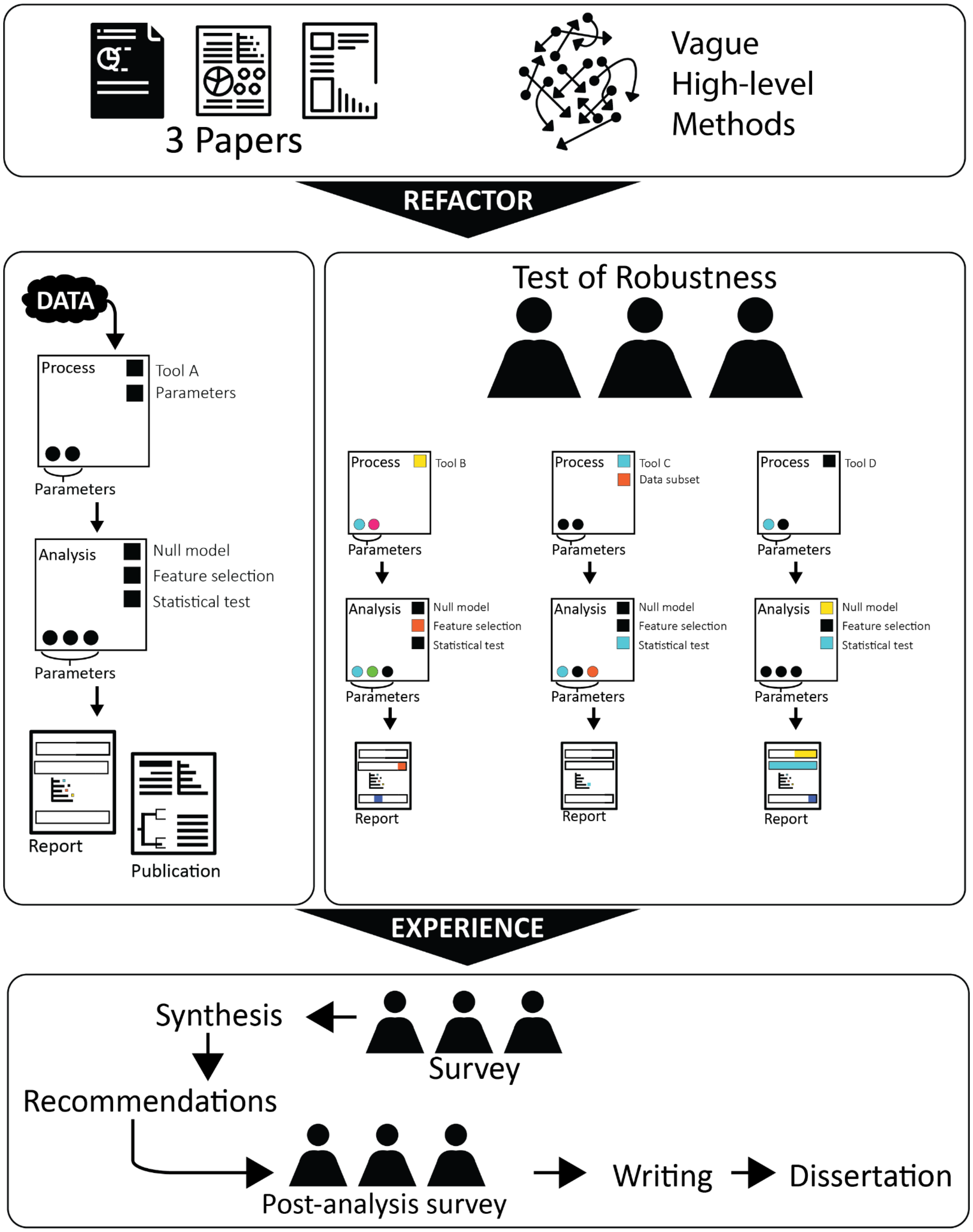


Figure 2: X outlines the study design abstract. Three papers without source code, with often vague or high-level methods sections, were refactored -prepared for robustness testing by providing organized and reproducible source code that could be used to conduct robustness testing. The refactored code is available in Github. The refactoring process is meant to examine what is necessary for consumption by reviewers, in particular, what pain points are involved in preparing data for tool swaps.

Study design diagram illustrating tests of robustness and evaluation phases

3.0.1 Paper selection

Identifying Candidate Papers Candidate papers refer to studies that will undergo the tests of robustness. The candidate papers should be peer-reviewed life science research manuscripts, as opposed to software or tool papers. These should be recent publications (>=2010) with at least nominal citation count (>= 5 citations/year). To accomodate a refactor, these papers should have a strong in-silico analytic focus, with substantial workflow and report components, as well as figures, tables, and test statistics to serve as targets. They must include open data (e.g. SRA or TCGA/GDC level 1, HMP), and either no open code or minimal refinement. To make this a decent exercise, no workflows are preferable. Of particular interest are papers that feature highly parameter-sensitive approaches such as deep learning. The reproducibility challenges of deep learning are more complex than simpler approaches - being highly sensitive to random seeds and often the inclusion of non-deterministic steps.

Pipeline framework selection Pipeline frameworks provide means of abstracting file transformations common to bioinformatic analyses. Modern frameworks consist of domain specific languages, which provides syntax used within full-featured programming languages, and configuration-based frameworks, which rely on formatted files with limited inline scripting to represent tools and steps of a workflow. There are four popular pipeline frameworks and languages used today - Snakemake, a DSL that relies on Python and uses a wildcard syntax to relate file inputs and outputs. Nextflow, a DSL in Groovy which uses abstracted channels to organize the flow of files through a pipeline rather than rely on filenames, Workflow Description Language, a JSON-based language developed at the Broad Institute that runs on a engine called Cromwell, and Common Workflow Language, a consortium-developed YAML-based language that runs on a number of graphical workbench portals in addition to command-line usage. CWL is the most verbose of these, but also offers the highest level of descriptive syntax for tools. It may be too verbose and heavyweight for rapid development and was therefore set aside for this exercise. WDL is very popular for genomic applications that rely on the Genome Analysis Toolkit (GATK), ENCODE pipelines, and other sequencing applications. Nextflow has a very strong community dedicated to building reusable pipelines, nf.core. Snakemake is popular among Python users and has a low barrier to entry. Because DSLs offer more flexibility in terms of rapid implementation and metadata management, they were used for the refactors. Snakemake was chosen for the Leiby paper as Sunbeam was already written in Snakemake. It was also used for Funnell et al due to the amount of "business logic" involved in sample management, some of which overlapped with Leiby. Nextflow was chosen for the Dominissi paper in order to attract nf.core users and test the workflow library approach to robustness testing.

Portal selection Workflow portal to accommodate these analyses. To our knowledge, there are no free workflow portals - SevenBridges Genomics, DNANexus, and Terra - are all for profit, but more importantly not often used in institutions without close relationships to those companies.

Terra, a portal from the Broad Institute, uses the Workflow Description Language. The vast majority of public workflows are from the Broad itself. A grant application to dispatch these reproductions on BioDataCatalyst - designed to encourage public workflows on such portals - was submitted, but was rejected because the research was not novel.

Computational environment All the pipelines were suited toward running on Amazon web services or Google cloud platform. AWS offers a very convenient integrated development environment, Cloud9, which enables users to access an editor, terminal and file browser from the web.

3.0.2 Selected Papers

The following papers were selected for the refactors and tests of robustness based on various qualities they brought to the table. Leiby et al's "Lack of detection of a human placenta microbiome in samples from preterm and term deliveries" introduces a high decision-entropy area c16s and metagenomics) combined with a clear but important result of interest - that of the sterile placenta. Dominissini's "Topology of the human and mouse m6A RNA methylomes revealed by m6A-seq" was chosen because of it's highly disputed citation record. Finally Funnell et al. "CLK-dependent exon recognition and conjoined gene formation revealed with a novel small molecule inhibitor" was chosen because it targeted an area that has been traditionally underserved by toolsets (read-through splicing events) and utilized two types of sequencing.

Leiby et al Leiby et al \cite{Leiby2018-lf} employs a multi-modal approach to studying a long-standing question of whether the human placenta inherits a maternal colony of bacteria (microbiome) or is sterile. Conditions include vaginal and Caesarean section delivery, preterm or full term birth. Positive controls were taken from mother's saliva.

16S rRNA qPCR'ed samples were sequenced on the Illumina MiSeq platform. 16S represents a small section of the ribosomal RNA that evolves at an appropriate rate to compare bacterial species, and is a standard and well-established method of microbiome diversity studies. The majority of this analysis is done in Dada2 \cite{Callahan2015-eu}, and Qiime1 \cite{Caporaso2010-rh}.

A shotgun metagenomic analysis was performed on the samples as well. This amplifies genes from all cells in a sample, including background host tissue. Metagenomic analysis is arguably a more rapidly changing area of microbial bioinformatics. The majority of the metagenomic analysis was performed with Kraken \cite{Wood2014-zm}.

The findings in this paper, while addressing an issue having important implications for health. Unlike the majority of scientific papers, this one features a "negative result" as its primary finding. This has some implications for the role reproductions and tests of robustness can play in a review - since most times an author will be hypothesizing for specific microbial composition changes while here the authors are simply claiming a sterile placenta. Though no replications have been performed, subsequent studies \cite{De_Goffau2019-zm} have largely confirmed the findings in this paper,

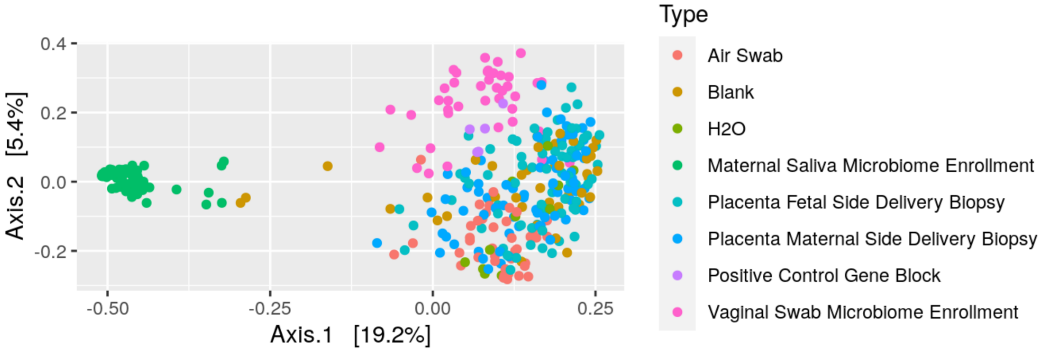
Graphics Type ‘SHAPE’ is not supported yet. Please insert it as image.

Graphics Type ‘SHAPE’ is not supported yet. Please insert it as image.

Original (Figure 1A) and reproduced (Figure 1B) bacterial abundance plots

Graphics Type ‘SHAPE’ is not supported yet. Please insert it as image.

Figure 2B



Original (Figure 2A) and reproduced PCoA (Figure 2B) of unweighted UniFrac distances, for all samples

As evidence in Figures 1A, 1B and 2A and 2B, the reproduction is not identical to the original, and warrants further investigation. As described below, missing details in the methods section may have contributed to these discrepancies.

Issues in the Leiby refactor To review verbiage, we consider a reproduction to essentially mean a third party repeats an analysis with readily available source code. A refactor implies either the source code is missing or it is substantially replaced and reconfigured to high reproducibility standards. As mentioned in the proposal, papers were chosen based on having little to no source code. This was explicitly decided in order to essentially force the refactor to closely examine the challenge of confronting decision entropy presented by inadequate methods sections. While the refactor involves a lot more reverse engineering and guesswork, it eliminates the possibility of glossing over impenetrable sections of source code which may behave in an unpredictable fashion. It can also detect insidious errors that arise from various common bugs such as:

- assumptions of column or row order (typical slicing bug in R)
- Assumptions of mistaking index ordinal for cell value
- Assumptions in calling for indices instead of cell value (Python)
- cell shifts (as happened in Potti scandal)

Leiby et al presents typical challenges in terms of reproducing a paper with no source code and little in the way of supplemental in silico methods section. The tools and methods employed, while not exotic, still require a thorough reading of tutorials in order to reproduce these steps even before addressing bigger issues of sample and parameter selection. Overall about 60 hours of labor were required to reproduce this paper. Dependency management and decay in the availability of dependencies were caused by a number of factors, namely the dependence of python2 for Qiime1 required it be isolated from other tools. Qiime1 also had numerous third-party dependencies that were deprecated or entirely missing (uclust implementation). Many scripts within Qiime1 attempt to fetch dependencies by unorthodox means but are unable to find them, so I had to preempt that process within the conda and pip universe. A significant amount of time was spent in what could be considered "data cleaning" tasks - mainly the alignment of naming schemes between SRA metadata schemes, files, and the sample metadata contained in the supplemental tables included with the paper. Significant time was also spent finding a way to generate a multiple sequence alignment and tree building suitable to use with Phylotree within a reasonable time. Several tree building steps took on the order of hours and occupied nearly 100GB of RAM. Building the Kraken indices used by Sunbeam required over 8 hours of processing time and nearly 1TB of disk space. Because of the vague methods it wasn't exactly clear what operations were performed by Dada2, Qiime1, Phylotree, RAXML. There was significant overlap and some software appears to take a more heuristic approach appropriate to large sequencing runs while others are exhaustive.

It would not be reasonable for a peer reviewer to engage in this level of reproduction for the sake of analysis, though such forensics have been done in the past on papers with no source code.

Causes of decision entropy The Leiby paper r

- Inadequate detail - which premade SILVA freeze and stringency (97/99 etc) was used
- Unclear choices when overlap in function are presented between DADA2 and Qiime steps
- Treatment of redundant or ambiguous sequences

- Chimeric sequences
- The effect of quality trimming \cite{Mohsen2019-er} on analyses in Qiime is significant.
- Unknown granularity of genus counts
- The cycle of threshold (Ct) qPCR data was not available to reproduce figure
- SILVA version was not given
- Operational Taxonomic Unit (OTU) vs Amplicon sequence variant (ASV) approaches. The OTU approach uses a degree of sequence identity to form clusters. This prevents sequence data with small errors, to not be misclassified as novel species,though it can potentially cluster distinct species. Within OTU clustering are reference-free approaches, in which sequences are clustered de novo without a reference database, and reference-based clustering approaches which rely on prebuilt taxonomic databases such as SILVA, RDP, or Greengenes. Amplicon sequence variant (ASV) approaches such as the one implemented in Dada2 uses exact nucleotide patterns to distinguish marker sequences \cite{Callahan2015-eu}. In the Leiby paper the early taxonomic assignments are handled by Dada2 but later measures of beta-diversity are calculated in Qiime using a reference-based OTU approach.

Unknown which are conscious decisions, which are known practices that have been long established best practices when default parameters are appropriate.
Dominissini/m6a
The second paper candidate for a test of robustness was selected based on its profile as a paper that had a very high number of contrasting citations in scite.ai, an index that performs lexicographic full-text analysis on phrases flanking citations in scientific literature. In scite.ai, the vast majority of citations are neutral or "mentioning", some are "supportive", and a few are "contrasting". This assignment is performed solely by a machine-learning model based on the verbiage in those citing articles.

A highly-cited paper from 2010, Dominissini et al.'s "Topology of the human and mouse m6A RNA methylomes revealed by m6A-seq", received 13 "contrasting" citations in scite.ai, indicating some groups found different mRNA splicing in METTL3 knockdown cells, number of peaks, peak location, overall methylation levels. This paper is one of the first to develop a means of detecting a type of post-transcriptional modification, the methylation of adenine that has been implicated in expression and splicing and implicated in cancer and neurological diseases. While the wet lab advances in this paper were profound, the bioinformatic analysis was fairly conventional.

The use of a paper with these replication issues, post-publication reviews allows the advantage of hindsight, which would not be available in a real-world situation. For the purposes of a proof of concept we can nonetheless examine if our approach could have identified such issues using robustness tests.

Workflow library approach The experience of the Leiby microbiome paper indicated most reviewers would tend to make fairly conservative changes to the pipeline rather than completely upend tools, the latter requiring a more intimate knowledge of intermediate steps than could be afforded in limited time. An alternate approach was adopted to address this: rather than attempt to faithfully reproduce this paper using its original analysis stack, have used a couple off-the-shelf pipelines to reanalyze this data.

The nf-core is a library of 50 Nextflow-based pipelines. These pipelines address a wide range of common analyses in bioinformatics including germline and somatic variant calling, RNA-Seq, ChIP-Seq, ATAC-Seq, to support a number of sequencing-based analyses. In particular, the workflows `https://github.com/eQTL-Catalogue/rnaseq` and `https://github.com/kingzhuky/meripseqpipe` were evaluated as appropriate. In order to prepare reviewers to quickly engage these, a manifest generator was developed to produce input manifests for these two pipelines. No reproduction code was provided to the reviewers, in order to ensure they used nf-core modules and new tools.

Decisions in epitranscriptomic studies

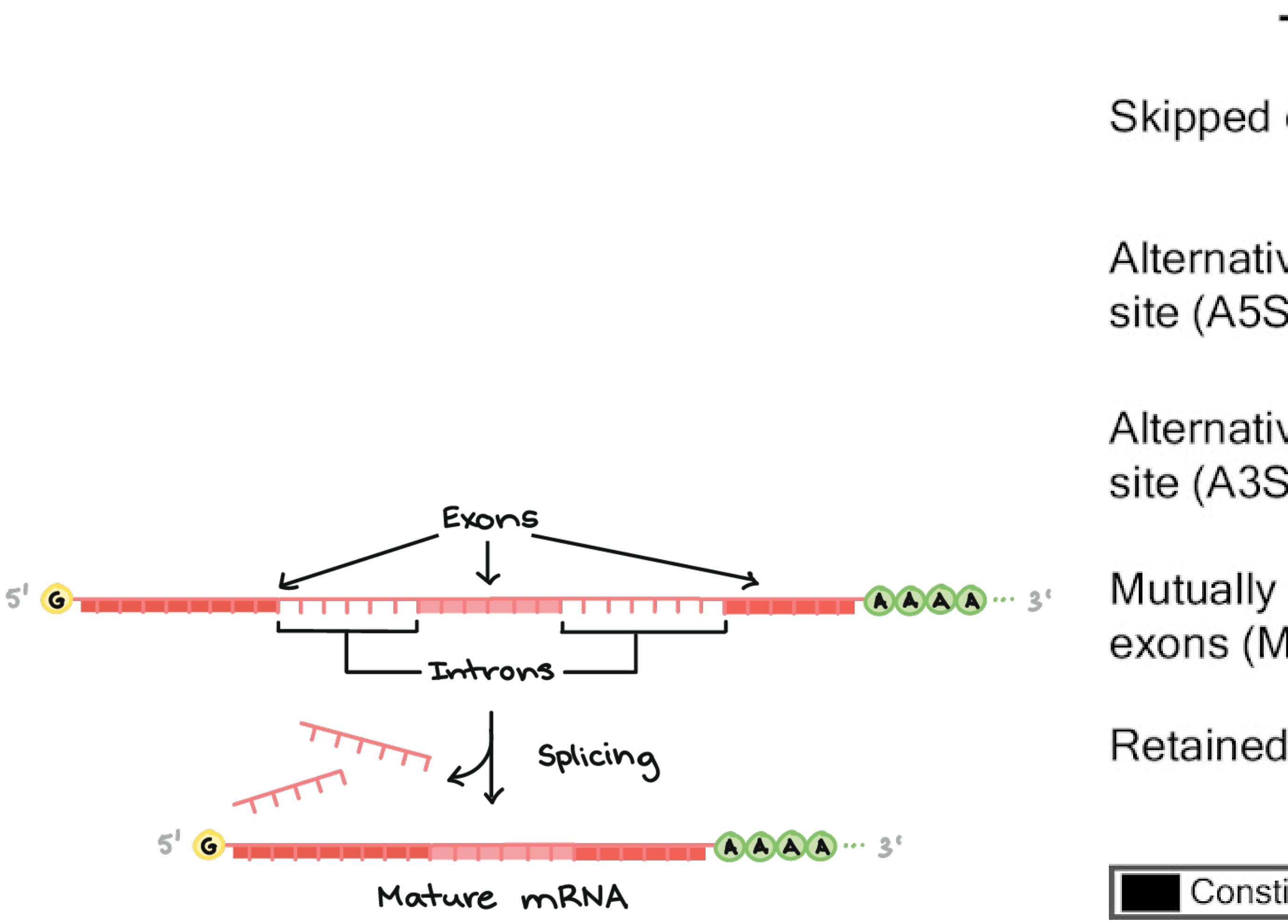
3.0.3 Funnell/CLK

Funnell et al "CLK-dependent exon recognition and conjoined gene formation revealed with a novel small molecule inhibitor" (2017), investigates the effect of CDC-like kinase (CLK) inhibition. Alternative splicing is a fundamental biological process by which a multitude of mature messenger-RNA (mRNA) transcript isoforms are created from pre-mRNAs in eukaryotes. This occurs typically by splicing together (or skipping) exons or exon segments. Kinases are ubiquitous proteins that phosphorylate residues and are central to cell signaling. Because alternative splicing is mediated by RNA-binding proteins that interact with spliceosomes in the nucleus, kinases can affect both the quantity and variety of mRNA isoforms.

The authors of this paper created a novel molecule, T3, to inhibit CLK2, a known splicing-related kinase, in order to examine its effect on splicing patterns. They discovered that T3 produces more alternative splicing events, the skipped exons where enriched for RNA-binding motifs (DNA patterns that are used for recognition by RNA-binding proteins), and perhaps most interesting, a dose-response increase in conjoined genes (see below). Conjoined genes, or "transcription-induced chimeras" are the results of either readthrough events in which the RNA polymerase continues transcription into the next downstream gene, or somehow

This paper was selected because the bioinformatic tools to investigate differential splicing and isoform reconstruction are rapidly in flux and have changed considerably since its publication. Isoform detection and estimation took something of a back seat to simpler "percent-spliced-in" exon-centric differential splicing during the short-read era. Because genes are long (mean 3522 bp) and sequencing-by-synthesis (i.e. Illumina) reads are relatively short, transcript reconstruction must take an indirect approach of inference. This can involve de-novo assembly, which is computationally expensive.

Secondly, the group employed both short (Illumina MiSeq) and long read (Pacbio SMRT) sequencing, which further complicates analysis and leveraging these mixed reads is an ongoing area of research. Finally, the phenomenon of trans-splicing, read-through events where the transcripts of two genes are conjoined into single transcripts, is unusual and itself not a typically reported measure of transcriptome analysis tools. It remains something of a mystery how the conjoined genes in this analysis were detected using MISO package as this is not a listed alternative splicing type in its documentation nor are there other examples in the literature of using MISO in this manner \cite{Katz2010-ts}.



A) The mechanism of alternative splicing to produce different mRNA isoforms, and B) the primary different types of alternative splicing events

Approach: Advisor Model Tests-of-Robustness Due to the age of the manuscript and some of the tools within a direct reproduction of this paper was not attempted, but instead three external advisors (YX, EL, were asked how they would investigate the phenomena described. I then implemented their suggestions and compared them to the results in the manuscript, a "naive" test of robustness. I term this proxy approach, in which a dedicated analyst performs the software development and implementation of the "advisor model", and its advantages and disadvantages to reviewer-implemented tests of robustness are discussed below.

4 Results

The results refer to results of the participant survey and critical first-order scientific observations drawn from performing tests of robustness, rather than assessments of the exercise itself.

4.0.1 Survey Results

A survey questionnaire was distributed to each participant to collect views on reproducibility and specific gaps in reproducibility with regard to reproducibility-enabled peer review. This survey was conducted simultaneously with the robustness exercise. Professional demographics and overall familiarity with reproducibility concepts, knowledge and usage level software, daily engagement with reproducibility best practices. Several questions were used verbatim from Baker et al's "1,500 scientists lift the lid on reproducibility" and State of Open Data 2019\cite{Digital_Science2019-iu} in order to assess any drift in attitudes, and the results are mostly in line with those. While an N of 10 respondents is not an adequate sample size from which to draw firm conclusions, the survey can be viewed as a decent filter for the questions themselves (many questions exhibited low entropy). More interesting were ranking-style questions that forced a participant to prioritize.

One exception is a question lifted directly from Baker, ranking factors by how much they contribute to a failure to reproduce results. The top five reasons listed were fraud, pressure to publish, insufficient oversight, insufficient peer review, and selective reporting. This varies somewhat from the Baker results (CHECK), and differs from the “party line" that a lack of details is responsible. The top three implying an intentional or nefarious intent to deceive, while more innocuous reasons of poor practices, mistakes, lack of detail and bad luck scored lower.

Some questions were designed to tease out a preference for replication vs robustness. Given replication is far more well-known concept in reproducibility, it was assumed it would be more warmly received, however votes were split evenly between them, with two favoring replicability, two robustness, and 4 stating these were equally important.

While 90% felt it was more (50%) or equally (40%) important that a scientific finding replicate with new data than with existing data.

Six participants were unaware of preregistrations (Q8.3)

Participants were also divided other where they would avoid journals that did not accept preprint (Q8.4)

In-line responses Attitudes toward the open data, data sharing, need for increased recognition, and the difficulty in obtaining raw data from others, and the effect of missing methods (Q7.5), difficulty identifying paper figure provenance (Q7.8), and glossing of computational details (Q7.10) were overwhelmingly assentive. Most agreed runnable analyses would be useful for peer review (Q9.2) Less pronounced were problems with label figures (Q7.6) and the role of sequencing providers(Q7.7).

In terms of ranking large categories of existing technologies as to how they contribute to reproducibility (Q6.2), all respondents identified dependency managers and containers, and lightweight pipeline frameworks, and notebooks as contributing “ a lot" or “a great deal", while workbenches, code commenting received slightly less enthusiasm.

The most important questions for this thesis concerned metadata. Question 7.4 revealed that “parameters used in an analysis" was viewed as most lacking, with raw omics, runtime environments, and lab protocols tied, and bioinformatic tool metadata trailing. Q7.11 asked respondents to rank which metadata would help improve collaborative review most – metadata about raw data beat out tools and statistical tests, with metadata about other papers (by topic or method) judged less important.

Perhaps the most divided question was “do you find too much information about files is stored in filenames" (Q7.12), with 4 strongly agreeing and 2 either strongly or somewhat disagreeing.

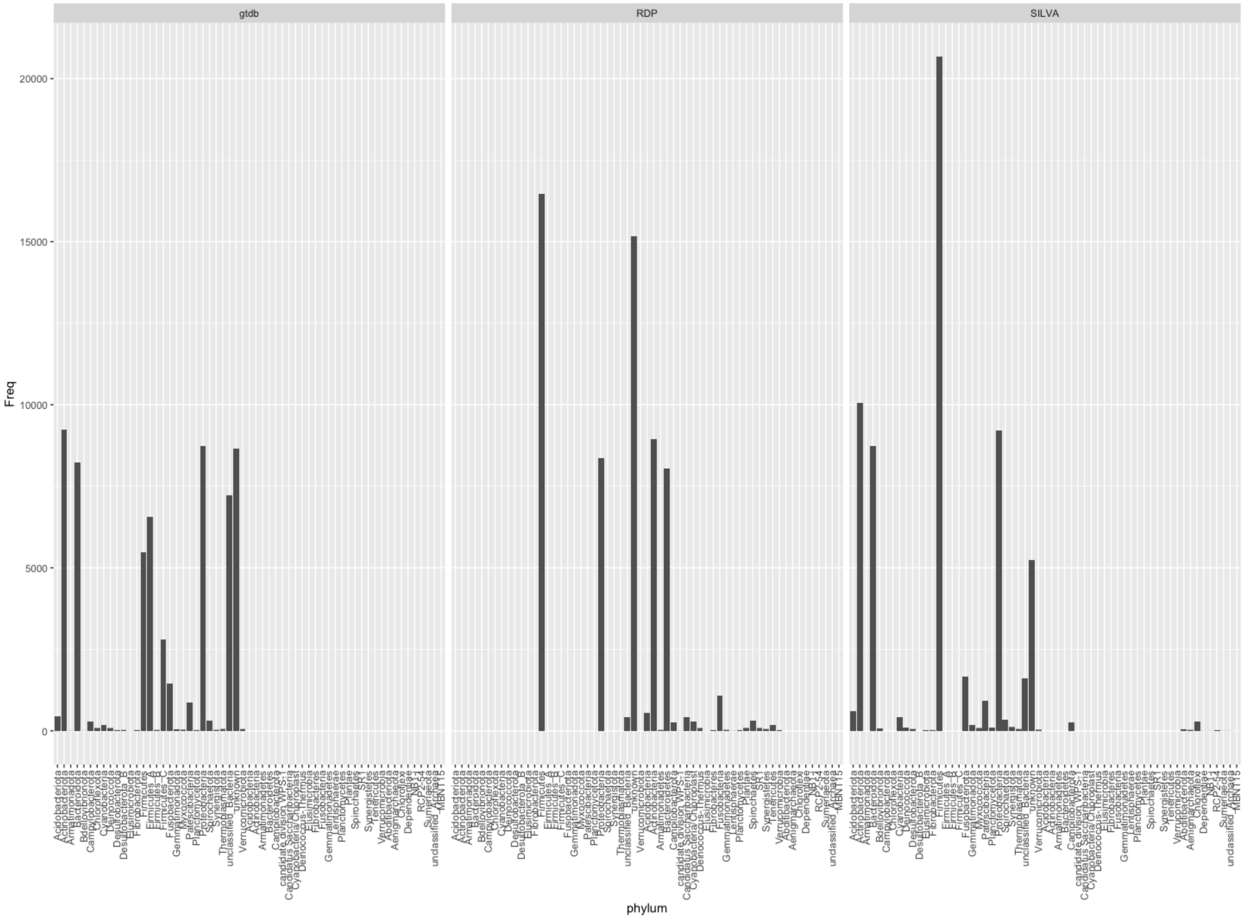
See Appendix for Full Survey Results

4.1 Robustness Testing Results

4.1.1 Leiby/placenta

Participant I is a tenure-track assistant professor with extensive experience in microbiome studies. During the entry interview, IB expressed confidence in the approach taken by Leiby et al but less confidence over the rigor of microbiome papers in general.
IB conducted a number of permutations involving clustering algorithms, reference databases, and alterations of forward-only or forward-reverse alignments in uclust.

Participant II Participant II is a bioinformatics software engineer with authorship on several microbiome papers. Participant II performed several swaps of the "pooled" vs "consensus" chimera removal step in Dada2. Chimeras refer to artefactual sequences composed of two or more parent sources as a result of PCR amplification errors \cite{De_la_Cuesta-Zuluaga2016-fr}. Participant II performed iterations of terms of taxonomic assignment and sequence tabulation using the Ribosomal Database Project (RDP), Genome Taxonomy Database (GTDB), and RefSeq, and SILVA 16S databases



Differences in phyla assigned by DECIPHER using three different 16S rRNA databases

Participant II produced iterations of parameters sent to DECIPHER and dada2 - namely choice of 16s database, chimeric removal, and randomized taxonomic classifications performed by DECIPHER showing substantial differences in choices between gtdb, RDP, and SILVA 16s databases. Part of these differences can be discrepancies between naming conventions (Firmicutes vs Firmicutes A/B/C), and large differences to the assignment to the "unknown" phylum. The participant did not follow-up to test if these assignments varied systematically between treatment groups.

5 Issues in the refactor

The three participants were asked to collaborate on developing a high-level description of metadata that would have assisted in a first pass review of a microbiome manuscript.

Minimal data to reproduce a microbiome paper: - FASTQs
- Mapping (samples' metadata) file
- 16S Database, including clustering identity (e.g. Greengenes clustered at 97% identity)
- Taxonomic database (kraken, kaiju, ...)
- Annotation database (NCBI-NR, swissprot, uniprot, kegg mapping, ...)
- host reference genome (if applied, preferably a link to the same or an upload on zenodo etc.)

Minimal list of software/methods to reproduce a microbiome paper:

- FASTQ primer and low-qual removal software (trimmomatic, cut-adapt, qiime)
- FASTQ merge software (pandaseq, vsearch, etc)
- FASTQ host-filtering process (blast, bwa, bowtie, minimap)
- 16S "Pipeline" (qiime1, qiime2, dada2, mothur, etc)
- OTU picking method (uclust, usearch, vsearch, etc)
- OTU picking strategy (de novo, closed-reference, open-reference)
- Programs/R libraries to process OTU/ASV tables (phyloseq, etc)
- Setting a seed under R or python or other approaches if possible to reproduce the run.
- QIIME tracks provenance, so similar information (sessionInfo() from R)

Additional list (other data and methods that should also be included if used):

- Primers (if not using standard Illumina V3-V4 primers)
- If Longreads are used, then definitely need the signal level information (either fast5 or subread bams) and the process to generate them. There's still a lot of variation between the various kits used and data quality seems to be improving day by day.
- Fastq barcode splitter (if FASTQs are barcoded)
- Taxonomy assignment/classification method
- Dereplication method
- Clustering method
- Chimera removal method
- Denoise method
- Assembly method (if not amplicon)

$*$ all methods should declare program version and parameters used

Dominissini/m6a
Background
Dominissini et al "Topology of the human and mouse m6A RNA methylomes revealed by m6A-seq" is a highly cited (>2000 citations on GS) paper which explores a type of RNA modification called N6-Methyladenosine or (m6A). Adenosine methylation is the most prevalent post-transcriptional modification in RNA \cite{Zhang2019-uh}, and has been identified as a core biological regulatory mechanism and dysregulation has been implicated in a number of diseases, particularly cancer and neurological disorders \cite{Jiang2021-kv}.

Dominissini et al is one of the first to comprehensively map m6a sites using meRIP and also examined the effect of m6a methylation on transcription using a knockdown of a known acetylation complex component METTL3 using HepG2 cells, a human liver cancer cell line that is very common in splicing studies. Another experiment in this paper uses mouse cell lines

This particular paper was chosen for a test of robustness based on a methodical search using scite.ai, a text-mining and lexicographic tool designed to measure the context surrounding citations . Scite.ai uses deep learning models to classify citations as supporting, contradicting or "contrasting", or merely neutral or "mentioning" \cite{Nicholson_undated-ry}. The vast majority of classifications in scite.ai are neutral.

A scite.ai search revealed a number of papers that contained a high number of contrasting statements and also had accessible data in the NCBI Sequence Read Archive. A Twitter poll was conducted to gauge interest in these candidate papers, and Dominissi et al was a clear favorite among the 14 respondents. Dominissini et al has 175 supporting, 2600 mentioning, and 13 contrasting statements as of June 2021. The flanking text in all of the contrasting statements leaves little doubt that they are in fact, contrasting, which suggests there may be some false negatives in the neutral category (i.e. the classification algorithm is conservative).

A number of contrasting statements are garnered from review articles that summarize later m6a publications which could be loosely considered replications. Because Dominissini et al. featured one of the first uses of m6A-seq aka MeRIPseq (methylated RNA immunoprecipitation sequencing) and associated peak calling, RNA-Seq, overlaps, in both human and mouse cells, there are a number of downstream replications that are relevant. Contrasting statements included disputes about the level of mRNA splicing in METTL3 knockdown cells, number of peak, peak location, and overall methylation levels.

The contrasting citations are important in that the instructions given to participants were instructed to use them as guidelines for further examination. While this type of post-publication replication hindsight would not be available in a traditional peer-review model.

The workflow-library approach It was decided to accelerate the robustness exercise for this manuscript using "off-the-shelf" components, rather than modifying an attempt at a faithful reproduction. This was decided for three reasons. First, from the experiences with Leiby et al, as none of the participants were able to progress past the 16S section of the paper. This meant that the reproduction of the metagenomic components of the paper were done without proper robustness testing, though a number of lessons were learned in the process. Secondly, the workflow-library approach offers some interesting advantages - such pipelines are well tested, reflect updated mainstream popular tools and approaches, a large user base for support, often come with built-in reference sets, often have multiple implementation choices built-in, and are designed to work on a number of computing environments out of the box. There are disadvantages that would make some papers inappropriate to be robustness tested in this manner - difficulty in customization, a lack of exotic or cutting edge protocols, and the need to conform to file manifests that may not match an specific experimental layout. However, these disadvantages did not seem to be insurmountable for this particular exercise. Finally, the age of this paper (11 years) made it somewhat impractical or desirable to reproduce faithfully.

Nf.core is a collection of prebuilt analysis workflows using the Nextflow pipeline framework \cite{Ewels2020-rf}. Over 47 pipelines are either released or under development in nf.core. Nf.core has a highly organized curation and testing process, with a preliminary vetting, guidelines for software development including formatting, comments, and code organization. Finally, the nf.core community maintains an active Slack channel, making it easy to identify beginners or students who have the time and interest to participate in this exercise.

In order to prepare participants to use nf.core for tests of robustness - manifest generators were developed for two pipelines identified as crucial - RNA-Seq, the most mature of pipelines in nf-core, and meripseqpipe, an unreleased pipeline with applicable tools.
As identified in scite.ai, many later experiments failed to replicate some of the global changes identified in Dominissini.

A review by Widalgo et al \cite{Widagdo2018-vw} identified differences between the METTL knockdown experiments in mice conducted by Dominissi and two subsequent studies and a comprehensive and deeply sequenced gradation knockdown performed by Ke et al \cite{Ke2017-lh} in mice.

Some of these discrepancies may be due to species differences, some may be due to cell lines, but in no case were subsequent analyses harmonized to remove the analytic components as a confound.

This result suggests that quantitatively little methylation or demethylation occurs in cytoplasmic mRNA. In addition, only ∼10% of m 6 As in CA-RNA are within 50 nucleotides of 5 or 3 splice sites, and the vast majority of exons harboring m 6 A in wild-type mouse stem cells is spliced the same in cells lacking the major m 6 A methyltransferase Mettl3.

It was noted that an open post-publication peer review site, PubPeer, identified the Domin-nisi samples as being very contaminated with mycoplasma as identified by a survey of SRA \cite{Olarerin-George2015-hc}. This suggests a number steps should be taken to avoid the deposition of contaminated samples pre-submission, and begs the question of what role in silico peer review has in that process.

Reviewer 1 To examine the RNA-Seq data, one participant ran the RNA-Seq samples through the nf.core RNA-Seq pipeline.

Log-fold shrinkage is a feature of modern differential expression packages that reduces the apparent log-fold changes of genes or transcripts that appear to be artifactually inflated. Apeglm shrinkage applies a heavy-tailed Cauchy prior distribution for effect size, in order to lower variance for genes with low read counts or high variance \cite{Zhu2019-wo}. The use of lfc shrinkage became default behavior with DESeq2, but Dominissini et al used the original DESeq, in which this shrinkage treatment was optional. In practice this may have resulted in inflated DEG counts.

Reviewer 2 To examine the possible effect of workflow on the lack of replication by Ke et al, a participant was interested in performing a mini-replication within this exercise. This participant reported Ke et al contained too much variety of paired-end and single-end reads to work easily with nf.core RNA-Seq, so the user switched to Wu et al. (CITE), a mouse METTL3 knockdown. This allowed the comparison with the previously calculated human METTL3 knockdown data (the users were in contact through a Slack channel dedicated to this project within nf.core). It also allowed the study of the overlap of mouse m6a peaks reported in Dominissini et al.

Reviewer 3
Despite the preparation of the meripseqpipe module in Nextflow, no volunteer was available to perform the peak calling exercise which would have evaluated the controversial Figure 4 in the Dominissini paper.

Issues in the Dominissini refactor The Dominissini refactor consisted of merely generating run manifests for the RNA-Seq and meripseqpipe modules. Without the benefit of hindsight it would have been very difficult to identify crux issues with this paper, being the first of its kind to use m6a-seq.

5.0.1 Funnell/clk

Issues in the Funnell refactor Funnell et al consists of a mix of 170 Illumina and Pacbio libraries, with a variety of treatments (T3 at 4 doses, various siRNA knockdowns), human cancer cell lines (HCT116,184-hTert), and stranded/unstranded library preps. The panoply of sequence files required the development of a metadata management library ("metautils.py") to to assist in generating both Snakemake targets and the appropriate input manifests for all the software requested by the expert advisors.

To accelerate the processing and take advantage of other QC outputs, a WDL-based workflow based on the GTEX RNA-Seq pipeline was used on a private workbench portal hosted at Truwl.com.

- The specification of which libraries are stranded/unstranded RNA is not located anywhere in the metadata except deeply embedded in sample names ("Untreated HCT116 whole transcriptome (unstranded)"). While not fatal, this added an additional step to the parser which would not be useful for other projects. A universal SRA manifest-generator would have to accommodate these types of edge cases.

- The use of older bas.h5 PacBio reads required an archived tool "bash5tools.py" which only worked with Python 2.7, which was deprecated at the end of 2020. This required a separate Conda environment.

Several installation and runtime issues were experienced with rMATS-Iso and SUPPA. While rMATS is production-level software used by hundreds of researchers, rMATS-Iso is still a beta distribution, and was selected by one of the reviewers in order to evaluate its potential.

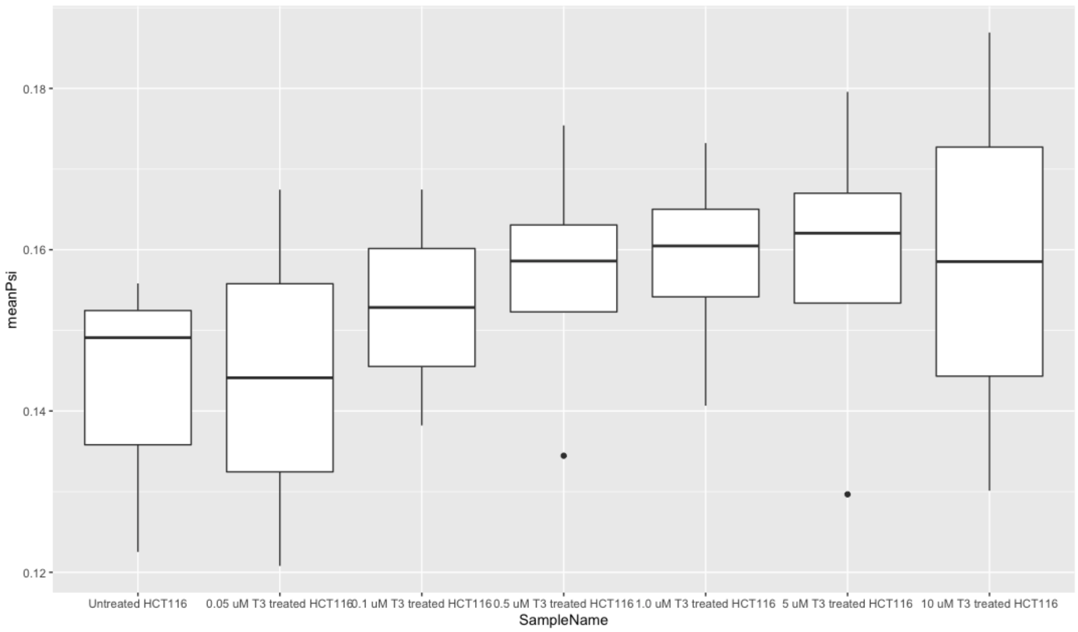
The lack of widespread user testing for rMATS-Iso led to some lack of a base from which to draw proper parameters for the older-style PacBio reads included with the Funnell paper.

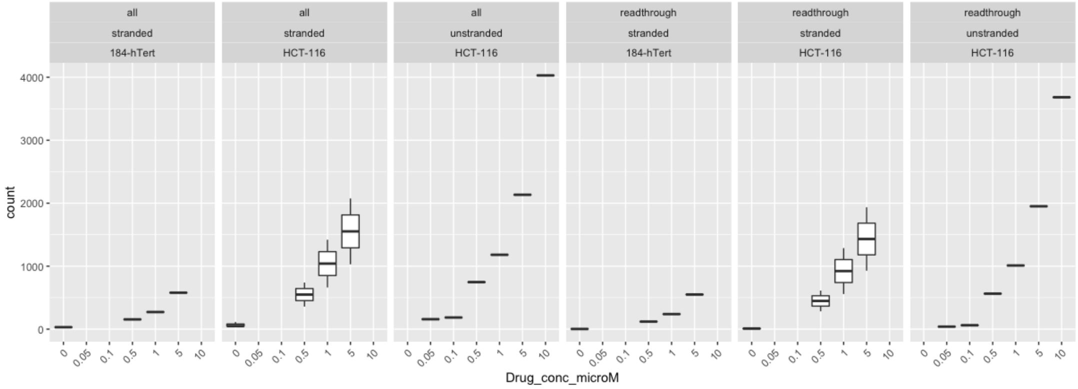
SUPPA utilizes pseudo-alignments generated against the transcriptome (typically in Salmon or Kallisto). As such the consistency between transcript FASTA files and gene models (GTF) must be preserved. One bug encountered was the additional transcript identifiers found in definition lines caused errors in SUPPA which were not reported anywhere. Another cryptic bug appeared with low numbers of mapped reads, which forced the inspection of

The initial mapping qualities for Illumina reads using the default k-mer index of Salmon (31) were very low (<50%). Upon closer inspection of the fastq files, many reads were shorter than 45bp, indicating some quality trimming was done prior to submission to SRA. This was not indicated in any SRA metadata. An index length of 17 was chosen to accommodate these shorter reads based on advice in help forums.

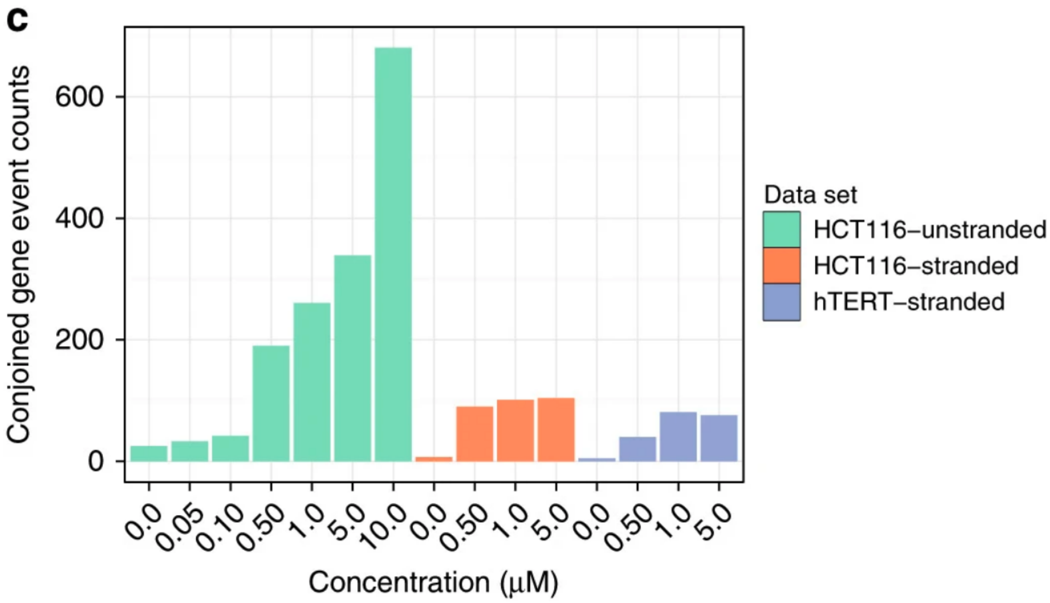
Very low mapping rates continued to appear for the PacBio reads. It was noted that "consensus generated" reads at EBI were mapping better than h5 extract fastq files. Upon closer inspection of the manuscript, there were suggestions that the PacBio reads, which constituted 60 of the 169 sequence files, were mainly used in ad-hoc confirmatory basis.

,
One problem with having so many similar tools is that many auto-fetched roughly, but not identical, reference files.





Counts of fusions by Arriba largely confirm the monotonic dose response, with the vast majority of fusions being classified as readthrough events



6 Discussion

6.1 Research questions - are tests of robustness a Validating the Theory of Robustness Testing

The theory of robustness testing presented here is not well vetted within the life sciences, and the term robustness itself is more likely to be used in the context of methods than analyses, and is almost never discussed in the context of peer review. There is a precedent from other fields, namely multiverse analysis in psychology \cite{Steegen2016-zx} which is defined as alternatively processed data sets corresponding to a large set of reasonable scenarios. Note this is different than triangulation, which involves interrogating a scientific phenomena using a multi-omics approach \cite{noauthor_undated-xz}. This dissertation attempts to measure the feasibility of this approach from a logistical standpoint rather than attempting to quantify the effectiveness of robustness testing in comparison to conventional peer review, as the latter seems largely dependent on the source papers.

6.1.1 Common and differing refactor issues

Before commencement the thought that the refactor issues would be centered around dependency management. These are largely resolved by bioconda, which provides dependencies for nearly all tools used in these analyses.

Participants were encouraged to accept a full Cloud9 IDE environment in the Amazon Web Services, which would provide the file browser, terminal, and code editor in one web application that can be accessed anywhere and retains state.

Notebooks do not yet play as big a role in bioinformatics as in data science, due to the large number of bulk operations that have typically been involved. However knitr/RMarkdown reports were generated for all three analyses. As an exercise two of these were ported to CodeOcean.

6.1.2 Relationship to benchmarking

The relationship of robustness testing - in whihch benchmarking
Three possibilities exist for analyses that fail a test of robustness:

- The effect is weak or the scientific phenomena requires a particular set of tools and settings to observe
  - Generally these will be explainable a priori but may involve detecting effects specific to a certain system - such as a peculiar genomic locus
  - The analyst engaged in some form of QRP such p-hacking, HARKing, multiple modeling
  - There was a bug in the initial code that was avoided using an alternate strategy
  - The effect is strong but other tools in the robustness test are simply inappropriate, and only the analysis used will reveal the effect

6.1.3 Comparison of Approaches

The tests of robustness were conducted using three different strategies: refactor alterations, a workflow library, and supervised approaches.

Unfortunately, the papers were not round-robin divided among the three approaches. In retrospect, this would have eliminated some of the confounds surrounding this study.

The faithful reproduction and refactor, applied to the Leiby microbiome paper, generated fairly detailed explorations of parameters but little in the way of major tool swaps. In essence participants did not feel emboldened to completely dismantle the workflow. Some obvious low-hanging fruit would have been major upgrades to Qiime2 or MOTHUR (as opposed to Qiime) and Kraken2 (as opposed to Kraken). None of the participants chose to focus on the metagenomic portions of the analysis.

The workflow library approach was conducted in order to accelerate the process of evaluation both for the developer and participants and liberate the participants to . This approach forced the use of state-of-the-art tools but was not able to directly interrogate all the approaches used by the author as the library of nf.core workflows is wide but not exhaustive. The workflow library approach produced arguably the single most revealing or interesting test of robustness (#BD), but that success could be attributed to the talents and aptitude of the reviewer and glaring problems with the original manuscript. In addition to encouraging refactors, this approach can more likely reveal systematic labeling errors and bugs that can remain hidden in existing code submissions and are likely to be missed by reviewers.

The supervised approach posed advantages in terms of having a technician who was familiar with the sample layout combined with senior third party experts who, while unable to dedicate hours to a test of robustness. In both CLK and m6a these sample metadata and the generation of tool manifests proved very unwieldy, with several combinations of treatments and file types, and posed the single biggest challenge to the quick implementation of refactors. These are not intellectual or scientific challenges, but merely data cleaning steps which favor a dedicated professional. In data science circles these data cleaning experts are a key member of a data science team, and may someday serve a role in the peer review process.

Administrative issues

Communication of goals and expected deliverables As tests of robustness are a new concept, communicating the goals to potential reviewers is crucial, as they will have no past exposure to the practice. This study suffered from a lack of communication with some participants, though others were more receptive. Packaging of tests of robustness ranged wildly. Reviewers in the Leiby returned essentially intermediates, while reflecting a large number of permutations, were difficult to interpret without taking them downstream. Many participants were understandably reluctant to interpret their findings, not clear if the robustness tests represented support, contradiction, or simply noise.

6.2 Recommendations

The following recommendations are derived from the results and discussion above. Some of these recommendations are specifically geared toward the possibility of implementing reproducibility-enabled peer review as a reality, however unlikely, while others are more general guidelines for reproducibility that may prove useful in other contexts.

For clarity we can organize these along the analytic stack comprised of input data, tools, workflows, notebooks, and publications.

6.2.1 Input data

Open data / Open code For the purposes of this study it was decided to use papers with no available source code, mostly as a mechanism to learn about what is required to produce a usable study for a test of robustness. In a real world scenario, lack of data or code is unacceptable. As reproducibility standards increase, it seems inevitable that some researchers will attempt to use privacy as a shield against scrutiny. While the public release of identifiable data is a valid concern, there are proper mechanisms for maintaining

6.2.2 Tools Workflows Notebooks

Expose resource metadata Some steps in the analysis, particularly the building of the Kraken index for Leiby, took many hours to complete. Other tools, such as Mintie, were so slow as to be impractical for high throughput analysis. For the quick dispatch of tests of robustness in the liTop level running time and resource specifications CPU/Memory/Running Time estimates for each step of an analysis.

Adoption of benchmarking frameworks tags into tool sets In the case of parameter sweeps, it became quickly clear that Leiby participants were not prepared to design an entire framework for evaluating the results of their changes, and simply sent in the code and intermediates they generated instead of collecting metrics. One reason for this is the relative difficulty of building benchmarking frameworks for each such exercise, a problem often experienced in data science where exhaustive hyperparameter exploration of machine learning models is the norm. One possible solution that is emerging are benchmarking frameworks such as Databrick's MLFlow, which integrates with standard programming languages to provide decorator syntax to mark inputs and outputs of interest, where they can be displayed in a local or remotely hosted web interface. The use of benchmarking frameworks is relatively unknown in science, but may provide a means of accelerating robustness testing, especially if prepackaged with tools.

Potential of recommendation engines for tests of robustness One solution to the "tool finding problem" are tool recommendation engines, which associate either manuscript text or workflow provenance traces to patterns of tool usage. Halioui et al mined workflows by manually curating 300 articles

It has been used extensively in tool recommenders \cite{Palmblad2019-uk}, tool registries \cite{Hillion2017-wg}, and within pipeline frameworks and workflow languages \cite{Bedo2019-ip,Amstutz2015-fa}. In the context of workflows, certain tool combinations tend to be chained in predictable usage patterns driven by application; these patterns can be mined for tool recommender software used in workbenches \cite{Kumar2019-xq}. For better or worse, this reduces the need for workbench developers to manually annotate tools with ontologies, replacing them with a machine learning black box.

Bridging the gap between notebooks and pipeline frameworks Distinguishing high-level choices from low-level details while still permitting that the devil is in the details. Statistical ontology to categorize steps in a workflow that are essentially one of

- Group & matching samples
- Removing samples
- Normalization
- Transformation

- Clustering
- Glue steps

Increasing the speed of reanalysis by focus on identifying individual findings Increasing the quality and decreasing selective bias of tool benchmarks - allowing or favoring tools that are up front and honest about their strengths and weaknesses rather than selective datasets. I found a dataset which performs well with my assumptions, did I overfit, well here's how my tool performs with other sets.

Greater enforcement of prerequisite and intermediate data types as workflow sanity checks - the BioSanity project Virtually every advance that has improved computational reproducibility in science has been motivated other than pure reproducibility. Dependency managers are largely software development tools, containerization was designed to spin up test instances in ecommerce, literate programming was designed to provide context, notebooks made literate programming interactive,

A common theme that has been presented here is that there is no structured format for understanding or communicating the rationale behind certain tool choices in the context of specific analyses. Bioinformatics still alarmingly relies on folk wisdom mixed in with largely biased tool papers, and a handful of well conducted benchmarking papers.

The EMBRACE Data And Methods (EDAM) ontology provides high-level descriptions of tools, processes, and biological file formats \cite{Ison2013-gm}. An elegant portal and API for the discovery of omics that utilizes this ontology, bio.tools, allows tool developers to annotate their tools according to common topics, operations, types of data and data formats. Given its capabilities, bio.tools is an underutilized resource. I have created over 60 bio.tools entries for popular bioinformatic tools that have been in existence for many years, suggesting the bio.tools usage may be low.

Bioinformatic pipelines have become a valued tool to aid reproducibility - as they facilitate the recording of processing and report steps using the incentives of automation, reentrancy, and reuse.

Quality control steps, including those inspecting files directly off of instrumentation and also the output of intermediates and statistical tests, are also an essential part of any solid analysis, but they require manual inspection.

In an otherwise automated workflow, manual pruning steps create an impediment to reproducibility. Often these are a necessary response to quality control (QC) steps that invalidate certain samples or input data out of the analysis. Removing samples upstream, either by omission or tagging, without any accompanying provenance information informing others of this decision, is a small step away from p-hacking and other undesirable behaviors. Furthermore, this manual step does not address new samples which may be introduced into the analysis, or reuse of the pipeline. Most bioinformatics QC software, such as FastQC

\cite{Andrews2010-bz} and MultiQC \cite{Ewels2016-uv}, is designed to produce reports but not filter. If a workflow is composed of interpreted steps inside a programming environment, such as R or Python, the task of filtering becomes trivial, but high-throughput analyses are more likely composed of compiled executables run on large files.

Another problem with pipeline frameworks is that they are designed with the assumption that intermediates are suitable as input for the next step, when in fact they may be corrupt, empty, or insufficient to converge. This allows pipelines to in essence "drive off a cliff" when they encounter such files.

There have been attempts to integrate semantic sanity checks in workflows, namely in WINGS \cite{Zheng2015-qc}, but these have largely focused on self-reported file type, rather than edge cases. For example, ensuring that the reported output of a step is an alignment, but not validating that the output file consists of more than just a header.

A solution I propose is a sanity checker which dispatches to various bioinformatic utilities (e.g. fastp, samtools, vcftools) in order to answer basic QC-related assertions:

```
./biosanity "is EDAM:format_1997 and numrecs > 100" –onfail die < myfile
```

Biosanity will methods typically stored within separate libraries into a common QC-centric focus in a command line tool. It will feature a limited syntax with basic conditionals and a finite number of reporting or exit strategies to cooperate with various pipeline frameworks. Biosanity will also be a gentle introduction to the use of ontologies for analysts who are not familiar with using them.

6.2.3 Publication and Peer Review Process

Develop strategies for overcoming code inertia Developers carry an internal conception of how to accomplish tasks, which makes it uncomfortable for them to dive into a stranger's code. In the Dominissini analysis, this was overcome by simply allowing developers to quickly triangulate the paper using off-the-shelf libraries, nf.core. This workflow-library strategy may prove effective only for the most generic of analyses. For robustness testing of newer approaches, original code will have to be used. This could have the undesirable effect of rewarding groups which produce opaque code, avoiding scrutiny through obfuscation. While basic data carpentry can play a role, incentivizing three main requirements, that have been recurrent issues throughout this analysis:

- software and dependencies should be installable through Bioconda
- pipelines should keep hard-coding parameters to a minimum
- samples identifiers both in the manuscript and workflow need to reflect their SRA identifiers

Publish with caveats One argument against paid peer review is that article processing charges (APCs) are not collected for work that is not accepted, leaving the journal responsible, or that authors would have to pay for each review process at each journal.

Harmonize preregistration, embargo, and reviewer access to data A non-trivial issue in the refactors was the use of internal file identifiers to identify samples. While SRA and EBI (the two primary centers for sequence submissions) maintain those names faithfully within uploads, the key identifier is always the SRR or "Run Accession". At present, upload of sequences to SRA is often a later step taken during manuscript submission, only required for final publication.

Ideally data should be made available to reviewers in the same way that readers will encounter if they choose to reproduce the paper. This ensures that reproducibility is not lost before publication, that data is in fact released (because it needs to be deposited for reviewers before submission), and that centralized quality control can be conducted by repositories that handle the greatest number of sequences.

Minor controversy erupted when it was revealed that a meta-analysis \cite{Edgar2021-lv} had redistributed data that had been mistakenly released by The Jackson Laboratory due to misconfigured changes in embargo period policy. It was revealed that 14,857 data sets were under embargo.

Domain-specific minimal reporting standards for studies All Leiby participants independently reported that a high-level of decision entropy, seemingly anecdotal choices in analytic strategy, and associated missing details in free-text methods were defining features of microbiome papers. As a follow-up, Leiby participants were requested to contribute to a checklist specifying "minimal information for a microbiome analysis".

Minimal Information for a Microbiome Analysis
- Details on study design
- The number and type of samples (e.g., strain for animals, demographics for humans)
- How samples were taken, including inclusion/exclusion criteria if they apply
- Identity of material being collected
- How the material was collected, stored, and transported to sequencing
- Time between data collection and sequencing

Minimal data to reproduce a microbiome paper:

- FASTQs
- Mapping (samples' metadata) file
- 16S Database, including clustering identity (e.g. Greengenes clustered at 97% identity)
- Taxonomic database (kraken, kaiju, ...)
- Annotation database (NCBI-NR, swissprot, uniprot, kegg mapping, ...)
- host reference genome (if applied, preferably a link to the same or an upload on zenodo etc.)

Minimal list of software/methods to reproduce a microbiome paper:

- FASTQ primer and low-qual removal software (trimmomatic, cut-adapt, qiime)
- FASTQ merge software (pandaseq, vsearch, etc)
- FASTQ host-filtering process (blast, bwa, bowtie, minimap)
- 16S "Pipeline" (qiime1, qiime2, dada2, mothur, etc)
- OTU picking method (uclust, usearch, vsearch, etc)
- OTU picking strategy (de novo, closed-reference, open-reference)
- Programs/R libraries to process OTU/ASV tables (phyloseq, etc) and their versions
- Setting a seed under R or python or other approaches if possible to reproduce the run.
- QIIME tracks provenance, so similar information (sessionInfo() from R)

Minimal list of analytical steps to report

- The number of samples included in the final analyses (e.g., those that passed QC)
- All outcomes-of-interest, and their number (if categorical) or range (if continuous)
- Details on whether and how outcomes-of-interest are pre-processed
- How lowly abundant OTUs/ASVs were filtered (called prevalence filtering)
- Whether and how diversity is measure
- Whether zeros were imputed or replaced, and how
- What transformation or normalization procedure was applied
- The covariates considered to be potential confounders, and criteria used to select them
- The number and type of statistical tests and presented in the results
- The number and type of statistical tests performed but not presented in the results
- The method used to adjust p-values

Additional list (other data and methods that should also be included if used):

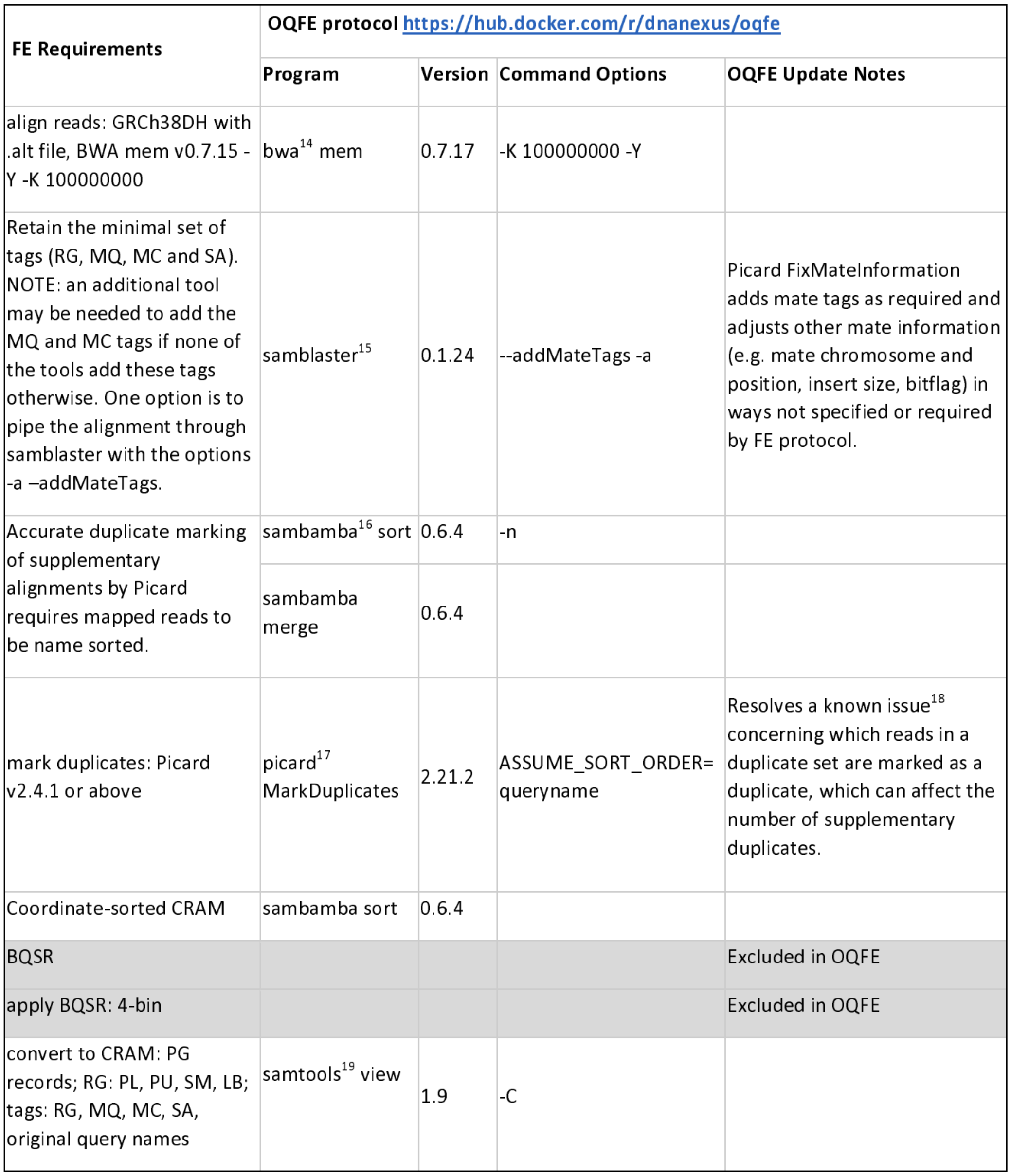
- Primers (if not using standard Illumina V3-V4 primers)
- If Longreads are used, then definitely need the signal level information (either fast5 or subread bams) and the process to generate them. There's still a lot of variation between the various kits used and data quality seems to be improving day by day.
- Fastq barcode splitter (if FASTQs are barcoded)
- Taxonomy assignment/classification method
- Dereplication method
- Clustering method
- Chimera removal method
- Denoise method
- Assembly method (if not amplicon)

∗ all methods should declare program version and parameters used

Another important trend is the emergence of reporting guidelines, essentially checklists, many of which are found in the EQUATOR network \cite{Simera2010-cd}, perhaps the most prominent being CONSORT (Consolidated Standards of Reporting Trials) originally from 1996 but updated in 2010 \cite{Schulz2010-bp}. Newer examples include STORMS (Strengthening The Organization and Reporting of Microbiome Studies), Strengthening the Reporting of Observational Studies in Epidemiology (STROBE). Such guidelines, while useful for authors, are rarely paired with metadata schema to allow them to be machine-readable.

Autogeneration of Requirement Summary Panels From a reproducible research perspective, it makes sense that these templates should be auto-generated from computational workflows, rather than manually entered. This would require a decorator or tagging scheme by which relevant metadata can be reliably identified even if still embedded as functional variables in workflows. In essence such a schema can guide an entity search engine to easily extract these pertinent details from workflows that conform to a markup standard, thereby saving authors from having to tediously fill out forms but also enabling some freedom of implementation.

Table 1



FE Requirements	OQFE protocol https://hub.docker.com/r/dnanexus/oqfe			
	Program	Version	Command Options	OQFE Update Notes
align reads: GRCh38DH with .alt file, BWA mem v0.7.15 -Y -K 100000000	bwa[14] mem	0.7.17	-K 100000000 -Y	
Retain the minimal set of tags (RG, MQ, MC and SA). NOTE: an additional tool may be needed to add the MQ and MC tags if none of the tools add these tags otherwise. One option is to pipe the alignment through samblaster with the options -a –addMateTags.	samblaster[15]	0.1.24	--addMateTags -a	Picard FixMateInformation adds mate tags as required and adjusts other mate information (e.g. mate chromosome and position, insert size, bitflag) in ways not specified or required by FE protocol.
Accurate duplicate marking of supplementary alignments by Picard requires mapped reads to be name sorted.	sambamba[16] sort	0.6.4	-n	
	sambamba merge	0.6.4		
mark duplicates: Picard v2.4.1 or above	picard[17] MarkDuplicates	2.21.2	ASSUME_SORT_ORDER= queryname	Resolves a known issue[18] concerning which reads in a duplicate set are marked as a duplicate, which can affect the number of supplementary duplicates.
Coordinate-sorted CRAM	sambamba sort	0.6.4		
BQSR				Excluded in OQFE
apply BQSR: 4-bin				Excluded in OQFE
convert to CRAM: PG records; RG: PL, PU, SM, LB; tags: RG, MQ, MC, SA, original query names	samtools[19] view	1.9	-C	

Requirement summary panels provide a mid-level organized table of a project's software requirements and parameters, existing as a middle conduit between raw code and methods sections. A key feature of these is that not every version and requirement is listed - various secondary and superficial tools would only distract from their pertinence. As of the present, all requirement panels are generated manually for publication, but in most cases it should be possible to autogenerate LaTeX or Markdown formatted panels from dependency management systems, given a domain-specific template.

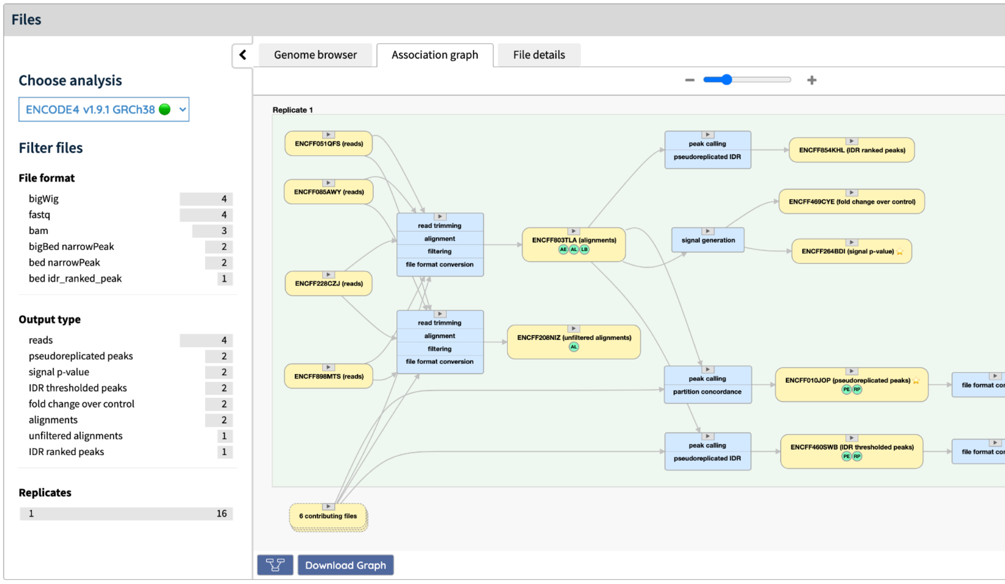
Standardization of cohort-dependent intermediates Some analyses with sensitive or identifable data will supply deidentified intermediates, rather than raw data. In other instances, a data point may be tangentially or indirectly rely on private data. One example of private-by-association is the dependence on large identifiable genomic cohorts used in joint genotyping. Obtaining enough statistical signals that indicate a variant is artifactual (strand bias, call quality, mapping quality, max depth), requires several data points to achieve predictive ability under a Bayesian framework (a prior). Additional samples provide more statistical power to infer a variant is due to artifacts. Conversely, obtaining enough evidence that a variant is real also requires more data, only obtainable from additional samples. Sequencing artifacts are highly variable, instrument and library construction, and often specific to individual runs. While cohort dependent files will never acheive the same level of provenance that standard references or primary data, developing a system of reliably identifying cohorts is essential to assessment of bias in peer review.

Reducing format decay Harmonizing intermediate and final results from comparable software for comparison is essential to robustness tests. First-mover status of certain tools places them to enforce certain output formats which can take hold. It is inevitable that as a field matures certain new formats will become more accommodating, generalizable, performant, or parsable. This trend places the burden on authors to update their software to support newer formats either as a natively or through translator. Tool papers often force tool builders to port over older tool results in order to perform benchmarks, but these are essentially isolated or anecdotal incidents. There is no practice, formalized or otherwise, of preventing format decay.

Relating specific tool uses cases to individual studies The Funnell CLK paper revealed a number of unknowns in terms of the predicted behavior of both traditional differential splicing software, isoform discovery and abundance and estimation, and gene fusion detection for conjoined genes in particular. While various free-text searches may reveal appropriate articles, a Google Scholar search of "detection of conjoined genes" is unsatisfactory (and ironically points back to the CLK paper). The difficulty of unknown performance has a profound effect on the potential for reviewers to quickly identify alternative tools to conduct robustness testing.

Paying for reproducibility-enabled peer review The concept of compensating peer reviewers for their time and effort is not new - and was a common practice prior to 1980 . A recent proposal, the $450 Movement, has garnered the attention of the broader scientific community \cite{noauthor_2021-ok} and has spawned a debate over the feasibility, ethics, and possible side-effects of this idea has been discussed in a number of forums \cite{Vines2021-bh}. Reproducibility and the replication crisis is often discussed hand-in-hand with paid peer review - the gist being that terse or low-quality reviews are at least in part responsible for the proliferation of irreproducible results. Virtually none of this discussion has focused specifically on reproducibility-enabled in silico peer review. The typical peer review takes 5 hours to complete \cite{Mark_Ware2015-bx}. While participants in this thesis project were not asked how many hours they spent on their analyses, personal direct communications with several participants in the unsupervised refactor and workflow library groups indicate at least 20 hours were required to complete this task. A casual twitter poll was conducted for this thesis to gauge what authors would "expect for their money", of the 585 votes, improvement in turnaround speed (70.4%), length or comprehensiveness of reviews (19.1%), help with English (2.2%), and help with analysis (8.2%).

Improving conceptual-level DAGs Distinguishing useful provenance from spaghetti DAGs. ENCODE provides some of the best DAGs in the form of high-level association graphs, and these tend to be more understandable than the spaghetti DAGs


Files

Choose analysis

ENCODE4 v1.9.1 GRCh38

Filter files

File format

bigWig	4
fastq	4
bam	3
bigBed narrowPeak	2
bed narrowPeak	2
bed idr_ranked_peak	1

Output type

reads	4
pseudoreplicated peaks	2
signal p-value	2
IDR thresholded peaks	2
fold change over control	2
alignments	2
unfiltered alignments	1
IDR ranked peaks	1

Replicates

1	16

Walsh et al discuss guidelines necessary for peer review of machine learning techniques to infer protein function. \cite{Walsh2016-rp}

- Data quality and representativeness - low quality data sets introducing noise that is liable to be overfit by models (learned noise). Data set metadata about the training sets must be examined by peer reviewers. Representativeness refers to the size and diversity of the reference sets

Addressing data leakage in peer review
Data leakage compromises the whole purpose of cross validation and test-sets, and is especially prone in biology due to the difficulty in assessing the independence of data points.

6.2.4 Applicable Metadata Standards

The review paper "The Role of Metadata in Reproducible Computational Research" exhaustively compiled a list of applicable metadata standards for reproducible research, some of these standards appear applicable to tests of robustness.

BioCompute Objects\cite{Simonyan2017-st}	Workflow	Provides lab and runtime parameter standards for workflows and tools	
GoGetData	Data	Data as a dependency tool	
EDAM\cite{Ison2013-gm}	Tool	Bioinformatic tool ontology	
BioConda\cite{Dale2017-aj}	Tool	Dependency resolution	Used heavily
labelschema	Tool	Docker label metadata standard	Used
Common Workflow Language\cite{Peter2016-vi}	Workflow	pipeline framework language	Too heavyweight
Workflow Description Language\cite{Voss2017-ro}	Workflow	pipeline framework language	Used
Drake\cite{Landau_undated-yt}	Analysis	Analysis pipeline framework	R-only
OBCS\cite{Zheng2016-cu}	Analysis	Statistical ontology	
CodeMeta\cite{Jones2016-dz}	Analysis	Code metadata standard	
Bagit\cite{Kunze2018-pn}	Analysis	Analysis file archival standard	Used
ReproZip\cite{Simonyan2017-st}	Analysis	Analysis file archival standard	
DataPackageR\cite{Finak2018-ai}	Analysis	Analysis archival standard	R-only
Binder\cite{Jupyter2018-md}	Analysis	Containerized notebook live viewer	
YesWorkflow\cite{McPhillips2015-um}	Analysis	Analysis pipeline markup	
Manubot\cite{Himmelstein2019-dt}	Publication	Automated git-driven publications	
RRID\cite{Bandrowski2015-qu}	Publication	Resource tags for papers	
Research Objects\cite{Bechhofer2010-lr}	Publication	Metadata describing and bundling the entire experiment	

6.3 Conclusion

This dissertation investigates the feasibility of using tests of robustness in the context of reproducibility enabled *in silico* peer review. Tests of robustness involve manipulating software tools, parameters, and statistical models with the intention of evaluating the underlying validity of an existing analysis. This practice is presented as a natural extension of technological advances in the last 5 years combined with growing awareness and case studies of reproducibility and issues of scientific quality.

The research problem was investigated through an unique human research mixed-model in which participants were engaged in the reproducibility-enabled peer review process, either actively or expert advisors, using high-impact genomic manuscripts selected to elicit review findings. Pre- and post-hoc surveys were also conducted to measure attitudes about reproducibility and the tests of robustness. The development of the materials for these tests, bioinformatic pipelines which I designed, were also explicitly intended to evaluate the process. This practice of refactoring papers without source code demonstrated many weaknesses of conventional scientific publishing standards.

Three papers - on the microbiome, m6a methylation, and CLK-induced splicing changes were approached by different means as difficulties and opportunities revealed themselves. It was discovered, perhaps not surprisingly, that reviewers would be reluctant to manipulate complex pipelines beyond parameter exploration to overhauls of core tools, and so findings were limited to such parameter sweeps, and were largely inconclusive. A workflow library approach was conducted for the m6a paper, by which participants were asked to use off-the-shelf pipelines to triangulate inspection of a paper with known problems. This approach appeared ostensibly more successful, though the paper choice remained a confounding factor. One participant discovered The final paper involved a mixed exotic combination of both long and short reads and an unusual phenotype - conjoined genes, making it better suited toward attempting swaps with latest tools. Experts were consulted and I performed the tests of robustness independently.

Some of these tests may have proven useful if they were conducted at the time of peer review.

6.4 Material Outcomes

Prior to this proposal I composed a review article, book chapter, an online case study catalog, and finally a prospectus that set the foundation for some of the ideas described in this proposal. In "A Review of Bioinformatic Pipeline Frameworks" \cite{Leipzig2017-hv} I attempted to classify existing software solutions for handling serial and parallel abstracted bioinformatic workflows to process sequence data and metadata. I contend that existing frameworks differ on three key dimensions: using an implicit or explicit syntax, using a configuration, convention or class-based design paradigm and offering a command line or workbench interface. While not an absolute requirement for computational reproducibility, the use of pipeline frameworks encourages reproducible research by abstracting file transformation steps into a parameterized and configured sequence. Such frameworks offer cohesion between tools and data and analysis, and encourage reuse and robustness through the advantages of easy extensibility and scalability, particularly through the cloud. As of this date, the review has received 231 citations.

A follow-up to this review is the book chapter "Computational Pipelines and Workflows in Bioinformatics" \cite{Leipzig2019-ag}. In this review I explored the ecosystem that has evolved around workflows - toolkits, ready-made pipelines, and pipeline frameworks. Future directions of dependency management and configuration, cloud computing, containerization, and notebooks. Of particular relevance to this is a discussion into the possibilities of semantically encoded workflows and linked data, including existing solutions built on the PROV-O ontology \cite{Belhajjame2013-at,Missier2013-ea}. A discussion on the difficulties of encoding and binding upstream experimental metadata to end results that emerged from this chapter informs much of this proposal.
The Role of Metadata in Reproducible Computational Research (CITE), exhaustively explores metadata standards that are applicable to reproducible computational research, organized along the analytic stack, and accompanied by numerous source code examples. It was accepted into Cell Patterns for an August 2021 edition. As a preprint it collected 4 citations.
Awesome Reproducible Research \cite{Leipzig2019-am} is a Github repository storing my research into case studies and other reproducible research resources that I have collected over my studies. It has 151 stars and has received contributions from 9 users.

6.5 References

7 Vita

Jeremy Leipzig

Education

Wake Forest University – Bachelor of Science in Biology 1997

North Carolina State University – Master of Computer Science 2003

Publications

```
## The following objects are masked from 'package:BiocGenerics':
##
##     combine, intersect, setdiff, union
## The following object is masked from 'package:biomaRt':
##
##     select
## The following objects are masked from 'package:stats':
##
##     filter, lag
## The following objects are masked from 'package:base':
##
##     intersect, setdiff, setequal, union
metadata<-read.csv("metadata/metadata.csv")

# for my notes: Oo1Zafe3Qox3
```

nrow(metadata) were obtained

7.0.1 Breakdowns

By platform

```
metadata %>% group_by(Platform) %>% summarize(samples=n(),median_reads=media
## # A tibble: 2 × 3
##   Platform    samples median_reads
##   <chr>         <int>        <dbl>
## 1 ILLUMINA        109       795864
## 2 PACBIO_SMRT      60        81741
```

By cell line HCT116 is a human colorectal carcinoma (i.e. malignant or “transformed") cell line. 184-hTERT-L2 cell line is derived from human mammary epithelial cells immortalized by transduction with hTERT.

```
metadata$cell_line<-as.vector(str_extract_all(metadata$SampleName,'(HCT116|184-hTert)',simplify=T
metadata %>% group_by(cell_line) %>% summarize(samples=n()) %>% arrange(-samples)
## # A tibble: 2 × 2
##   cell_line samples
##   <chr>       <int>
## 1 HCT116        161
## 2 184-hTert       8
```

By treatment Various silencing transfections were tested on CLK itself, splicing factors such as U2AF2 and SRSF9, TIA1 and CPEB RNA-binding proteins siCLK, siCPEB ,siDAZA, siHNRNPH, siKHDRBS1, siLIN28A, siELAVL1, siHNRNPC, siHNRNPF, siU2AF2, siTIA, siSRSF, siSRRM, siSFPQ, siSAMD4B, RNA recognition motif (RRMs) and splicing enhancers are also tested.

```
metadata %>% group_by(SampleName) %>% summarize(samples=n()) %>% arrange(-samples)
```

```
## # A tibble: 90 × 2
##    SampleName                   samples
##    <chr>                        <int>
## 1 0.5 uM T3 treated HCT116          24
## 2 5 uM T3 treated HCT116            24
## 3 Untreated HCT116                  24
## 4 1.0 uM T3 treated HCT116           4
## 5 0.05 uM T3 treated HCT116          2
## 6 0.1 uM T3 treated HCT116           2
## 7 0.5 uM T3 treated 184-hTert        2
## 8 1.0 uM T3 treated 184-hTert        2
## 9 10 uM T3 treated HCT116            2
## 10 5.0 uM T3 treated 184-hTert       2
## # ... with 80 more rows
```

7.0.2 Alignment and rMATS-ISO

Alignment was performed with STAR against hg38 using GTEX pipeline settings. The alignments themselves were run on the Truwl.com platform.

```
STAR –runMode alignReads \
–outSAMtype BAM SortedByCoordinate \
–limitBAMsortRAM ${bytes} \
–readFilesCommand zcat \
–outFilterType BySJout   –outFilterMultimapNmax 20 \
–outFilterMismatchNmax 999   –alignIntronMin 25 \
–alignIntronMax 1000000   –alignMatesGapMax 1000000 \
–alignSJoverhangMin 8   –alignSJDBoverhangMin 5 \
–sjdbGTFfile GRCh38_star/genes.gtf \
–genomeDir GRCh38_star \
–runThreadN ${cpus} \
–outFileNamePrefix ${sample}. \
–readFilesIn ${sample}_1.fastq.gz ${sample}_2.fastq.gz
```

RMATS-ISO was run using the gencode.v28.annotation.gtf, roughly equivalent to the Ensembl gtf.

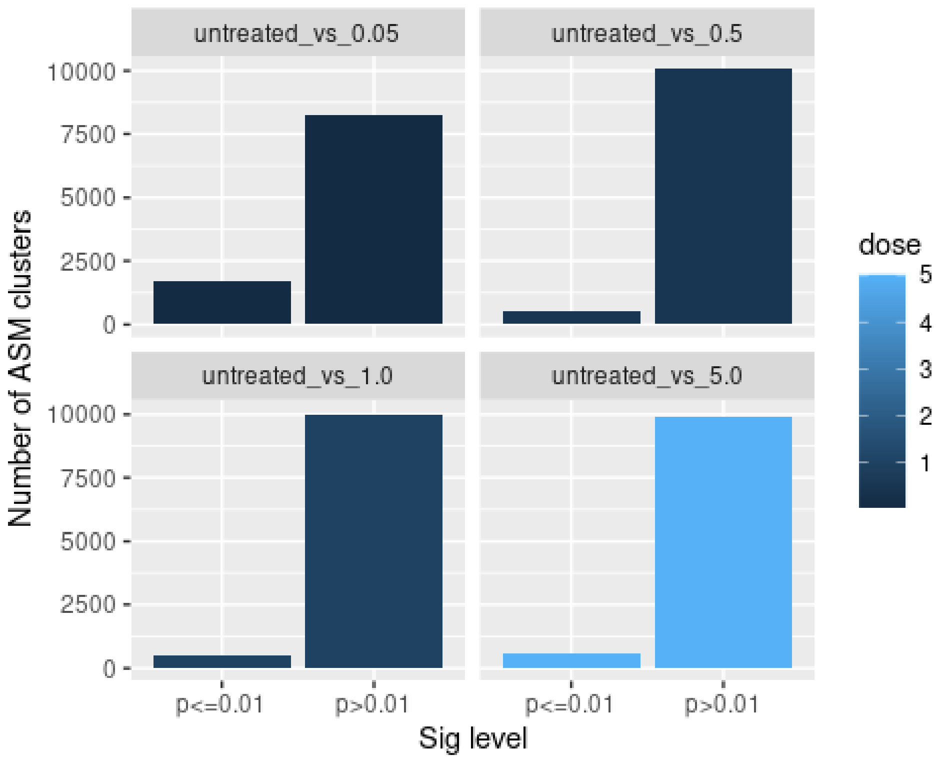
7.0.3 RMATS-EM output

The following columns are returned from rMATS-EM:

```
[1] "ASM_name"                         "total_isoforms"                    "total_exons"
"total_read_count_group_1"       "total_read_count_group_2"

"p_value"                          "test_statistic"                    "isoform_inclusion_group_1"
"isoform_inclusion_group_2"      "isoform_inclusion_constrained"

"variance_group_1"                 "variance_group_2"                  "variance_constrained"
"dirichlet_parameter_group_1"    "dirichlet_parameter_group_2"

"dirichlet_parameter_constrained"  "paired_isoform_pvalues"            "isoform_index"
"merge_info"
```

7.0.4 Dose dependent splicing patterns of T3 in HCT116

```
pthresh<-0.01
doses<-c('0.05','0.5','1.0','5.0')
control<-"untreated"
em_all<-NULL
for(dose in doses){
em<-read.table(paste0("results/iso_",control,'_vs_',dose,'/EM_out/EM.out'),comment.char
= ”,strip.white = TRUE, header=TRUE)
em$dose<-as.numeric(dose)
em$dosestr<-paste0(control,'_vs_',dose)
if(!is.null(em_all)){
em_all<-rbind(em_all,em)
}else{
em_all<-em
}
}

#set p_value of 0 to a token dummy minimum, display only highly significant assemblies
#ggplot(em_all %>% filter(!is.na(p_value)) %>% filter(p_value<=0.01) %>% rowwise()
%>% dplyr::mutate(p_value = max(p_value,1e-16)),aes(-log(p_value)))+geom_histogram(binwidth=1)+
ggplot(em_all %>% dplyr::filter(!is.na(p_value)) %>% rowwise() %>% mutate(sig=ifelse(p_value<=pthr
of ASM clusters")+xlab("Sig level")+facet_wrap(.~dosestr)
```



```
em_all %>% dplyr::filter(!is.na(p_value)) %>% rowwise() %>% mutate(sig=p_value<=pthresh)
%>% group_by(dose,sig) %>% summarize(cnt=n()) %>% reshape2::acast(dose~sig) ->
sig_table
## `summarise()` has grouped output by 'dose'. You can override using the `.groups` argument.
## Using cnt as value column: use value.var to override.
dimnames(sig_table)[[2]]<-c(paste0("p>",pthresh),paste0("p<=",pthresh))
sig_table<-cbind(sig_table,total=rowSums(sig_table))
sig_table<-cbind(sig_table,sig_frac=round(sig_table[,2]/sig_table[,3],2))
fold_change<-c(0,sapply(1:(nrow(sig_table)-1),function(x){(sig_table[x+1,2]/sig_table[x+1,3])/(sig_ta
sig_table<-cbind(sig_table,fold_change=fold_change)
knitr::kable(sig_table)
```

	p>0.01	p<=0.01	total	sig_frac	fold_change
0.05	8228	1698	9926	0.17	0.0000000
0.5	10063	516	10579	0.05	0.2851292
1	9953	516	10469	0.05	1.0105072
5	9888	585	10473	0.06	1.1332879

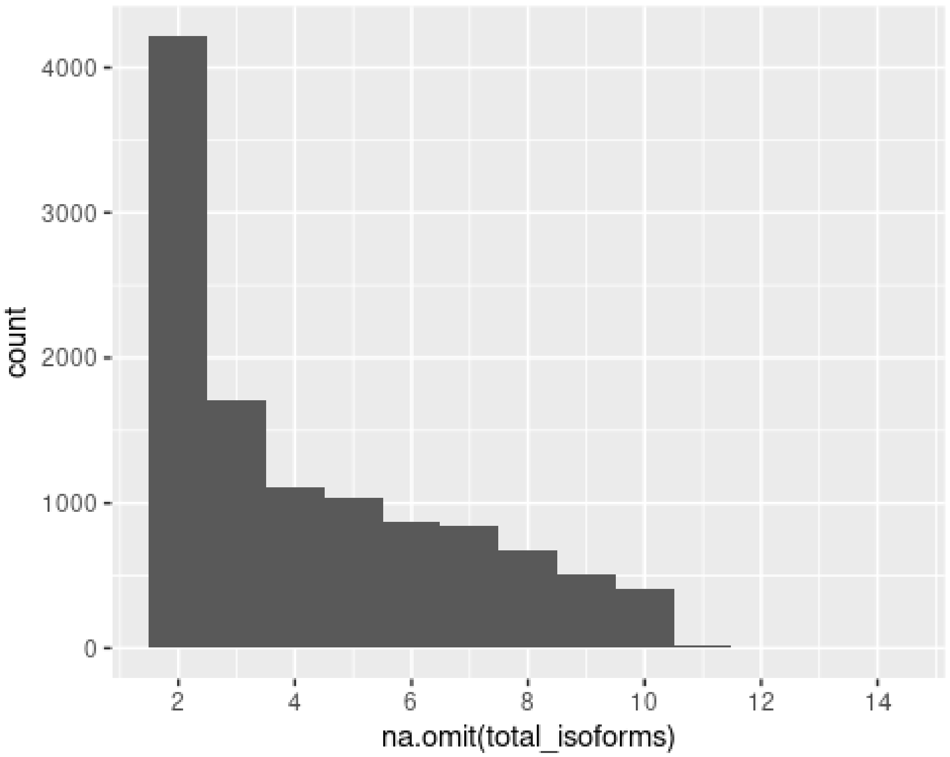
The number of assemblies in which significant AS events ($p<0.01$) were observed as a fraction of all assemblies was highest in the untreated_vs_1.0 group, although the greatest fold increase occurs at 0.5uM 1.1332879 reported in the paper as 4.1.

7.0.5 Clusters of interest in T3 0.5

```
dose<-c('0.5')
control<-"untreated"
em<-read.table(paste0("results/iso_",control,'_vs_',dose,'/EM_out/EM.out'),comment.char
= ”,strip.white = TRUE, header=TRUE)
coor<-read.table(paste0("results/iso_",control,'_vs_',dose,'/ISO_classify/ISO_module_coor.txt'),comm
= ”,strip.white = TRUE, header=FALSE,sep=":",col.names = c("chr","strand","start","end"))
gene<-read.table(paste0("results/iso_",control,'_vs_',dose,'/ISO_classify/ISO_module_gene.txt'),comm
= ”,strip.white = TRUE, header=FALSE,sep="_",col.names= c("asm","hugo","ensg"))
type<-read.table(paste0("results/iso_",control,'_vs_',dose,'/ISO_classify/ISO_module_type.txt'),comm
= ”,strip.white = TRUE, header=FALSE)
#typesummary<-
```

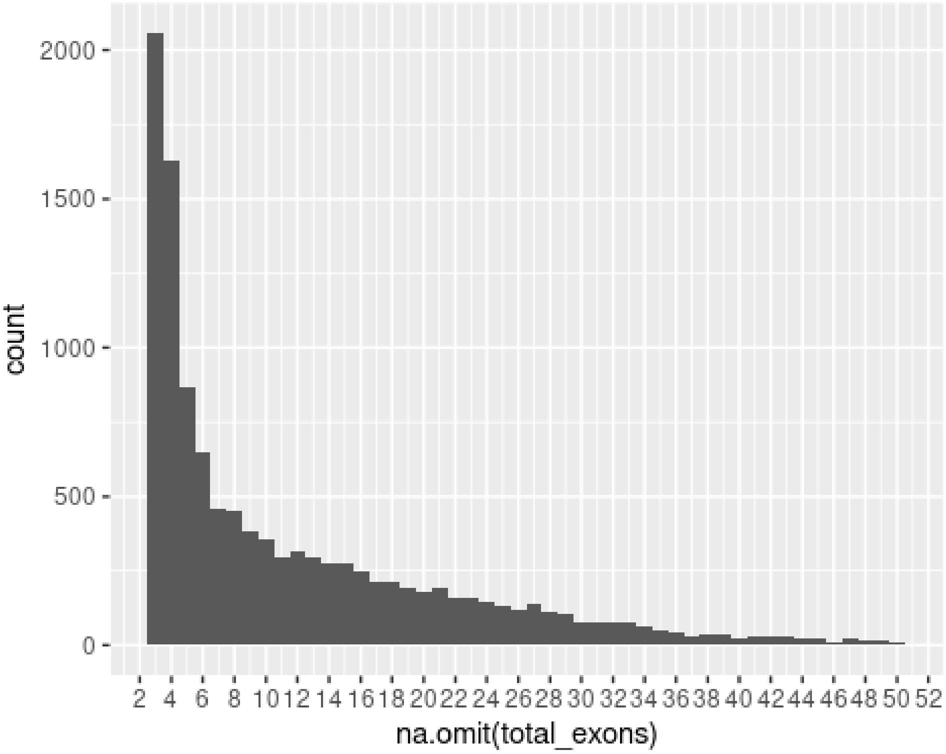
7.0.6 Number of isoforms in T3 0.5 clusters

```
ggplot(em,aes(na.omit(total_isoforms)))+geom_histogram(binwidth=1)+scale_x_continuous(breaks
= scales::pretty_breaks(n = 10))
```



7.0.7 Number of exons in T3 0.5 clusters

```
ggplot(em,aes(na.omit(total_exons)))+geom_histogram(binwidth=1)+scale_x_continuous(breaks
= scales::pretty_breaks(n = 25))
```



7.0.8 Top 10 simple events

Look at only those clusters with 3 or fewer isoforms, 4 or fewer exons and rank by the lowest paired isoform pvalue among them.

```
strmin<-function(x){
if(is.na(x)){return(NA)}
if(x=='NA,NA'){return(NA)}
return(min(as.numeric((str_split(x,',',simplify = TRUE)))))
}

em %>% dplyr::filter(total_isoforms<=3,total_exons<=4) %>% arrange(p_value) %>%
head(n=10) -> simple_events
knitr::kable(em %>% dplyr::filter(test_statistic==max(em$test_statistic,na.rm = TRUE)))
```

A	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
A	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Table 1: Top by test statistic

```
em %>% dplyr::filter(test_statistic==max(em$test_statistic,na.rm = TRUE)) %>% pull(ASM_name)
%>% as.character() -> top_hit
```

```
str_replace(top_hit,’#’,”) -> top_hit_nohash
gene %>% dplyr::filter(asm==top_hit_nohash)
##       asm hugo            ensg
## 1 ASM6705 GAPDH ENSG00000111640
coords<-coor[which(em$ASM_name==top_hit),]
```

7.0.9 Find conjoined genes

```
library(TxDb.Hsapiens.UCSC.hg38.knownGene)
txdb<-TxDb.Hsapiens.UCSC.hg38.knownGene
genes_gr <- genes(txdb)
##   1613 genes were dropped because they have exons located on both strands
##   of the same reference sequence or on more than one reference sequence,
##   so cannot be represented by a single genomic range.
##   Use ’single.strand.genes.only=FALSE’ to get all the genes in a
##   GRangesList object, or use suppressMessages() to suppress this message.
#don’t want overlapping genes
reduce(genes_gr) -> genes_reduced
ir<-IRanges(start=coor$start,end = coor$end)
coor_gr<-GRanges(seqnames = coor$chr,ranges=ir,strand = coor$strand)
GenomicRanges::findOverlaps(subject=genes_reduced,query=coor_gr) -> hits

#this is my apporac to find clusters that span more than one gene
as.data.frame(hits) %>% group_by(queryHits) %>% summarize(nhit=n()) %>% dplyr::filter(nhit>1)
%>% pull(queryHits) -> cg

#now with the subset of ranges that span more than one gene hit up teh original txdb so
we can get real gene ids
cg_gr<-coor_gr[cg,]
GenomicRanges::findOverlaps(subject=genes_gr,query=cg_gr) -> cg_hits

cgranges<-genes_gr[subjectHits(cg_hits),"gene_id"]
```

There are only 25 such conjoined genes identified with clusters that span more than two genes.

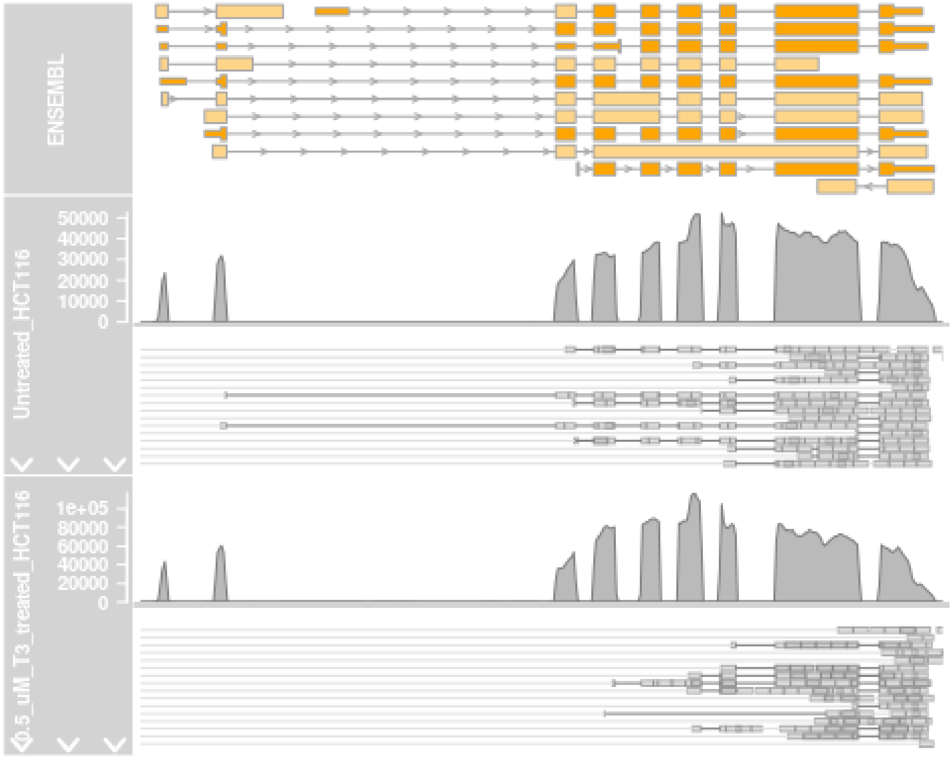
```
#get teh gene ids
txtable = biomaRt::select(txdb, keys=cgranges$gene_id, columns=columns(txdb), keytype="GENEID")
## ’select()’ returned 1:many mapping between keys and columns
library(org.Hs.eg.db)
hgnc_names<-biomaRt::select(org.Hs.eg.db, cgranges$gene_id, "SYMBOL")
## ’select()’ returned 1:1 mapping between keys and columns
cgranges$hgnc<-hgnc_names$SYMBOL
knitr::kable(cgranges)
```

seqnames	start	end	width	strand	gene_id	hgnc
chr3	9933822	9945413	11592	+	78987	CRELD1
chr3	9917074	9933630	16557	+	84818	IL17RC
chr6	41789896	41895361	105466	-	25862	USP49
chr6	41905354	41921139	15786	-	9477	MED20
chr6	85607785	85643792	36008	-	10492	SYNCRIP
chr6	85676637	85678748	2112	-	26799	SNORD50A
chr6	85650491	85678932	28442	-	387066	SNHG5
chr6	85505496	85615234	109739	-	57231	SNX14
chr6	85677589	85677658	70	-	692088	SNORD50B
chr7	27106184	27140225	34042	-	3200	HOXA3
chr7	27128507	27130780	2274	-	3201	HOXA4
chr7	27141052	27143681	2630	-	3202	HOXA5
chr7	27145396	27150603	5208	-	3203	HOXA6
chr7	141764097	141765197	1101	+	50831	TAS2R3
chr7	141778442	141780819	2378	+	50832	TAS2R4
chr8	1801125	1801192	68	+	100500912	MIR3674
chr8	1817231	1817307	77	+	693181	MIR596
chr12	49002274	49018807	16534	-	5571	PRKAG1
chr12	49018975	49059774	40800	-	8085	KMT2D
chr13	27255064	27255135	72	+	26771	SNORD102
chr13	27255401	27255526	126	+	619499	SNORA27
chr15	50354959	50356034	1076	+	100129387	GABPB1-AS1
chr15	50360329	50360410	82	+	100616396	MIR4712
chrX	45746157	45746266	110	-	407006	MIR221
chrX	45747015	45747124	110	-	407007	MIR222

Top hit: ASM6705

```r
dose="0.5"
#treated
metadata %>% dplyr::filter(str_detect(SampleName,dose)) %>% dplyr::filter(str_detect(SampleName,'T
%>% dplyr::filter(Platform=='ILLUMINA') %>% filter(str_detect(SampleName,'HCT116'))
%>% dplyr::pull(Run) -> treated
metadata %>% dplyr::filter(str_detect(SampleName,'Untreated')) %>% dplyr::filter(Platform=='ILLUM
%>% dplyr::filter(str_detect(SampleName,'HCT116')) %>% dplyr::pull(Run) -> untreated

#aws s3 cp s3://clk-splicing/SRP091981/SRR5009487.Aligned.sortedByCoord.out.bam SRP091981/
atreated<-"SRR5009487"
auntreated<-"SRR5009474"

plot_window<-function(asm,type){
```

```
stringr::str_replace(asm,’#’,”) -> asm_nohash
gene %>% dplyr::filter(asm==asm_nohash)
coords<-coor[which(em$ASM_name==asm),]
afrom <- coords$start - 100
ato <- coords$end + 100
chr <- as.character(coords$chr)
treatedName <- metadata %>% dplyr::filter(Run==atreated) %>% pull(SampleName) %>%
as.character() %>% stringr::str_replace_all(’ ’,’_’)
untreatedName <- metadata %>% dplyr::filter(Run==auntreated) %>% pull(SampleName)
%>% as.character() %>% stringr::str_replace_all(’ ’,’_’)
if(type==’gviz’){
treatedTrack <- AlignmentsTrack(paste0("./SRP091981/",atreated,".Aligned.sortedByCoord.out.md.bam
isPaired = TRUE,name = treatedName)
untreatedTrack <- AlignmentsTrack(paste0("./SRP091981/",auntreated,".Aligned.sortedByCoord.out.md
isPaired = TRUE, name = untreatedName)
options(ucscChromosomeNames=TRUE)

bmt <- BiomartGeneRegionTrack(genome = "hg38", name="ENSEMBL", chromosome =
chr, start = afrom, end = ato,biomart=biomaRt::useMart(biomart="ensembl",dataset="hsapiens_gene_e
, stacking = "squish")
plotTracks(list(bmt,untreatedTrack,treatedTrack), from = afrom, to = ato, chromosome =
chr) #, type = c("coverage","sashimi"))
}else{
sashimiPlot<-paste0("rmats2sashimiplot –b1 ",paste0("./SRP091981/",atreated,".Aligned.sortedByCoord
–b2 ",paste0("../SRP091981/",auntreated,".Aligned.sortedByCoord.out.md.bam")," -c ",chr,":",coords$st
–l1 ",treatedName," –l2 ",untreatedName, " –exon_s 1 –intron_s 5 -o sashimiout")
cat(sashimiPlot)
}
}

plot_window(top_hit,"gviz")
```


ENSEMBL
Untreated_HCT116
50000
40000
30000
20000
10000
0
0.5_uM_T3_treated_HCT116
1e+05
80000
60000
40000
20000
0