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## A field guide for the compositional analysis of any-omics data

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<b>Abstract:</b>	<p>Next-generation sequencing (NGS) has made it possible to determine the sequence and relative abundance of all nucleotides in a biological or environmental sample. Today, NGS is routinely used to understand many important topics in biology from human disease to microorganism diversity. A cornerstone of NGS is the quantification of RNA or DNA presence as counts. However, these counts are not counts per se: the magnitude of the counts are determined arbitrarily by the sequencing depth, not by the input material. Consequently, counts must undergo normalization prior to use. Conventional normalization methods require a set of assumptions: they assume that the majority of features are unchanged, and that all environments under study have the same carrying capacity for nucleotide synthesis. These assumptions are often untestable and may not hold when comparing heterogeneous samples (e.g., samples collected across distinct cancers or tissues). Instead, methods developed within the field of compositional data analysis offer a general solution that is assumption-free and valid for all data. In this manuscript, we synthesize the extant literature to provide a concise guide on how to apply compositional data analysis to NGS count data. In doing so, we review zero replacement, differential abundance analysis, and within-group and between-group coordination analysis. We then discuss how this pipeline can accommodate complex study design, facilitate the analysis of vertically and horizontally integrated data, including multi-omics data, and further extend to single-cell sequencing data. In highlighting the limitations of total library size, effective library size, and spike-in normalizations, we propose the log-ratio transformation as a general solution to answer the question, "Relative to some important activity of the cell, what is changing?". Taken together, this manuscript establishes the first fully comprehensive analysis protocol that is suitable for any and all -omics data.</p>
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# A field guide for the compositional analysis of any-omics data

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## Abstract

Next-generation sequencing (NGS) has made it possible to determine the sequence and relative abundance of all nucleotides in a biological or environmental sample. A cornerstone of NGS is the quantification of RNA or DNA presence as counts. However, these counts are not counts *per se*: the magnitude of the counts are determined arbitrarily by the sequencing depth, not by the input material. Consequently, counts must undergo normalization prior to use. Conventional normalization methods require a set of assumptions: they assume that the majority of features are unchanged, and that all environments under study have the same carrying capacity for nucleotide synthesis. These assumptions are often untestable and may not hold when comparing heterogeneous samples. Instead, methods developed within the field of compositional data analysis offer a general solution that is assumption-free and valid for all data. In this manuscript, we synthesize the extant literature to provide a concise guide on how to apply compositional data analysis to NGS count data. In highlighting the limitations of total library size, effective library size, and spike-in normalizations, we propose the log-ratio transformation as a general solution to answer the question, “Relative to some important activity of the cell, what is changing?”

## Introduction

The advent of next-generation sequencing (NGS) has allowed scientists to probe biological systems in unprecedented ways. For an ever decreasing sum of money, it is possible to determine the sequence and relative abundance of all nucleotide fragments in a sample [47]. NGS works by sequencing a population of DNA fragments, including reverse transcribed RNA isolates. In addition to its general use for variant discovery and genome assembly, NGS is used to quantify relative abundances of (a) RNA species from tissue (RNA-Seq) [47], (b) organism diversity from the environment (metagenomics) [77], (c) RNA species from the environment (meta-transcriptomics) [6], and (d) regions of the genome targeted by a protein (ChIP-Seq) [50], among others. Recently, improvements in the sequencing protocols have allowed for these measurements to be carried out at the single-cell level, with single-cell RNA-Seq being the most mature technology. Most applications share an analogous procedure whereby DNA or RNA are isolated from samples, optionally filtered by size or other property [29], converted to a cDNA library of nucleotide fragments, sequenced on a sequencer, and then mapped to a reference to quantify relative abundance. Since all data derive from the same assay, one might expect that they would undergo the same analysis. However, this is not true: rather, methods tailored for one mode of data do not generalize to another (e.g., RNA-Seq methods have inflated false discovery rates (FDR) when applied to metagenomics data [68, 28]).



Fernandes et al. posited that the analysis of all NGS data can be conceptually unified by recognizing the compositional nature of these data [18]. By “compositional”, we mean that the abundance of any one nucleotide fragment is only interpretable relative to another. This property emerges from the sequencer itself; the sequencer, by design, can only sequence a fixed number of nucleotide fragments. Consequently, the final number of fragments sequenced is constrained to an arbitrary limit so that doubling the input material does not double the total number of counts. This constraint also means that an increase in the presence of any one nucleotide fragment necessarily decreases the observed abundance of all other transcripts [8], and applies to bulk and single-cell sequencing data alike. It is especially problematic when comparing cells that produce more total RNA than their comparator (e.g., high c-Myc cells which up-regulate 90% of all transcripts without commensurate down-regulation [38]). However, even if a sequencer could directly sequence every RNA molecule within a cell, the cells themselves are compositional because of the volume and energy constraints that limit RNA synthesis, as evidenced by the observation that smaller cells of a single type contain proportionally less total mRNA [48].

Compositional data only carry relative information. Consequently, they exist in a Simplex space with one fewer dimensions than components. Analyzing relative data as if they were absolute can yield erroneous results for several common techniques [2, 22, 57] (also demonstrated in the Supplementary Information). First, statistical models which assume independence between features are flawed because of the mutual dependency between components [74]. Second, distances between samples are misleading and erratically sensitive to the arbitrary inclusion or exclusion of components [3]. Third, components can appear definitively correlated even when they are statistically independent [53]. For these reasons, compositional data pose specific challenges to the differential expression, clustering, and correlation analyses routinely applied to NGS data, as well as other data that measure the relative abundance of small molecules (e.g., spectrometric peak data [19]). For compositional NGS data, each sample is called a “composition” and each nucleotide species is called a “component” [22, 57].

There are three general approaches to analyzing compositional data. First, the *normalization-dependent* approach seeks to normalize the data in order to reclaim absolute abundances. However, normalizations depend on assumptions that may not hold true outside of tightly controlled experiments. For example, popular RNA-Seq normalization methods assume that most transcripts have the same absolute abundance across samples [61, 5], an assumption that does not hold for the high c-Myc cells discussed above [38]. Second, the *transformation-dependent* approach transforms the data with regard to a reference to make statistical inferences relative to the chosen reference [2]. Third, the *transformation-independent* approach performs calculations directly on the components [46] or component ratios [25].

The latter two approaches constitute compositional data analysis (CoDA). Unlike normalization-based methods, CoDA methods will generalize to all data, relative or absolute. In this article, we describe a unified pipeline for the analysis of NGS count data, with all parts fully capable of modeling the uncertainty of lowly abundant counts. First, we show how existing CoDA software tools can be used to draw compositionally valid and biologically meaningful conclusions. Second, we illustrate how these methods can accommodate complex study design, facilitate the analysis of horizontally integrated multi-omics data, and accommodate machine learning applications. Third, we show how compositionality can systematically bias results if ignored. Finally, we conclude with a discussion of key problems associated with spike-in normalization, and show how the CoDA framework applies specifically to single-cell sequencing data.

## Methods

### Overview of pipeline

Our pipeline uses software tools made freely available for the R programming language. It begins with an unnormalized “count matrix” generated from the alignment and read-mapping of a sequence library. Details regarding quality control, assembly, alignment, and read-mapping are beyond the scope of this article, and have been covered extensively elsewhere (e.g., [14, 20]). This count matrix records the number of times each feature (e.g., transcript or operational taxonomic unit [OTU]) appears in each sample. Most software return measurements as integer counts, although some use continuous values (e.g., Salmon quasi-counts [51]) or another proportional unit (e.g., transcripts per million (TPM) [71]). For many CoDA methods, units have no importance.

However, small counts carry more uncertainty than large counts, and our pipeline can model this directly. Therefore, we recommend using unadjusted “raw counts”. TPM can also be used with CoDA methods, but can bias the modelling of small counts if the library size differs greatly between samples. Otherwise, the data should not undergo further normalization or standardization, and must never contain negative values. Figure 1 provides a schematic of our unified NGS pipeline.

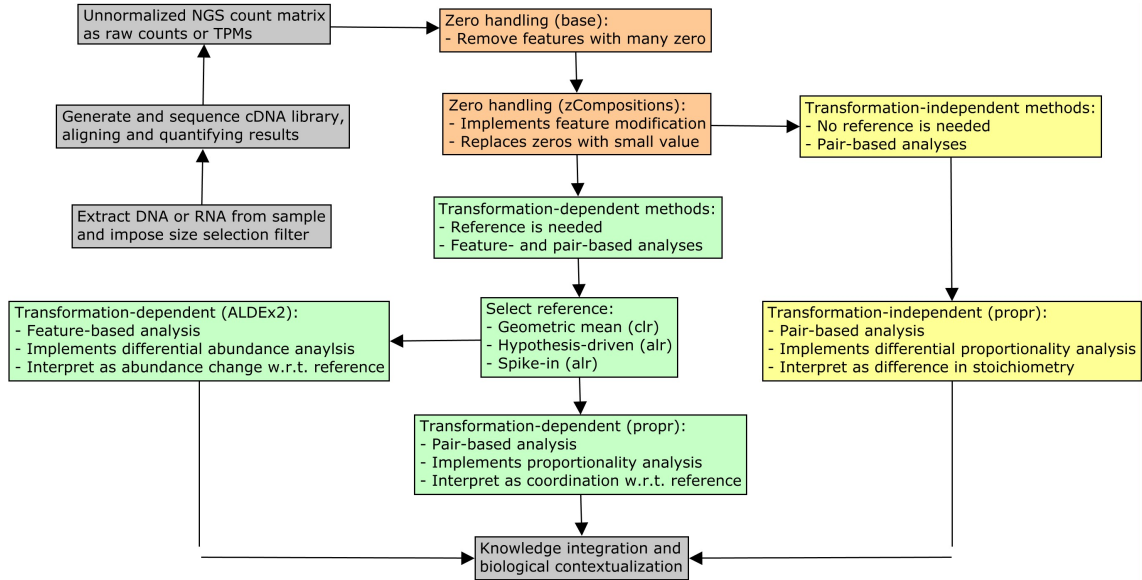


Figure 1: This figure illustrates how our unified NGS pipeline might sit within a larger workflow. Colored boxes indicate procedures that would apply to any relative data set. In orange, we describe the optional zero removal and modification steps presented in “Part 1: Zero handling”. In green, we describe the log-ratio transformation-dependent methods presented in “Part 2a: Transformation-dependent analyses”. This includes the differential abundance analysis of individual features and the proportionality analysis of feature pairs. In yellow, we describe the transformation-independent methods presented in “Part 2b: Transformation-independent analyses”. This includes the analysis of the differences in the log-ratio means of feature pairs. In gray, we describe other essential steps unique to the data type under study but not covered here.

## Data acquisition

To demonstrate the utility of our pipeline, we use publicly available time course data of the RNA and protein expressed by mouse dendritic cells following lipopolysaccharide (LPS) exposure, a potent immunogenic stimulus. RNA-Seq and mass spectrometry (MS) data were acquired already pre-processed to measure the relative abundance of 3147 genes in TPM-equivalent units [31]. The RNA-Seq and MS data had 28 overlapping samples, spanning 2 conditions with 7 time points and 2 replicates each.

```
# Read in the RNA-Seq data
rnaseq <- read.csv("rnaseq-x.csv", row.names=1)
rnaseq.annot <- read.csv("rnaseq-y.csv", row.names=1)

# Read in the Mass Spec HL data
masshl <- read.csv("masshl-x.csv", row.names=1)
masshl.annot <- read.csv("masshl-y.csv", row.names=1)

# We will subset Mass Spec to include timepoints
# with a corresponding RNA-Seq measurement
# (used in ‘Vertical Data Integration’)
inRNAandMS <- masshl.annot$Time %in% rnaseq.annot$Time
masshl <- masshl[,inRNAandMS]
masshl.annot <- masshl.annot[inRNAandMS,]
```

## New analyses

In presenting this workflow, we perform a new analysis of the Jovanovic et al. data in order to learn how mRNA transcript abundance and protein abundance change in response to LPS stimulation. This includes a relative differential abundance analysis, an analysis of gene-gene coordination, and an analysis of differential gene-gene coordination. In addition, we integrate the two data types with a differential proportionality analysis to evaluate how mRNA stoichiometry differs from protein stoichiometry in response to LPS treatment. Unlike the original analysis presented by Jovanovic et al., we do not use transcripts per million (TPM) normalization. Rather, we argue that TPMs re-cast an already compositional data set as yet another compositional data set (just with a different denominator). In the Supplementary Information, we show how TPMs introduce systematic errors. This is because when a reference is not explicitly chosen, an arbitrary reference is still implicitly present. We also include an appendix that benchmarks how several zero handling procedures impact proportionality and differential proportionality analysis.

## Software contributions

This workflow primarily uses three open source software packages, all of which are available for the R programming language. They include `zCompositions` [49], `ALDEx2` [17, 18], and `propr` [58, 16]. The reader can download these software from Bioconductor and CRAN.

```
install.packages("zCompositions")
install.packages("propr")
install.packages("BiocManager")
# Read '::' as 'the install function from the BiocManager package'
BiocManager::install("ALDEx2")
library(zCompositions)
library(ALDEx2)
library(propr)
```

In preparing this workflow, we have made several contributions to the compositional data analysis software universe. First, we present the new `propr::aldex2propr` function that integrates the `ALDEx2` and `propr` packages by calculating an average proportionality coefficient over `ALDEx2`-generated Monte Carlo instances. Second, we present the new `propr::updateCutoffs` function that permutes a false discovery rate across varying proportionality coefficient cutoffs. Third, we present the `propr::propd` function that implements the differential proportionality method described by Erb et al. [16], including an implementation of a zero handling procedure based on the Box-Cox transform. These new contributions make a complete compositional data analysis workflow possible.

## Benchmark validation

Although one can devise a “normalizing” reference by invoking a set of assumptions, we prefer an alternative framework that does not require any normalization. We use this framework because it provides a more general solution to the analysis of *-omics* data. As such, our proposed workflow could be used to analyze bulk RNA-Seq, single-cell RNA-Seq, metagenomics, metabolomics, lipidomics, and other data.

Although the software tools presented here do not normalize the data, they can be benchmarked against conventional methods by invoking the assumption that the explicit reference performs a kind of “log-ratio normalization”. Under these conditions, `ALDEx2` can identify differential abundance with high precision in RNA-Seq data [18, 55], and control false positive rates in highly sparse 16S metagenomics count data [68]. Meanwhile, proportionality analysis has been shown to outperform all 15 competing measures of association in single cell clustering and network inference tasks across 213 data sets [65]. Although differential proportionality analysis has not yet been benchmarked, it is formally related to an analysis of variance (ANOVA), a foundational test in most biological research. As a statistical test for significance, it is valid wherever an ANOVA is valid. We also include an appendix that benchmarks how several zero handling procedures impact proportionality and differential proportionality analysis.

## Part 1: Zero handling

### General strategies for zero handling

CoDA methods depend on logarithms which do not compute for zeros. Therefore, we must address zeros prior to, or during, the pipeline. Before handling zeros, the analyst must first consider the nature of the zeros. There exists three types of zeros: (1) *rounding*, also called *sampling*, where the feature exists in the sample below the detection limit, (2) *count*, where the feature exists in the sample, but counting is not exhaustive enough to see it at least once, and (3) *essential*, where the feature does not exist in the sample at all [45]. The approach to zero handling depends on the nature of the zeros [45]. For NGS data, a nucleotide fragment is either sequenced or not, and would not contain rounding zeros. Since there is no general methodology for dealing with essential zeros within a strict CoDA framework [45], we assume that any feature present in at least one sample could appear in another sample if sequenced with infinite depth, and thus treat all NGS zeros as “count zeros”. Others have also suggested that the essential zeros of NGS count data are sufficiently modeled as sampling zeros [62].

There are two general approaches to zero handling. In *feature removal*, components with zeros get excluded, yielding a sub-composition that can be analyzed by any CoDA method. Feature removal is usually appropriate when a feature contains many zeros, and can always be justified for essential zeros. In *feature modification*, zeros get replaced with a non-zero value, with or without modification to non-zeros. Analysts may choose one or both zero handling procedures, but should always demonstrate that the removal or modification of zero-laden features does not change the overall interpretation of the results.

### Feature modification with zCompositions

For “count zeros”, Martin-Fernandez et al. recommend replacing zeros by a Bayesian-multiplicative replacement strategy that preserves the ratios between the non-zero components [45], implemented in the `zCompositions` package as the `cmultRepl` function [49]. Alternatively, one could use a multiplicative simple replacement strategy, whereby zeros get replaced with a fixed value less than 1 in a compositionally robust manner. Here, we use `zCompositions` to replace zeros.

```
# Standard functions expect rows as samples
# so we will transpose the matrix
rnaseq <- t(rnaseq)
masshl <- t(masshl)

# Now we can replace zeros with a small value
# the ‘p-counts’ option has the function return
# pseudo-counts instead of proportions
library(zCompositions)
rnaseq.no0 <- cmultRepl(rnaseq, output = "p-counts")
masshl.no0 <- cmultRepl(masshl, output = "p-counts")
```

Many compositional software tools have their own built-in zero handling procedures. Although `zCompositions` is not necessarily better than these built-in procedures, we recognize that removing zeros right away has a practical advantage: by using `zCompositions` in combination with a log-ratio transformation, analysts can apply most conventional analyses to their compositional data right away. Since `zCompositions` empowers readers to use methods beyond the ones presented here, we decided to include it as the first part of our field guide. However, we recommend that readers look at our appendix which benchmarks how several zero handling procedures impact proportionality and differential proportionality analysis.

## Part 2a: Transformation-dependent analyses

### The log-ratio transformation

All components in a composition are mutually dependent features that cannot be understood in isolation. Therefore, any analysis of individual components is done with respect to a reference. This reference transforms each sample into an unbounded space where any statistical method

can be used. The centered log-ratio (**clr**) transformation uses the geometric mean of the sample vector as the reference [1]. The additive log-ratio (**alr**) transformation uses a single component as the reference [1]. Other transformations use specialized references based on the geometric mean of a subset of components (collectively called multi-additive log-ratio (**malr**) transformations [55]). One **malr** transformation is the inter-quartile log-ratio (**iqlr**) transformation which uses components in the inter-quartile range of variance [78]. Another, the robust centered log-ratio (**rclr**) transformation, only uses the non-zero components [42].

Importantly, transformations are not normalizations: while normalizations claim to recast the data in absolute terms, transformations do not. The results of a transformation-based analysis must be interpreted with respect to the chosen reference. Of these, the **clr** transformation is most common:

$$\text{clr}(\mathbf{x}_j) = \left[ \ln \frac{x_{1,j}}{g(\mathbf{x}_j)}, \dots, \ln \frac{x_{D,j}}{g(\mathbf{x}_j)} \right] \quad (1)$$

where  $\mathbf{x}_j$  is the  $j$ -th sample and  $g(\mathbf{x}_j)$  is its geometric mean. The other transformations replace  $g(\mathbf{x}_j)$  with a different reference.

The isometric log-ratio (**ilr**) transformation uses an orthonormal basis as the reference [13], and is preferred when a non-singular covariance matrix is needed [46]. When the basis is a branch of a dendrogram, the **ilr** offers an intuitive way to contrast one set of components against another set of components. These contrasts, called balances, have been used to analyze metagenomics data based on evolutionary trees [63, 76], but could be applied to any data if a similarly meaningful tree were available.

Each transformation implies its own reference(s). In most practical settings, the choice of transformation will depend on the preferred interpretation. An analysis of **clr** data will tell you how genes (or OTUs) behave relative to the per-sample average. An analysis of **alr** and **malr** data will tell you how genes (or OTUs) behave relative to one or more explicitly-chosen internal references. An analysis of **iqlr** data will tell you how genes (or OTUs) behave relative to the per-sample inter-quartile (“robust”) average. In a compositional framework, none of these are normalizations: each new variable is a log-ratio of the original variable divided by the reference, and therefore should get interpreted as a kind of within-sample log-fold difference. Although the difference between transformation and normalization may seem subtle, it can have a profound impact on the conclusions drawn from the analysis. Although the temptation will exist, one must never confuse the transformed data with absolute abundances.

## Differential abundance analysis with ALDEx2

Differential abundance (DA) analysis seeks to identify which features differ in abundance between experimental groups. The ALDEx2 package tests for DA in compositional data by performing univariate statistical analyses on log-ratio transformed data [17, 18]. It does so with a layer of complexity that controls for technical variation by finding the expectation of  $B$  simulated instances of the data, each sampled from the Dirichlet distribution. This procedure implicitly models the uncertainty of low counts while also handling zeros.

Importantly, ALDEx2 identifies DA *with respect to the chosen reference*. By default, this reference is the geometric mean of the composition. It is possible, if not likely, that the mean centers are not the ideal references; if so, differences in the transformed abundances would not reflect differences in the absolute abundances. On the other hand, if one could assume that the chosen reference did have fixed absolute abundance across all samples, then the log-ratio transformation can be benchmarked as a “log-ratio normalization” [57]. Under these conditions, ALDEx2 can identify DA with high precision in RNA-Seq data [18, 55], and control false positive rates in highly sparse 16S metagenomics count data [68]. However, the “log-ratio normalization” interpretation implies a similar assumption implied by other DA tools: that the majority of transcript species remain unchanged [33]. Alternatively, one could select an arbitrary reference based on a biological hypothesis to identify *relative DA*, even if the reference does not have fixed abundance across samples. Figure 2 shows how the chosen reference changes the interpretation of DA.

To run ALDEx2, the user must provide count data with integer values, a vector of group labels, and a reference. The reference could be “all” (for **clr**), “iqlr” (for **iqlr**), or one or more user-specified features (for **alr** or **malr**). Here, we use the geometric mean of two NF $\kappa$ B sub-units

as a hypothesis-based reference, chosen because LPS activates NF $\kappa$ B to control the transcription of other immune genes [54]. With this reference, up-regulation signifies that a gene's expression increases beyond that of NF $\kappa$ B, allowing for a clear biological interpretation. Table 1 lists 47 genes up-regulated relative to NF $\kappa$ B.

```
# Let's use Nfkb sub-units as alr reference
ref <- grep("Nfkb", colnames(rnaseq))

# ALDEx2 expects:
# 'reads': integer counts with columns as samples
# 'conditions': the experimental outcome
# 'denom': the log-ratio transform reference
library(ALDEx2)
conditions <- factor(rnaseq.annot$Treatment, levels = c("MOCK", "LPS"))
tt <- aldex(reads = t(ceiling(rnaseq)),
            conditions = conditions,
            denom = ref)

# ALDEx2 outputs a data.frame:
# 'we.eBH': the FDR-adjusted p-value
# 'effect': the effect size
# Below, we get the names of genes
# with relatively more abundance
# in the LPS group
tt.bh05 <- tt[tt$we.eBH < .05,]
up <- rownames(tt.bh05[tt.bh05$effect > 0,])
```



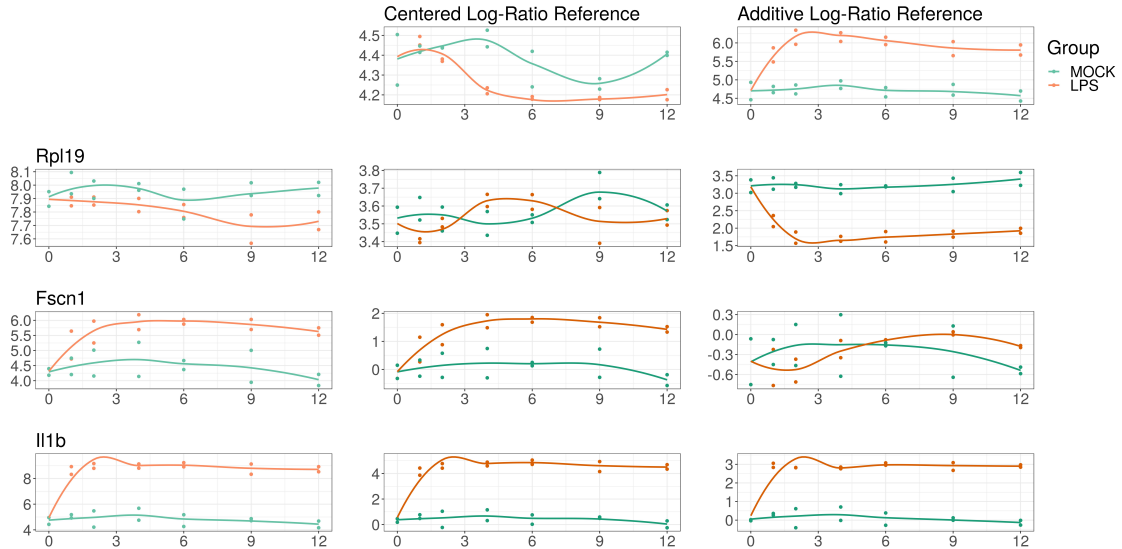


Figure 2: This figure illustrates how the interpretation of differential abundance depends on the reference chosen. On the left margin, we show the log-abundance of three genes (RPL19, FSCN1, and IL1B) for the LPS-treated cells (orange) and control (blue). For compositional data, these abundances carry no meaning in isolation because the constrained total imposes a “closure bias”. On the top margin, we show the log-abundance of two references: the geometric mean of the samples (a la the **clr**) and a hypothesis-based reference  $\text{NF}\kappa\text{B}$  (a la the **alr**). In the middle, we show the abundance of the log-ratio of the left margin feature divided by the top margin reference (equivalent to left margin minus top margin in log space). RPL19 alone appears more abundant in the control, but actually has equivalent expression when compared with the geometric mean; however, it has significantly higher expression in the control relative to  $\text{NF}\kappa\text{B}$ . On the other hand, FSCN1 alone appears more highly expressed in the LPS-treated cells, which remains true when compared with the geometric mean; however, it has equivalent expression relative to  $\text{NF}\kappa\text{B}$  (interpreted as  $\text{NF}\kappa\text{B}$  and  $FSCN1$  expression changing similarly in response to LPS stimulation). IL1B alone appears more highly expressed in the LPS-treated cells, which remains true when compared with the geometric mean and with  $\text{NF}\kappa\text{B}$  (interpreted as IL1B expression becomes even higher than  $\text{NF}\kappa\text{B}$  expression in response to LPS stimulation). Choosing a reference makes normalization unnecessary, but requires a shift in interpretation.

## Proportionality analysis with **propr**

Proportionality analysis is designed to identify feature coordination in compositional data [37, 15], without assuming sparsity in the association network [21, 34]. The **propr** package tests for the presence of feature coordination across all samples, irrespective of group label, by calculating one of three proportionality measures. Two of these have been shown to outperform all 15 competing measures of association in single cell clustering and network inference tasks across 213 data sets [65]. The default measure,  $\rho_p$ , resembles correlation in that it ranges from  $[-1, 1]$ . Like DA, proportionality analysis requires a reference.

```
# propr expects:
# 'counts': the data matrix with rows as samples
# 'metric': the proportionality metric to calculate
# 'ivar': the log-ratio transform reference
library(propr)
pr <- propr(counts = rnaseq.no0,
            metric = "rho",
            ivar = "clr")
```

The **propr** package offers two alternatives to zero handling. The `propr::aldex2propr` function will calculate the expected proportionality from the simulated instances generated by ALDEx2, again addressing the uncertainty of low counts [7]. The `alpha` argument will use a zero handling procedure

	Effect Size	Difference (between)	Difference (within)	Expected BH $p$ -value
I11b	4.7372	3.9576	0.6912	0.0000
Irg1	4.3462	3.8904	0.7888	0.0000
I11a	3.5950	3.8242	0.9037	0.0000
Cd40	2.2887	5.3325	2.0422	0.0000
Ifih1	2.2056	2.8529	1.1157	0.0000
Isg15	1.9678	4.4490	1.8330	0.0000
Oasl1	1.9304	5.6562	2.1200	0.0000
Ifit1	1.8317	5.6101	2.0773	0.0000
Ptgs2	1.6923	4.0869	2.0606	0.0002
Gbp5;Gbp1	1.6523	2.4494	1.2349	0.0000
Rsad2	1.4933	6.2747	2.4692	0.0001
Marcksl1	1.4886	1.0748	0.5740	0.0001
BC006779	1.4686	2.2184	1.2465	0.0001
Mndal	1.4163	2.1047	1.5182	0.0000
Parp14	1.3139	1.7655	0.9357	0.0002
Ifi205	1.2916	5.3159	3.4587	0.0026
Slc7a2	1.2883	1.3797	0.9920	0.0002
Ifit2	1.2292	5.4975	2.6744	0.0002
Clic4	1.2037	0.8486	0.5765	0.0003
Sp140	1.1612	1.0030	0.7385	0.0005
Cmpk2	1.1149	5.7323	2.1088	0.0003
Stat5a	1.0806	0.8666	0.6461	0.0017
Ifi47	1.0443	2.0495	1.5704	0.0030
Pyhin1	1.0152	1.9150	1.4752	0.0024
Ifit3	0.9978	4.7313	3.2116	0.0012
Ccl5	0.9962	2.0765	1.6671	0.0015
Acsl1	0.9937	1.0837	1.0073	0.0009
I11rn	0.9811	0.6795	0.6366	0.0017
Irgm1	0.9755	1.7076	1.0634	0.0094
IIGP;Iigp1	0.9588	3.5610	3.1760	0.0023
Rnf213;AK217856	0.9541	1.2867	1.0478	0.0041
Daxx	0.9118	1.1938	0.9013	0.0119
Flnb	0.8639	1.6654	1.8185	0.0122
Cd274	0.8299	0.6050	0.6354	0.0051
Trex1	0.8171	0.5647	0.6350	0.0090
Car13	0.7586	1.1455	1.2839	0.0140
Xaf1	0.7550	1.5118	1.4338	0.0214
Gbp3	0.7478	1.5118	1.4837	0.0128
Ehd1	0.7460	0.3648	0.4812	0.0078
Gm4902	0.7413	1.9614	1.7899	0.0151
Rasa4	0.7254	0.8805	0.9109	0.0478
Oas3	0.7089	1.5673	1.7756	0.0213
Serpib2	0.7048	1.7770	2.1734	0.0272
Dhx58;D11lgp2	0.6947	1.4875	1.6956	0.0425
Gbp2	0.6597	1.5376	1.7339	0.0212
Saa3	0.6291	1.0259	1.5384	0.0187
Sbds	0.5522	0.3107	0.5363	0.0443

Table 1: This table shows the 47 genes selected as significantly up-regulated by ALDEx2 when using the  $\text{NF}\kappa\text{B}$  sub-units as a reference. One can interpret this “up-regulation” to mean that the gene increases its expression in response to LPS stimulation more than  $\text{NF}\kappa\text{B}$ . All  $p$ -values correspond to the expectation of the Benjamini-Hochberg adjusted  $p$ -values computed from a Welch’s  $t$ -test over 128 simulated instances of the data. By choosing a reference that is relevant to the biological system under study, we can gain meaningful insights from the data without any need for normalization. In this table, between-group differences are the differences between the two conditions (defined for each Dirichlet instance), within-group differences are the maximum difference across Dirichlet instances (defined for each condition), and effect sizes are the ratio of the between-group differences to the maximum of within-group differences (defined for each Dirichlet instance). The columns “Effect size”, “Difference (between)”, and “Difference (within)” report the median effect size, median between-group difference, and median within-group difference, respectively.



based on the Box-Cox transform, a pragmatic approach that allows for essential zeros, but does not fall under the strict CoDA framework [24]. A Box-Cox transform with  $\alpha = 0.5$  appears to work well in simulations (see Appendix). For proportionality, we do not calculate parametric p-values. Instead, we permute the FDR for a given cutoff. From this, we choose the cutoff  $\rho_p > 0.45$  to control FDR below 5%. The package vignette describes several built-in tools for visualizing proportionality. Figure 3 shows the output of the `getNetwork` function.

```
# We can select a good cutoff for 'rho'  
# by permuting the FDR at various cutoffs  
# Below, we use [0, .05, ..., .95, 1]  
pr <- updateCutoffs(pr, cutoff = seq(0, 1, .05))  
pr@fdr  
  
# Let's visualize using a strict cutoff  
getNetwork(pr, cutoff = 0.9, coll = up)  
getResults(pr, cutoff = 0.9)
```

Proportionality depends on a log-ratio transformation and must get interpreted with respect to the chosen reference. Although proportionality appears more robust to spurious associations than correlation [37, 58], wrongly assuming that the reference has fixed absolute abundance across all samples could lead to incorrect conclusions [15]. We interpret **clr**-based proportionality to signify a coordination that follows the general trend of the data. In other words, these proportional genes move together as individuals relative to how most genes move on average.



Figure 3: This figure shows a network where edges indicate a high level of coordination between gene expression relative to the per-sample geometric mean. Node color indicates differential expression relative to NF $\kappa$ B. The connections between red nodes indicate genes whose expression increase more than NF $\kappa$ B in a coordinated manner. The connections between white nodes indicate genes whose expression increase the same amount as NF $\kappa$ B in a coordinated manner. The connections between blue nodes indicate genes whose expression either (a) up-regulate less than NF $\kappa$ B, (b) do not change absolutely, or (c) down-regulate, all in a coordinated manner. The high level of connectivity between all nodes suggests a strong coordinated response to LPS. Like correlated pairs, proportional pairs can have any slope in non-log space. Note that this network only shows highly coordinated events (where  $\rho_p > .9$ ).

## Part 2b: Transformation-independent analyses

The methods above depend on a log-ratio transformation to standardize the comparison of one gene's expression (or one pair's coordination) with another. However, by comparing the variance of the log-ratios (VLR) within groups to the total VLR, we do not need a reference to estimate between-group differences in coordination [16, 75]:

$$\text{VLR}^k(\mathbf{x}^g, \mathbf{x}^h) = \text{var} \left[ \ln \frac{x_{g,1}}{x_{h,1}}, \dots, \ln \frac{x_{g,N^k}}{x_{h,N^k}} \right]. \quad (2)$$

for group  $k$  with  $N^k$  samples, where  $\mathbf{x}^g$  and  $\mathbf{x}^h$  are component vectors. From this equation, we see that any normalization or transformation factor would cancel. The VLR ranges from  $[0, \infty)$ , where zero indicates perfect coordination. Otherwise, VLR lacks a meaningful scale [1]. As such, we

cannot compare the VLR of one pair to the VLR of another pair (hence why we used proportionality instead) [37, 58]. However, in differential proportionality, we compare the VLR for the same pair across groups [16].

Differential proportionality analysis is designed to identify changes in proportionality between groups [16], interpretable as a change in gene stoichiometry. The `propd` function tests for events where the proportionality factor (i.e., the magnitude of  $\frac{x}{y}$ ) differs between the experimental groups. This is measured by  $\theta_d$  which ranges from 0 to 1, where zero indicates a maximal difference between the groups. As above, users can permute the FDR and build a network, but can also calculate an exact p-value from  $\theta_d$  using the `updateF` function [16], with the optional application of `limma::voom` precision weights [35] and  $F$ -statistic moderation [66]. Precision weights eliminate the mean-variance relationship that affects the results for low counts, while the moderated statistic helps avoid false positive results in the case of few replicates. When testing the significance of multiple log-ratio pairs, it is absolutely necessary to correct the p-value for multiple testing. In addition, this function implements a zero handling procedure based on the Box-Cox transform, where  $\alpha = 0.5$  appears to work well in simulations (see Appendix). Figure 4 shows significant differentially proportional pairs containing NF $\kappa$ B in the log-ratio. Most of these companion genes were also called (relatively) differentially abundant by ALDEx2.

```
# propd expects:
# 'counts': the data matrix with rows as samples
# 'group': the class labels
library(propr)
pd <- propd(counts = rnaseq.no0,
            group = rnaseq.annot$Treatment)

# Calculate an exact p-value
pd <- updateF(pd)
getResults(pd)
```

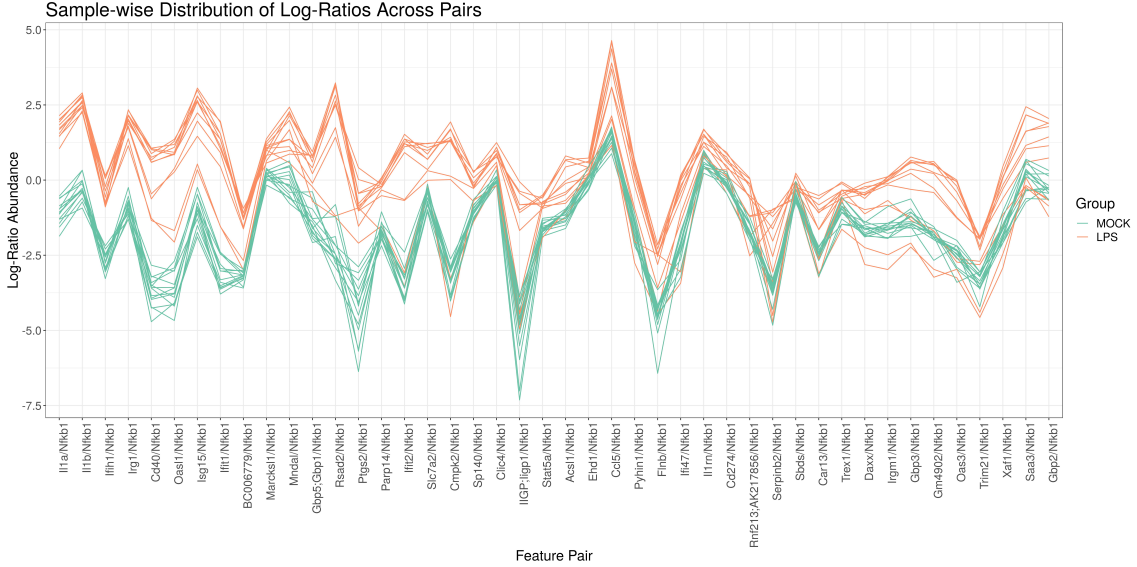


Figure 4: This figure shows a parallel coordinate plot of the log-ratio abundance (y-axis) of significant differentially proportional pairs that contain  $\text{NF}\kappa\text{B}$  in the log-ratio (x-axis). Each line represents a single sample, colored by group. Gene pairs toward the left of the x-axis have greater differences in the log-ratio means between groups (i.e., smaller  $\theta_d$  values). This plot only shows pairs for which the LPS-stimulated samples have different log-ratio means from the control (with the order of the numerator and denominator chosen such that the LPS average is always greater than the control average). It is not surprising that many of these significant pairs contain the same genes found by differential abundance analysis. Indeed, one can think of differential proportionality analysis as the differential abundance analysis of all pairwise log-ratios. Although pairs toward the right of the x-axis still have large differences in log-ratio abundance on average, some time points deviate from the trend. Indeed, this figure incidentally reveals a time-dependent process that we could test for specifically with models presented in “Complex study design”.

## Advanced applications

### Complex study design

Above, we used our pipeline to analyze the data as if samples belonged to one of two groups. This pipeline can also accommodate complex study designs with multiple covariates. For `ALDEx2`, we can supply a `model.matrix` R object to find the expectation of a linear model (instead of a  $t$ -test). On the other hand, proportionality is calculated for all samples regardless of class label, and so does not require a new procedure. Differential proportionality measures the difference in the log-ratio abundance between two groups. By design, it is an efficient implementation of the two-group ANOVA expressed by the formula  $[\log(\mathbf{x}_g) - \log(\mathbf{x}_h)] \sim \text{group}$ , for all combinations of features  $g$  and  $h$ . Thus, we can extend differential proportionality by modeling each pairwise log-ratio outcome as a function of any `model.matrix`. This may become computationally burdensome for high-dimensional data. When testing the significance of multiple log-ratio pairs, it is absolutely necessary to correct the p-value for multiple testing, for example by using the `p.adjust` function in R.

### Vertical data integration

We envision two general strategies for the vertical integration of compositional data. First, the *row join* strategy treats other *-omics* data as additional samples and models the *-omics* source as a covariate. This requires that all *-omics* sources map to the same features. For the RNA-Seq and MS data used here, both quantify the relative abundance of gene products. This allows us to use `ALDEx2` to find features where mRNA abundance changes more than protein abundance, relative to a common reference (and *vice versa*). Likewise, we can use proportionality analysis to find feature pairs where genes and proteins both have coordinated expression in response to LPS.

Finally, we can use differential proportionality analysis to find feature pairs with stoichiometric differences between a gene pair and its respective protein pair. Figure 5 shows some examples of differentially proportional pairs.

```
# Get LPS-treated cells only
rna <- rnaseq.no0[rnaseq.annot$Treatment == "LPS",]
pro <- masshl.no0[masshl.annot$Treatment == "LPS",]

# Join as single matrix
merge <- rbind(rna, pro)
group <- c(rep("RNA", 14), rep("Protein", 14))

# Run propd analysis
pd.ms <- propd(merge, group)
```

Second, the *column join* strategy, treats other *-omics* data as additional features. This strategy is more complicated, as it requires that each *-omics* source has its own reference. In practice, we should perform differential abundance analysis on each *-omics* source independently. For proportionality and differential proportionality analysis, we would need to log-ratio transform each *-omics* source independently, then column join them with `cbind`. Here, any proportionality occurring between features from different sources would be with respect to two references, and must get interpreted accordingly.

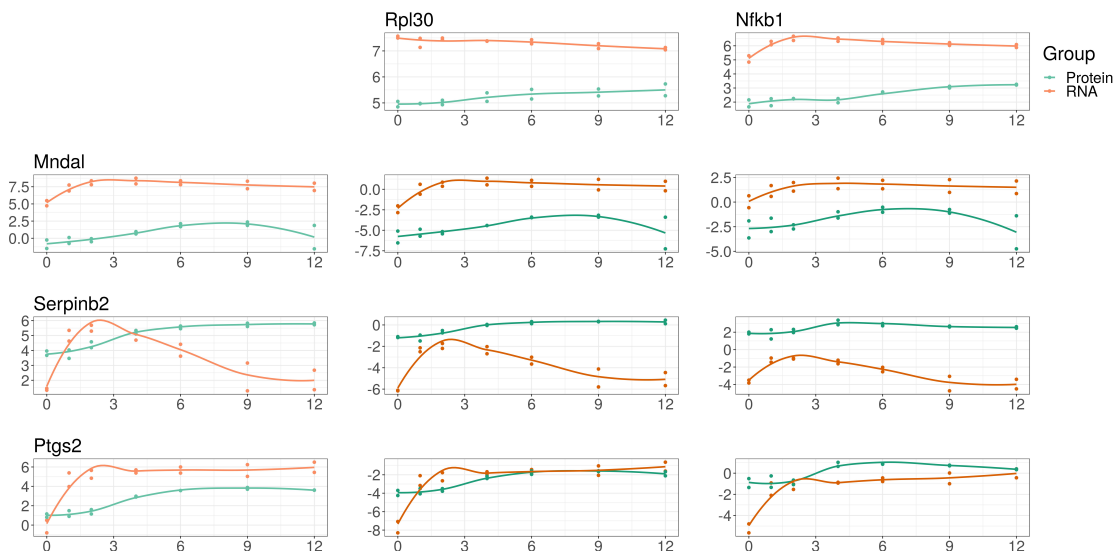


Figure 5: This figure compares mRNA abundance with newly synthesized protein abundance following LPS stimulation, illustrating the vertical integration of multi-omics data under a compositional framework. On the left margin, we show the log-abundance of three genes (MNDAL, SERPINB2, and PTGS2) as measured by RNA-Seq (orange) and mass spectrometry (blue). For compositional data, these abundances carry no meaning in isolation because the constrained total imposes a “closure bias”. On the top margin, we show the log-abundance of two references: RPL30 (chosen because its abundance is proportional to the geometric mean of the samples) and  $\text{NF}\kappa\text{B}$  (chosen based on the hypothesis). In the middle, we show the abundance of the log-ratio of the left margin feature divided by the top margin reference (equivalent to left margin minus top margin in log space). MNDAL alone appears to exist more as mRNA than protein, which remains true when compared with both references. This suggests that MNDAL is translated with lower efficiency than RPL30 and  $\text{NF}\kappa\text{B}$ . On the other hand, SERPINB2 alone appears to exist as mRNA and protein similarly on average; however, it actually exists more as protein than mRNA when compared with both references. This suggests that MNDAL is translated with greater efficiency than RPL30 and  $\text{NF}\kappa\text{B}$ . PTGS2 alone appears to exist more as mRNA than protein, but this difference is less apparent when compared with both references. This suggests that PTGS2 is translated with a similar efficiency to RPL30 and  $\text{NF}\kappa\text{B}$ . By choosing a reference shared between two multi-omics data sets, we can perform an analysis of vertically integrated data without any need for normalization.

## Horizontal data integration

The term “mega-analysis” describes a single analysis of samples collected across multiple studies [72]. Batch effects pose a major barrier to mega-analyses. Here, we consider two types of batch effects. The first affects all genes within a sample proportionally (e.g., due to differences in sequencing depth). A log-ratio transformation will automatically remove this batch effect. The second affects only some genes within a sample (e.g., due to differences in RNA depletion protocols). This requires explicit modification of the corrupted features. If needed, one could apply standard batch correction tools, normally applied to normalized data, to the transformed data instead (c.f., the moderated log-link *sva* in [36]).

## Clustering and classification

Most distance measures lack sub-compositional dominance, meaning that it is possible to reduce the distance between samples by adding dimensions [3]. When clustering compositions, methods that rely on distance, like hierarchical clustering, also lack sub-compositional dominance [44]. Instead, one should use the Euclidean distance of **clr** transformed compositions (called the Aitchison distance) [44]. Other statistical methods used for clustering, like PCA and t-SNE, also compute distance and should also get **clr** transformed prior to analysis. When clustering components, one could use the proportionality metric  $\phi_s$  as a dissimilarity measure [58]. The  $\phi_s$  proportionality metric, like the  $\rho_p$  proportionality metric, is defined for **clr**-transformed data. If the geometric mean center changes drastically across samples, some proportional pairs may not be proportional in an absolute sense. We refer the reader to the sub-section, “Proportionality analysis with **propr**”, for further explanation.

How best to classify compositional data remains an open question, but **ilr** transforming the data prior to model training would grant the data favorable properties, as done for linear discriminant analysis [70]. Alternatively, one could train models on the log-ratios themselves, though this may not scale to high-dimensional data. Recently, balances have been used for feature selection and classification [60, 56], where they achieve both accuracy and interpretability [10].

## Selected topics

### Closure bias and the implicit reference

NGS count data measure relative abundances because of the arbitrary limit imposed by the cell, the environment, and the sequencer. This is sometimes called the “constant sum constraint” because the sum of the relative abundances must equal a constant. Anything that introduces a constant sum constraint is a kind of “closure”; all closures irreversibly make a data set relative (i.e., “closed”). One could think of a cell (in the case of RNA-Seq) or the environment (in the case of metagenomics) as natural closures, and sequencers as technical closures.

Total library size normalizations, like TPM, are not normalizations at all: they are actually yet another closure, imposing the constant sum constraint of transcripts per million. TPMs do not convert closed sequencing data into an “open” unit such as concentration. Analyzing TPMs as if they were concentrations is theoretically flawed, and can substantially affect the modeling of cellular processes. Our own analysis indicates that in Jovanovic et al., mRNA translation rates could have been systematically over-estimated due to compositional bias. In the Supplementary Information, we show that at the latest time point, the error compared to normalized data is around 13% in the control condition, reaching 35% in LPS-stimulated samples. This bias is due to the closure operation: if the analyst does not select a reference, the estimates must get interpreted with regard to the unknown and immeasurable “closure bias”. Since the magnitude of this closure bias can be large for samples that range widely in terms of nucleotide synthesis capacity, a reference should always be used when modeling the univariate features of compositional data. If a reference is not chosen, then the closure bias acts as an “implicit reference” that makes interpretation impossible.

### Count compositions and low-count imprecision

Closed count data differ from idealized compositional data because additive variation affects small counts more than large counts [58]. As such, the difference between 1 and 2 counts is not the same as the difference between 1000 and 2000 counts. Moreover, NGS experiments often have many



more features than samples, leading to severe under-estimation of the technical variance; indeed, the technical variance can be much larger than the biological variance at the low-count margin [17]. “Count zero” features are those that are observed as a non-zero value in at least one sample, and thus are expected to be observed at or near the margin in other samples. While not intuitive, the distribution of the relative “count zero” values is quite large and spans many orders of magnitude [23]. In addition, the expected value of a “count zero” feature must be greater than zero because a value greater than zero was observed in at least one sample.

As mentioned above, the “count zero” values can be modified to give a point-estimate of their expected value, but this leads to under-estimation of their true variance since we are estimating the expected value of the feature. In the approach instantiated in the `aldex.clr` function used by the `ALDEx2::aldex.ttest`, `ALDEx2::aldex.effect`, and `propr::aldex2propr` functions, a distribution of “count zero” values is determined by sampling from the Dirichlet distribution (i.e., a multivariate generalization of the  $\beta$  distribution). Another way to think about the Dirichlet distribution is a multivariate Poisson sampling with a constant sum constraint. The distribution of relative abundances near the low-count margin can be surprisingly wide, both as estimated by sampling from the Dirichlet distribution, and as observed in real data [23]. By sampling from the Dirichlet distribution, we get a set of multivariate probability vectors, each of which is as likely to have been observed from the underlying data as the one actually observed from the sequenced sample. From this, ALDEx2 and `propr` can account for low-count technical imprecision (which can be much larger than the biological variation) by reporting the expected values of a test statistic instead of the point estimate [17].

## Spike-in “log-ratio normalization”

Transformations are not normalizations because they do not claim to recast the data in absolute terms. However, if one were to choose a set of references with *a priori* known fixed abundance across all samples, one could use this “ideal reference” to normalize the data (something we call a “log-ratio normalization” [57]). The use of spike-in controls, consisting of multiple synthetic nucleotide sequences with known absolute abundance, may offer one such option. For RNA-Seq, the External RNA Controls Consortium (ERCC) spike-in set consists of 92 polyadenylated RNA transcripts with varying length (250-2000 nt) and GC content (5-51%) with a  $10^6$ -fold range in abundance [30]. The spike-in set is added to a standardized amount of purified RNA in equimolar concentrations, then both the spike-in and target transcripts are processed together to create a cDNA library. Since 23 of the ERCC transcripts are designed to have the same absolute abundance, one could use their geometric mean as a reference to recast the data in absolute terms. Similarly, one could spike-in a known quantity of bacteria cells or synthetic plasmids to standardize the abundance of PCR-amplified metagenomics samples [67, 69].

However, two important assumptions underly the use of spike-ins for normalization. First, it is assumed that the spike-in and target sequences have the same *capture efficiency of RNA conversion*, in that they are both equally affected by the technical biases of cDNA library creation. Second, it is assumed that the spike-ins are *calibrated to the number of RNA molecules per cell*. In other words, it is assumed that the amount of spike-in is added per molecule of RNA *and* that each cell yielded the same number of RNA molecules. The latter is a particular issue for bulk RNA-Seq due to the technical difficulty of adding an appropriate amount of spike-in at a cell population level [59]. However, even when controlling for technical variation, cells may produce less total RNA in one of the experimental groups [38] or over time [41]. In this case, standardizing the spike-in to the *total amount of input RNA* will invalidate this assumption. Without standardizing the spike-in to the *total number of cells*, it is impossible to reclaim absolute abundances (i.e., in units of transcripts per cell) [11]. Even if it were possible to standardize spike-ins to the total number of cells, the interpretation may be difficult if the cells within a single batch produce varying amounts of total RNA.

Beyond ERCC spike-ins, several other spike-ins have been proposed. For RNA-Seq studies, example spike-ins include sequins [26, 12], control plasmid spiked-in genomes [64], and isoform-specific spike-in RNA variants [52]. For metagenomics studies, example spike-ins include exogenous bacteria [67] and sequins [27]. It is beyond the scope of this field guide to compare and contrast all of the different spike-ins. However, we must emphasize that *if the spike-ins are calibrated to the total weight of input RNA, they do not automatically normalize the data to absolute abundances*. The reason for this follows logically from how spike-ins work: when spike-ins are added at a

fixed proportion to an arbitrary mass of RNA, sequencing will return counts at the same fixed proportion. As such, spike-ins only tell us the *amount of RNA sequenced*. However, the term “absolute abundances” refers to the *amount of RNA present in the biological sample* (e.g., in units of transcripts/cell for RNA-Seq or bacteria/L for metagenomics). Therefore, spike-ins will normalize to absolute abundances if and only if the amount of RNA sequenced is equal to the amount of RNA present in the biological sample. Even if the difference between the absolute RNA and the input RNA – which we call  $\delta$  – is proportional, this  $\delta$  must be the same for *all* samples. Otherwise, the  $\delta$  becomes yet another a closure bias that could introduce systematic errors. In this case, spike-in “normalization” causes the same problem as TPM “normalization”: the analyst has transformed their old compositions into new compositions under the mistaken belief that the new compositions are absolute concentrations. Before using spike-in normalization, the analyst should critically evaluate their protocol to assess whether they can safely assume that  $\delta$  is fixed for all samples. On the other hand, a transformation with respect to an internal reference is not affected by global differences in  $\delta$ .

## Single-cell RNA sequencing

Single-cell RNA sequencing (scRNA-Seq) resembles bulk RNA-Seq, except that the RNA of individual cells are captured and barcoded separately prior to building the cDNA library [4]. This RNA capture step involves a non-exhaustive sample of the total RNA which acts as another closure operation to make the data relative. The sequencer would then re-close the already closed data. Interestingly, if the sequence libraries were then expressed in TPMs, the per-million divisor would act as yet another closure of the data. For these reasons, scRNA-Seq resembles other NGS count data in that each sample is a composition of relative parts. Like other NGS count data, it is impossible to estimate absolute RNA abundance without a per-cell spike-in reference.

scRNA-Seq analysis is described as being more difficult than bulk RNA-Seq analysis for two reasons. First, scRNA-Seq library sizes vary more between samples [40]. This is due to differences in the *capture efficiency of RNA extraction*, sequencing depth, and so-called “doublet” events where two cells get captured at once [40]. To address these differences in library size, the data are normalized by effective library size normalization or by reference normalization (via a set of house-keeping or spike-in transcripts). Effective library size normalization assumes that most genes are unchanged; this assumption is especially problematic for scRNA-Seq data because single-cell experiments study heterogeneous cell populations [39]. Reference normalization has limitations too. House-keeping genes may not have consistent expression at the single-cell level due to transcriptional bursting or tissue heterogeneity [39]. Meanwhile, scRNA-Seq spike-ins imply the same assumptions as bulk RNA-Seq: that the spike-ins and target sequences have the same *capture efficiency of RNA conversion* and that the spike-ins are *calibrated to the number of RNA molecules per cell*. The second assumption is problematic for scRNA-Seq because it implies that all cells were similarly affected by the *capture efficiency of RNA extraction* [39]. Since spike-ins are added to the lysis buffer, spike-in normalization can only reveal how much RNA was captured from the cell, not how much RNA was present in the cell: as such, spike-ins cannot normalize away differences in cell lysis efficiency (which are common, and an important cause of “dropout”) [32]. On the other hand, a transformation with respect to an internal reference is not affected by global differences in cell lysis efficiency. This is analogous to the discussion of  $\delta$  from the preceding sub-section.

Second, scRNA-Seq contains many zeros. Although some zeros are described as “biological zeros” (i.e., *essential zeros*) [73], most are described as “dropout zeros”. For “dropout zeros”, a zero is a missing value that occurs because the “mRNA molecules are not captured...at the same proportion” for all cells [4]. By this definition, “dropout zeros” are simply *count zeros* caused by non-exhaustive sampling. Since differences in cell lysis efficiency are an important cause of dropout, spike-ins cannot solve the dropout problem [32]. However, these “dropout” zeros are really no different than the under-sampling zeros found in metagenomics data (which are already handled by our pipeline [17]). However, if an analyst wishes to impute zeros, there exists imputation methods designed specifically for compositional data [43, 9].

## Discussion

Compositional data analysis (CoDA) provides a conceptual framework for studying relative data. In this paper, we present a collection of software tools designed for NGS count data that together



form a pipeline which unifies the analysis of all compositional data, including RNA-Seq, metagenomics, single-cell and spectrometric peak data. Unlike existing pipelines, ours does not seek to normalize the data to reclaim absolute abundances. Instead, it transforms the data with regard to a reference, allowing the analyst to study any relative data set without invoking the often untestable assumptions underpinning NGS data normalization.

The CoDA framework has evolved independently from much of the alternative techniques currently applied to NGS data. Interestingly, although not explicitly tailored for compositional data, the most rigorous of the NGS methods have converged on similar solutions for handling compositional bias. They rely on effective library size normalizations (and offsets) that make use of the (pseudo-counted) log-transformed data in a manner similar to log-ratio transformations. In CoDA, such transformations are explicitly derived to address the constrained nature of the data. From this perspective, explicit references and pairwise log-ratios apply to a broader range of experiments, including less well-controlled studies where effective library size normalizations may not work. The analysis of count compositions, especially the handling of low-count imprecision, has now reached a state of maturity that allows for NGS analysis without any loss of formal rigor.

An important aspect of CoDA is that it better quantifies the coordination between features than correlation, the latter of which is often spurious when the compositional constraint is ignored. Meanwhile, applying differential abundance analysis with respect to a reference remains valid even across the most widely varying conditions. For clustering and classification, the fully ratio-based Aitchison distance provides a superior inter-sample distance that is still under-appreciated in current applications. Last but not least, CoDA opens up new perspectives with respect to the integration of big multi-omics data sets where explicit references may play an important role in the future.

## **1 Declarations**

### **1.1 Ethics approval and consent to participate**

Not applicable.

### **1.2 Consent for publication**

Not applicable.

### **1.3 Availability of data and material**

All data and scripts are publicly available at <http://doi.org/10.5281/zenodo.3270954>.

### **1.4 Competing interests**

No authors have competing interests.

### **1.5 Funding**

Not applicable.

### **1.6 Authors' contributions**

TPQ outlined and drafted the field guide. TPQ, IE, GG, and MFR drafted the Selected Topics section. IE prepared the supplement. TPQ prepared the appendix. CN, MFR, and TMC supervised the project. All authors revised and approved the final manuscript.

### **1.7 Acknowledgements**

TPQ thanks Larry Croft for helpful discussions.

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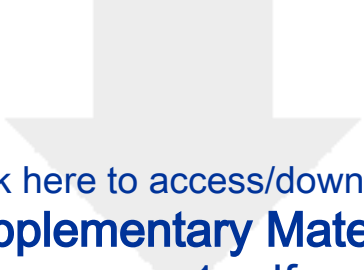
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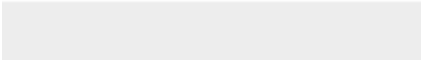



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July 10, 2019

Dear Editors,

Thank you again for granting us the opportunity to revise and resubmit our article titled, “A field guide for the compositional analysis of any-omics data”.

Below, we enumerate the specific changes that address the comments made by the reviewers.

We again thank the editor, and the reviewers, for their time.

Sincerely,

Thomas P. Quinn,  
Ionas Erb,  
Greg Gloor,  
Cedric Notredame,  
Mark F. Richardson, &  
Tamsyn M. Crowley

A detailed response to the reviewers is provided on following pages.

## Our response to Reviewer #1:

1. *Where are the "Methods" & "Results" sections? The whole paper reads like a review article and it is unclear from the text exactly what the authors have done here (as opposed to providing a summary of existing work in the field). Were any new experiments done? If so, the details should be provided. How was the software developed?*

This publication was stylized as a “workflow” paper that does summarize existing work in the field, but also presents a new reproducible analysis for readers to follow. We now include a “Methods” section that contains the sub-sections: “Overview of pipeline”, “Data acquisition”, “New analyses”, “Software contributions”, and “Benchmark validation”. In the “New analyses” section, we now write:

“In presenting this workflow, we perform a new analysis of the Jovanovic et al. data in order to learn how mRNA transcript abundance and protein abundance change in response to LPS stimulation. This includes a relative differential abundance analysis, an analysis of gene-gene coordination, and an analysis of differential gene-gene coordination. In addition, we integrate the two data types with a differential proportionality analysis to evaluate how mRNA stoichiometry differs from protein stoichiometry in response to LPS treatment. Unlike the original analysis presented by Jovanovic et al., we do not use transcripts per million (TPM) normalization. Rather, we argue that TPMs re-cast an already compositional data set as yet another compositional data set (just with a different denominator). In the Supplementary Information, we show how TPMs introduce systematic errors. This is because when a reference is not explicitly chosen, an arbitrary reference is still implicitly present. We also include an appendix that benchmarks how several zero handling procedures impact proportionality and differential proportionality analysis.”

In the “Software contributions” section, we now write:

“In preparing this workflow, we have made several contributions to the compositional data analysis software universe. First, we present the new `propr::aldex2propr` function that integrates the ALDEx2 and `propr` packages by calculating an average proportionality coefficient over ALDEx2-generated Monte Carlo instances. Second, we present the new `propr::updateCutoffs` function that permutes a false discovery rate across varying proportionality coefficient cutoffs. Third, we present the `propr::propd` function that implements the differential proportionality method described by Erb et al. (Erb et al. 2017). These new contributions make a complete compositional data analysis workflow possible.”

2. *It is noted that the authors published a review on a very similar topic last year (<https://academic.oup.com/bioinformatics/article/34/16/2870/4956011>); at first glance, it would seem that there is substantial overlap between the two papers.*

Indeed, this work is a spiritual successor to our review published last year. However, the motivation for this manuscript was not to re-review the literature: we set out to teach people how to perform a compositional data analysis, and to provide the tools for them to do it. As such, this manuscript is structured as a “workflow” paper. These have become increasingly common in bioinformatics research (e.g., <https://f1000research.com/articles/5-1281> or <https://f1000research.com/articles/5-2122>).

Nevertheless, there are several things that set this manuscript apart, for example:

- This manuscript includes a new and complete analysis of the Jovanovic et al. data set.
- This manuscript includes an estimate of the error in the published Jovanovic et al. measurements that comes about from ignoring compositional data.
- This manuscript discusses the zero replacement controversy and some solutions.
- This manuscript presents a compositional analysis where the reference is chosen because it is biologically meaningful, not because it is unchanged. This shows the reader how it is possible to draw coherent conclusions without assuming that there exists a discoverable set of non-DE genes.

- We present for the first time the `propr::aldex2propr` function which integrates the ALDEx2 and `propr` packages.
- We present for the first time the `propr::updateCutoffs` function which permutes a false discovery rate across varying proportionality coefficient cutoffs.
- We present for the first time an implementation of differential proportionality analysis (as `propr::propd`), along with methods for p-value calculation and visualization.
- In “Complex study design”, we describe for the first time how differential proportionality can be extended with a generalized linear model (glm).
- In “Vertical data integration”, we describe for the first time how differential proportionality could be used to integrate paired gene abundance and protein abundance data. We then perform this analysis on the Jovanovic et al. data and draw conclusions.
- We now include an appendix that benchmarks how several zero handling procedures impact proportionality and differential proportionality analysis.
- The review did not discuss vertical or horizontal data integration.
- The review did not discuss clustering or classification.
- The review did not discuss spike-ins.
- The review did not discuss single-cell RNA-Seq data.
- The review did not contain reproducible code for the readers to follow and use.

Together, these make our field guide a unique contribution to the literature. We hope the additional Methods sub-sections further clarify what sets this paper apart.

3. *A related concern is that the authors need to compare/benchmark their new software with previously published methods. It would be interesting to see how it performs compared to existing methods, eg. DESeq, edgeR, TMM, RUVg (to name only a few).*

We now explicitly reference previously published benchmark validation studies in a new sub-section “Benchmark validation”. In this sub-section, we write:

“Although the software tools presented here do not normalize the data, they can be benchmarked against conventional methods by invoking the assumption that the explicit reference performs a kind of “log-ratio normalization”. Under these conditions, ALDEx2 can identify DA with high precision in RNA-Seq data (Fernandes et al. 2014; Quinn et al. 2018), and control false positive rates in highly sparse 16S metagenomics count data (Thorsen et al. 2016). Meanwhile, proportionality analysis has been shown to outperform all 15 competing measures of association in single cell clustering tasks across 213 data sets (Skinnider et al. 2019). Although differential proportionality analysis has not yet been benchmarked, it is formally related to an analysis of variance (ANOVA), a foundational test in most biological research. As a statistical test for significance, it is valid wherever an ANOVA is valid. We also include an appendix that benchmarks how several zero handling procedures impact proportionality and differential proportionality analysis.”

4. *P 1, line 52 - should be "next-generation sequencing" (hyphenated) for consistency.*  
*P 2, lines 1-3. It is stated that all NGS applications involve alignment of reads to a reference. This statement ignores alignment-free methods, eg. k-mer based quantification and variant-calling. Whilst these may be beyond the scope of the paper, this should be stated upfront.*  
*P 6, line 21. The following sentence appears to contain a typo: "This figure illustrates how the interpretation of differential abundance with respect to the reference chosen". Please revise.*  
*P 12, line 15: "this latter procedures" - typo. Please revise.*  
*P 12, line 54. The authors state: "Moreover, NGS experiments almost always have many more features than samples". This statement would often not be true for single-cell RNA-seq experiments, which now analyse many thousands of cells.*

Thank you for these suggestions. We have made the recommended changes as follows:

- P 1, line 52 – Hyphen added.
- P 2, lines 1-3 – We changed “All” to “Most” and “aligned” to “mapped”.
- P 6, line 21 – It now reads, “This figure illustrates how the interpretation of differential abundance depends on the reference chosen.”
- P12, line 15 – Fixed.
- P 12, line 54 – We changed “almost always” to “often”.

5. *P 13, line 24: if mentioning the ERCC spike-ins, the authors should also mention other, more recent, synthetic spike-in controls for NGS, eg: Spliced synthetic RNA spike-ins for RNA-seq:*  
<https://www.nature.com/articles/nmeth.3958>  
<https://www.biorxiv.org/content/10.1101/080747v1.full>  
*Synthetic DNA spike-ins for genome sequencing:*  
<https://www.nature.com/articles/nmeth.3957>  
[https://jmd.amjpathol.org/article/S1525-1578\(16\)00046-5/fulltext](https://jmd.amjpathol.org/article/S1525-1578(16)00046-5/fulltext)  
*Synthetic microbial spike-ins for metagenome sequencing:*  
<https://www.nature.com/articles/s41467-018-05555-0>  
*P 13, lines 30-32. For this sentence - "Similarly, one could spike-in a known quantity of bacteria cells or synthetic plasmids to standardize the abundance of PCR-amplified microbiome samples." - the authors should also cite the following publication:*  
<https://microbiomejournal.biomedcentral.com/articles/10.1186/s40168-016-0175-0>

Thank you for the recommended readings. These challenged me to think more about the how spike-ins get used. Spike-ins are an important technological advance that may make it possible to get absolute abundances from NGS studies. However, to reclaim absolute abundances, they must get used properly. We now include another paragraph that further discusses the nuances of spike-in use:

“Beyond ERCC spike-ins, several other spike-ins have been proposed. For RNA-Seq studies, example spike-ins include sequins (Hardwick et al. 2016; Deveson et al. 2016), control plasmid spiked-in genomes (Sims et al. 2016), and isoform-specific spike-in RNA variants (Paul et al. 2016). For metagenomics studies, example spike-ins include exogenous bacteria (Stammler et al. 2016) and sequins (Hardwick et al. 2018). It is beyond the scope of this field guide to compare and contrast all of the different spike-ins. However, we must emphasize that *if the spike-ins are calibrated to the total weight of input RNA, they do not automatically normalize the data to absolute abundances*. The reason for this follows logically from how spike-ins work: when spike-ins are added at a fixed proportion to an arbitrary mass of RNA, sequencing will return counts at the same fixed proportion. As such, spike-ins only tell us the *amount of RNA sequenced*. However, the term “absolute abundances” refers to the *amount of RNA present in the biological sample* (e.g., in units of transcripts/cell for RNA-Seq or bacteria/L for metagenomics). Therefore, spike-ins will normalize to absolute abundances if and only if the amount of RNA sequenced is equal to the amount of RNA present in the biological sample. Even if the difference between the absolute RNA and the input RNA – which we call  $\delta$  – is proportional, this  $\delta$  must be the same for *all* samples. Otherwise, the  $\delta$  becomes yet another a closure bias that could introduce systematic errors. In this case, spike-in “normalization” causes the same problem as TPM “normalization”: the analyst has transformed their old compositions into new compositions under the mistaken belief that the new compositions are absolute concentrations. Before using spike-in normalization, the analyst should critically evaluate their protocol to assess whether they can safely assume that  $\delta$  is fixed for all samples. On the other hand, a transformation with respect to an internal reference is not affected by global differences in  $\delta$ .”

6. *P 14, lines 2-3. The authors should provide citation(s) for this sentence: "House-keeping genes may not have consistent expression at the single-cell level due to transcriptional bursting or tissue heterogeneity".*

Whoops! Fixed. This is stated in Lun et al. “Assessing the reliability of spike-in normalization for analyses of single-cell RNA sequencing data”. See: <https://genome.cshlp.org/content/early/2017/10/13/gr.222877.117>, paragraph 2.

7. *P 14, lines 3-6. The authors state: "Meanwhile, scRNA-Seq spike-ins imply an additional assumption beyond the two assumptions for bulk RNA spike-ins: they assume that the spike-ins and endogenous transcripts are similarly affected by the capture efficiency of RNA extraction [36], in that they are both equally affected by the technical biases of single-cell RNA extraction."*  
*I'm not sure that I understand this point. Aren't RNA spike-ins added to samples after RNA extraction; if so, how can they be affected by RNA extraction?*

Thank you. This is a nuanced point, and I failed to explain myself clearly. I struck this sentence and replaced it with the following text:

“Meanwhile, scRNA-Seq spike-ins imply the same assumptions as bulk RNA-Seq: that the spike-ins and target sequences have the same *capture efficiency of RNA conversion* and that the spike-ins are *calibrated to the number of RNA molecules per cell*. The second assumption is problematic for scRNA-Seq because it implies that all cells were similarly affected by the *capture efficiency of RNA extraction* (Lun et al. 2017). Since spike-ins are added to the lysis buffer, spike-in normalization can only reveal how much RNA was captured from the cell, not how much RNA was present in the cell: as such, spike-ins cannot normalize away differences in cell lysis efficiency (which are common, and an important cause of “dropout”) (Kolodziejczyk et al. 2015). On the other hand, a transformation with respect to an internal reference is not affected by global differences in cell lysis efficiency.”

8. *P 14, lines 14-15. The authors state that "dropout zeros" are caused by "the stochastic nature of gene expression...at the single-cell level".*  
*I doubt that this is correct; if stochastic gene expression led to a particular cell not expressing a given gene at a certain time, I would've thought that this would be a biological zero, not a dropout zero.*

Thank you. I re-reviewed the literature and agree that this definition is incorrect. I struck this sentence and replaced it with the following text:

“For “dropout zeros”, a zero is a missing value that occurs because the “mRNA molecules are not captured...at the same proportion” for all cells (AlJanahi et al. 2018). By this definition, “dropout zeros” are simply *count zeros* caused by non-exhaustive sampling. Since differences in cell lysis efficiency are an important cause of dropout, spike-ins cannot solve the dropout problem (Kolodziejczyk et al. 2015).”

## Our response to Reviewer #2:

1. *Omics analysis, and RNAseq in particular, account for the vast majority of current sequencing projects, and there are a number of popular analysis pipelines and R packages already that do a good job of analyzing this type of data in statistically sound ways. While this method is a useful addition to other analysis methods, the authors don't offer any reasons why this method should be chosen over any other, or even mention other methods. I don't necessarily think the authors need to show EdgeR or DESeq2 results or provide benchmarking (although that would be nice), however some discussion of what sets this pipeline apart from these other well known methods is needed.*

We agree. We now explicitly reference previously published benchmark validation studies in a new sub-section "Benchmark validation". In this sub-section, we write:

"Although the software tools presented here do not normalize the data, they can be benchmarked against conventional methods by invoking the assumption that the explicit reference performs a kind of "log-ratio normalization". Under these conditions, ALDEx2 can identify DA with high precision in RNA-Seq data (Fernandes et al. 2014; Quinn et al. 2018), and control false positive rates in highly sparse 16S metagenomics count data (Thorsen et al. 2016). Meanwhile, proportionality analysis has been shown to outperform all 15 competing measures of association in single cell clustering tasks across 213 data sets (Skinnider et al. 2019). Although differential proportionality analysis has not yet been benchmarked, it is formally related to an analysis of variance (ANOVA), a foundational test in most biological research. As a statistical test for significance, it is valid wherever an ANOVA is valid. We also include an appendix that benchmarks how several zero handling procedures impact proportionality and differential proportionality analysis."

2. *The introduction, overview and selected topics, in particular are very well written and accessible by researchers with little or no experience in -omics analysis. The authors have done a great job explaining why RNAseq data is difficult to analyse appropriately and have provided examples and intuition for the reader. To make the entire field guide equally useful to a relative novice, I would like to see this level of explanation added to several of the other sections. In particular,*
  - *in Part 1, the explanation for how zeros can differ is great, but there is little discussion of why the reader would choose to do this before analysis or as part of the analysis*
  - *in Part 2a 'The log-ratio transformation', the differences between several types of transformation are well-explained, however, with the exception of ilr, there are no guidelines for why the reader would choose one over another. This information would also be useful on page 12, line 47 where it is made clear that the choice of reference determines whether the data is interpretable.*
  - *in instances where you show multiple lines of code, as in lines 38-44 on page 5, it would be useful to provide more comments to explain the function of the code*
  - *line 16, page 12 "This latter procedures implies a reference and must get interpreted accordingly." Why does that metric imply a reference, and how does that change the interpretation?*

- Part 1: Please see our response to Comment 8.

- Part 2a: We added another paragraph to Part 2a: “Each transformation implies its own reference(s). In most practical settings, the choice of transformation will depend on the preferred interpretation. An analysis of **clr** data will tell you how genes (or OTUs) behave relative to the per-sample average. An analysis of **alr** and **malr** data will tell you how genes (or OTUs) behave relative to one or more explicitly-chosen internal references. An analysis of **iqlr** data will tell you how genes (or OTUs) behave relative to the per-sample inter-quartile (“robust”) average. In a compositional framework, none of these are normalizations: each new variable is a log-ratio of the original variable divided by the reference, and therefore should get interpreted as a kind of log-fold change. Although the difference between transformation and normalization may seem subtle, it can have a profound impact on the conclusions drawn from the analysis. Although the temptation will exist, one must never confuse the transformed data with absolute abundances.”
  - We added more comments to all code chunks.
  - line 16, page 12: We now replace this line with, “The  $\phi_s$  proportionality metric, like the  $\rho_p$  proportionality metric, is defined for **clr**-transformed data. If the geometric mean center changes drastically across samples, some proportional pairs may not be proportional in an absolute sense. We refer the reader to the sub-section, “Proportionality analysis with **propr**”, for further explanation.”
3. *The code and data provided by the authors via zenodo has several minor errors, and also doesn't produce figure 3 as shown in the text. For Figure 3, the figure isn't printed out as a jpg like the others, which is a minor concern, however the code itself (line 120) seems to produce a plot with an entirely different topology than the one shown in the article.*

Figure 3 in this manuscript is a 3D rendering of the figure from the supplemental script. However, we have now updated the supplemental script to produce the same 3D figure.

4. *Lines 31, 32 and 33 don't run as written, and need to be changed to 'output = "p-counts"'*

Ah, yes. This is due to a recent **zCompositions** package update. Fixed.

5. *The code would be much more user friendly if all of the library() calls were moved to the top of the R script so the user can tell what packages need to be installed. Even better would be to provide the install code as if statements or comments in the first few lines of the script file*

We agree. Fixed.

6. *The filenames used in the code don't match the ones used in the code callouts in the text, which is confusing*

We agree. The filenames now agree.

7. *JovanovicMsML-counts.csv (massml) is a provided datafile, and is loaded in, subset and transposed in the R code, but doesn't get used for any analysis or explained anywhere in the code or text*

Fixed. We no longer import unused files.



8. *It is very unclear when and why the authors have chosen to use the zero replaced versus raw data. In both the text and R code, they begin the analysis by using zCompositions to replace zeros in the data and make the matrix 'rnaseq.no0'. However in the next step, they use the raw data as input for ALDEx2. Since ALDEx2 can do it's own zero handling and arguably does a better job, this seems like a reasonable choice, however it makes it unclear why the zCompositions step was included. In subsequent steps, the authors use propr for both the transformation dependent and independant analyses, and note that propr has a function for ingesting the ALDEx2 simulated instances and using it's superior zero handling, however in both propr steps they use the rnaseq.no0 matrix to run the analysis. There doesn't seem to be any reason to do this, or any comparison to show that the rnaseq.no0 matrix and ALDEx2 simulated instances produce similar results. Unless there are specific instances where the zCompositions zero handling gives better results, it seems like a superfluous step in the pipeline. It should either be dropped, or have explanation added.*

We now include an appendix that benchmarks how several zero handling procedures impact proportionality and differential proportionality analysis.

We now include an explanation for why zCompositions is included in the workflow: “Many compositional software tools have their own built-in zero handling procedures. Although zCompositions is not necessarily better than these built-in procedures, we recognize that removing zeros right away has a practical advantage: by using zCompositions in combination with a log-ratio transformation, analysts can apply most conventional analyses to their compositional data right away. Since zCompositions empowers readers to use methods beyond the ones presented here, we decided to include it as the first part of our field guide.”

9. *On page 13, lines 41-43 the last sentence of the paragraph is confusing. It might help to break it into two sentences, or to specify that the 'even then' is referring back to the spike-in.*

Thank you. We agree. We added a second sentence that reads: “Even if it were possible to standardize spike-ins to the total number of cells, the interpretation may be difficult if the cells within a single batch produce varying amounts of total RNA.”

10. *In the Complex study design section, the authors suggest "modeling each pairwise log- ratio outcome as a function of any model.matrix". Wouldn't this pose a multiple testing problem? How would you address that given that it is more difficult to devise an appropriate simulation sampling strategy on complex designs?*

Each pairwise log-ratio is a unique test that yields one p-value. Therefore, it is absolutely necessary to adjust the false discovery rate for multiple testing. We now make this clear in the text: “When testing the significance of multiple log-ratio pairs, it is absolutely necessary to correct the p-value for multiple testing, for example by using the `p.adjust` function in R.”

For  $p$  genes, there are about  $p^2/2$  log-ratios. Yet, we have found that many pairs remain significantly differentiated, even after a conservative FDR adjustment. Note that an FDR-adjustment is built into the `propr::propr` function.

11. *If it is possible to alter the colors, Figure 3 would be much easier to read (and more color blind friendly) if the lines were black and the nodes were the only colors.*

Sorry, this color scheme is hard-coded into the software package.

**Errata:**

1. We changed “Second, it is assumed that the same amount of spike-in is added per total amount of RNA” to read more clearly: “Second, it is assumed that the spike-ins are *calibrated to the number of RNA molecules per cell*. In other words, it is assumed that the amount of spike-in is added per molecule of RNA *and* that each cell yielded the same number of RNA molecules.”