Gene expression
BSDE: barycenter single-cell differential expression for case–control studies

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Abstract

Motivation: Single-cell sequencing brings about a revolutionarily high resolution for finding differentially expressed genes (DEGs) by disentangling highly heterogeneous cell tissues. Yet, such analysis is so far mostly focused on comparing between different cell types from the same individual. As single-cell sequencing becomes cheaper and easier to use, an increasing number of datasets from case–control studies are becoming available, which call for new methods for identifying differential expressions between case and control individuals.

Results: To bridge this gap, we propose barycenter single-cell differential expression (BSDE), a nonparametric method for finding DEGs for case–control studies. Through the use of optimal transportation for aggregating distributions and computing their distances, our method overcomes the restrictive parametric assumptions imposed by standard mixed-effect-modeling approaches. Through simulations, we show that BSDE can accurately detect a variety of differential expressions while maintaining the type-I error at a prescribed level. Further, 1345 and 1568 cell type-specific DEGs are identified by BSDE from datasets on pulmonary fibrosis and multiple sclerosis, among which the top findings are supported by previous results from the literature.

Availability and implementation: R package BSDE is freely available from doi.org/10.5281/zenodo.6332254. For real data analysis with the R package, see doi.org/10.5281/zenodo.6332566. These can also be accessed thorough GitHub at github.com/mqzhanglab/BSDE and github.com/mqzhanglab/BSDE_pipeline. The two single-cell sequencing datasets can be download with UCSC cell browser from cells.ucsc.edu/?ds=ms and cells.ucsc.edu/?ds=lung-pf-control.

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Supplementary information: Supplementary data are available at Bioinformatics online.

1 Introduction

Single-cell RNA sequencing (scRNAseq) aims at profiling the gene expression in every cell of a given sample, by sequencing their genomes, transcriptomes or proteomes. As such, it overcomes the limitation of the bulk analysis and enables researchers to inspect the spatial-temporal details of a biological procedure with high resolutions. With this technology, the type and the life-cycle status of each cell can be observed and traced. Due to the complex nature of biological procedures, to better understand the mechanisms behind, single-cell sequencing is instrumental in detecting cell heterogeneity, finding rare cell types, selecting specialized biomarkers and characterizing rare molecular features at the cellular level (Giladi and Amit, 2018).

One common strategy for understanding the intrinsic and extrinsic biological processes in scRNAseq is to detect the differentially expressed (DE) genes. Through such analyses, the signal from a certain cell type can be isolated and examined. Yet, there are some challenges. For example, scRNAseq data are highly heterogeneous and usually come with a large number of zero counts, which complicates statistical modeling and analysis.

In a bulk RNAseq analysis, the overall expression level is point estimated by a count (Category A of Fig. 1). Alternatively, the single-cell data, which contain more information, are represented as an empirical distribution over counts, where each cell contributes a count (Categories B–D of Fig. 1). Naturally, there could be two levels of comparison: cell level and individual level.

First, two cell types from an individual can be compared through their distributions. There are already quite a few methods available from the literature for this purpose, including off-the-shelf statistical tests, such as Mann–Whitney U-test, as well as purpose-built parametric and non-parametric methods, such as SCDE (Kharchenko et al., 2014), MAST (Finak et al., 2015), scDD (Korthauer et al., 2016), EMDomics (Nabavi et al., 2016), D3E (Mengqi Zhang et al., 2016), Monocle (Trapnell et al., 2014; Qiu et al., 2017), SINCERA (Guo et al., 2015), edger (Robinson et al., 2010), DESeq2 (Love et al., 2014), DEsingle (Miao et al., 2018) and
SigEMD (Wang and Nabavi, 2018), to name a few. In terms of the taxonomy given in Figure 1, most of them fall into Category B, except for those originally designed for bulk analysis (edgeR and DESeq2) that belong to Category A.

Further, DE analysis can be performed between two groups of individuals, which is the focus of our paper. In contrast to bulk analysis, existing methods for this type of comparison are scarce; see Zhang et al. (2022) for a recent proposal. Some earlier proposals, such as MUSCAT (Crowell et al., 2020) and aggregateBioVar (Thurman et al., 2021), are based on summarizing counts from certain single-cell sequences into a “pseudo-bulk” RNASeq (Category C of Fig. 1). Then, methods from Category A are immediately applicable to these summarized counts; however, as we will see from simulations, summarization discards distributional information and hence cannot detect nuanced differential expressions. At the moment, arguably the most common approach towards such a comparison is based on mixed-effect models. For example, the hurdle model (e.g., Finak et al., 2015), which specifies a logistic regression model for the expression rate and a linear model for the logarithmic non-zero expression, can be fitted with a mixed effect. Fixed effects are fitted on the case/control indicator (along with other covariates), and random effects are fitted by introducing individual-level random intercepts; see Velmeshev et al. (2019, Supp. Mat.) for such an analysis on autism data. Yet, performing valid statistical inference (e.g., testing DE at a prescribed significance level) for the fitted mixed-effect model can be challenging. As we will show in Section 3.2, due to the presence of random effects, standard likelihood ratio tests are typically inapplicable to these settings. Further, the suitability of the hurdle model is limited by its parametric assumptions, which may not hold in real data.

To adapt DE analysis to conventional case-control studies, we propose barycenter single-cell differential expression (BSDE), which performs comparison in two stages. The first stage is to aggregate individual-level distributions into a case group distribution and a control group distribution by finding the corresponding Wasserstein barycenters. The second stage is to compare the two group-level distributions in terms of their Wasserstein distance. The Wasserstein barycenter and distance are defined nonparametrically in terms of optimal transportation of probability measures, which does not rely on restrictive parametric assumptions. The type-I error can be readily controlled with a permutation P-value or its Monte Carlo approximation.

It is worth mentioning that BSDE, by design, differentiates itself from other methods by comparing distributions instead of simple summary statistics of distributions (e.g., mean). To illustrate the differences, we propose a taxonomy of current methods as shown in Figure 1. We also note that recently Wasserstein distance (or the earth mover’s distance) has been introduced for differential expression analysis; see, e.g., Nabavi and Beck (2015), Nabavi et al. (2016) and Wang and Nabavi (2017, 2018), which employ the distance as a test statistic for comparing distributions. However, we argue that our method goes one step further in utilizing the tools from optimal transportation—the case and control distributions themselves are aggregated from the individual level as their respective Wasserstein barycenters.

2 Materials and methods

We propose BSDE, a nonparametric procedure based on optimal transportation of probability distributions. For two distributions, their Wasserstein distance (also known as the earth mover’s distance) is defined as the minimal cost (in terms of some cost/loss function) of ‘transporting’ the mass of one distribution to the other. Throughout, we will focus on the Wasserstein distance \( W_2 \) between distributions \( P \) and \( Q \), defined as

\[
W_2(P, Q) = \inf_{
\begin{align*}
\gamma & 
\in \mathcal{P}(P\times Q) \\
\mathbb{E} 
\left[
\|X - Y\|^2 
\right] 
\end{align*}
\]
where the loss function $c(x, y) = ||x - y||^2$ is the square Euclidean distance. The set $\Pi(P, Q)$ refers to the set of couplings between probability distributions $P$ and $Q$, i.e., the set of bivariate distributions with $P$ and $Q$ as margins. Although other loss functions $c(x, y)$ can be considered in principle as well, the square loss is a safe default choice (similar to being the default for regression problems) and its properties are well studied.

Defined as such, it can be argued that the Wasserstein distance is more informative than other definitions of distance or divergence (e.g. total variation, Kullback–Leibler) between distributions as it takes account of the metric information captured by the loss function, which in our context, translates to the difference in expression levels.

This further induces a notion of average for a set of distributions. Let $P_1, \ldots, P_n$ be a collection of distributions on metric space $\mathcal{X}$. Their Wasserstein barycenter is defined as the minimizer to

$$
\min_{\mu} \sum_{i=1}^{n} W_2^2(\mu, P_i), \quad \mu \in \mathcal{P}(\mathcal{X}),
$$

where $\mathcal{P}(\mathcal{X})$ is the set of probability measures on $\mathcal{X}$. Note that the square loss is strongly convex and the Wasserstein barycenter is uniquely defined (Agueh and Carlier, 2011, Proposition 3.5). Compared to the arithmetic average of distributions, the Wasserstein barycenter, by additionally using the metric information, better aligns with our intuition of an averaged distribution; see Figure 2B for an illustration.

Wasserstein distance and barycenter enjoy many appealing properties (Villani, 2009) and find applications in various domains, including image processing (Gramfort et al., 2015), computer graphics (Rabin et al., 2011), and very recently, computationally biology (Schiebinger et al., 2019).

As depicted in Figure 2C, BSDE proceeds in two stages.

1. Distribution aggregation. Suppose there are $l$ cases and $n$ controls. Let $P_1, \ldots, P_l$ be the empirical histograms of case data and $Q_1, \ldots, Q_n$ be the empirical histograms of control data. The histograms are typically built on the count data under the commonly used $x \rightarrow \log(x + 1)$ transform to reduce skewness. Further, to ease computation, the histograms are built with a common set of breakpoints. Let $\hat{P}$ and $\hat{Q}$ be the respective Wasserstein barycenters:

$$
\hat{P} = \arg\min_{\mu} \sum_{i=1}^{l} W_2^2(\mu, P_i),
\hat{Q} = \arg\min_{\nu} \sum_{j=1}^{n} W_2^2(\nu, Q_j),
$$

where $\mu$ and $\nu$ are minimized over 1D probability distributions, which, without loss of generality, can also be restricted to the set of histograms with the given breakpoints. In practice, to speed up computation, entropy-regularized versions of $\hat{P}$ and $\hat{Q}$ are computed with the Sinkhorn–Knopp matrix scaling algorithm (Benamou et al., 2015), for which we use the implementation provided by Python package POT (Flamary et al., 2021).

2. Distribution comparison. Our test statistic is simply taken to be the Wasserstein distance between the two aggregated histograms:

$$
\lambda = W_2(\hat{P}, \hat{Q}),
$$

which is computed with the fast Greenkhorn algorithm (Altschuler et al., 2017).

We reject the null-hypothesis of no differential expression between case and control for larger values of $\lambda$. We use permutation to control the type-I error. Under the null hypothesis, the case and control labels can be permuted without changing the distribution of statistic $\lambda$. In fact, under the null, the statistics computed under permutations are exchangeable. The $P$-value can be approximated by taking a large number of random permutations. Let $\lambda^{(i)}, i = 1, \ldots, N$ be the statistic computed from $N$ (e.g. $N = 1000$) random permutations. The $P$-value is approximated as

$$
p = \frac{1 + \sum_{i=1}^{N} I(\lambda^{(i)} \geq \lambda)}{N + 1},
$$

where $I(\cdot)$ is the indicator function.

The method is implemented in R package BSDE, available from https://github.com/mqzhanglab/BSDE.

3 Results

In what follows, we compare BSDE with a number of competing methods on simulated and real datasets.
3.1 Methods for comparison
In view of the taxonomy given by Figure 1, we consider the following methods for comparison.

1. MAST (glm, Finak et al., 2015), a mixed-effect model for the cell-level DE analysis. We fit the model in R with
   \[ \text{MAST::glm(formula = \sim \text{diagnosis}, \text{scRNAseq} \text{data})} \]
   and conduct inference with \text{MAST::lrtTest(b0, ‘diagnosis’)}, where \text{diagnosis} represents the case/control label.

2. DESeq2 (Love et al., 2014), a state-of-the-art method for bulk RNAseq analysis. We treat the sum of raw counts from all cells of each individual as a ‘bulk count’.

3. DESeq2 (Love et al., 2014), a state-of-the-art method for bulk RNAseq analysis. We treat the sum of raw counts from all cells of each individual as a ‘bulk count’.

4. VarBind (Mann–Whitney U-test on the subject (pseudo-bulk) level).

5. MAST (mixed effect, Finak et al., 2015), a slightly variation of the previous method. The model is fitted with
   \[ \text{MAST::glm(formula = \sim \text{diagnosis} + \text{1-ind}, \text{scRNAseq} \text{data})} \]
   and the test is called with \text{MAST::lrtTest(b1, ‘diagnosis’)}. Here, \text{ind} represents the individual label and \text{diagnosis} represents the case/control label. Though not recommended by the software manual, this type of analysis is seen from the literature (e.g. Schirmer et al., 2019, Velmeshev et al., 2019).

3.2 Simulations
3.2.1 Simulation protocol
The simulation protocol is illustrated in Figure 2D. We generate 3000 genes from a particular type of cell of \( n \) n cases and \( n \) n controls from which we select \( m \) m cells (m = 5, 10, 20), with each subject having \( m \) m cells (m = 50, 100, 200, 400). We simulate the basic parameters (mean, dispersion and dropout) by drawing from a distribution fitted with a reference dataset, the scRNAseq data on single-nuclei genomics of autism (Velmeshev et al., 2019). Given a set of basic parameters, expression levels are simulated from a zero-inflated negative binomial model.

More concretely, considering simulating the expression levels of gene \( i \). For each individual \( j \), we estimate parameters \( \mu_j \) (mean), \( \phi_j \) (dispersion), \( z_j \) (dropout rate) and \( \sigma_j \) (cell level variability) from the reference dataset (on the logarithmic scale). To capture the variability of these parameters across individuals, we fit a four-variate Gaussian distribution. Then, the expression level of gene \( i \) on the \( k \)th cell of individual \( j \), denoted by \( Y_{ijk} \) in Figure 2D, is simulated from a zeroinflated negative binomial model \( \text{ZINB}(\mu_{ijk}, \phi_j, z_j) \), where \( \mu_{ijk} \) (mean), \( \phi_j \) (dispersion), \( z_j \) (dropout rate) and \( \sigma_j \) (cell level variability) are drawn from the four-variate Gaussian (on the logarithmic scale) and further \( \mu_{ijk} \sim \mathcal{N}(\mu_i, \sigma_i^2) \) for each cell \( k \).

3.2.2 Types of differential expression
We introduce four types of DE in our simulated data, where the size of each type is controlled by a factor \( r \); see Figure 2A.

1. Mean DE: The size factor is varied from \( \mu = 1.1, 1.2, 1.5, 2.4 \). Parameters \( (\mu, \phi, z) \) are specified relative to \( (\mu, \phi, z) \) as
   \[ \mu = \frac{\mu}{r}, \quad \phi = \frac{\phi}{r}, \quad z = z \]
   such that case and control variances are the same.

2. Variance DE: The size factor is varied from \( \sigma = 1.1, 1.2, 1.5, 2.4 \). Parameters \( (\mu, \phi, z) \) are specified relative to \( (\mu, \phi, z) \) as
   \[ \mu = \mu, \quad \phi = \frac{\phi}{r}, \quad z = z \]
   and \( z = z \) such that the mean remains the same.

3. Proportion DE: The size factor is varied from \( \frac{\mu}{r} = 0.6, 0.7, 0.8, 0.9 \). Counts are simulated from either mixture
   \[ \text{rZINB}(\mu, \phi, z) + (1 - r) \text{ZINB}(\mu, \phi, z) \]
   or the component-swapped \( (1 - r) \text{ZINB}(\mu, \phi, z) + r \text{ZINB}(\mu, \phi, z) \).

4. Multimodality DE: The size factor is varied from \( r = 0.1, 0.2, 0.3, 0.4 \). Counts are simulated from either a two-component mixture \( \frac{1}{2} \text{ZINB}(\mu, \phi, z) + \frac{1}{2} \text{ZINB}(\mu, \phi, z) \), or its single-component counterpart \( \text{ZINB}(\mu, \phi, z) \). The parameters are related by
   \[ \mu = \frac{\mu}{r}, \quad \phi = \frac{\phi}{r}, \quad z = z \]
   such that the mean and the variance are unchanged.

3.2.3 Results
We compare BSDE with competing methods in terms of (i) the type-I error under the null hypothesis of no differential expression between case and control and (ii) the detection power under the four types of differential expression considered. The significance level is chosen to be 0.05. The power is defined as the proportion of \( P \)-values no more than 0.05. For more details on the simulation, the reader is refereed to https://github.com/mqzhanglab/BSDE_pipeline.

The results are presented in Figure 3, where rows correspond to different settings of \( n \times m \); additional settings can be found in the Supplement. From the left panel, we can see that BSDE and the subject-level Mann–Whitney U-test are the only two methods that control the type-I error at the nominal level. In particular, as mentioned in Section 1, the uncorrected likelihood ratio-based inference for MAST fails to control the type-I error due to the presence of random effects; the model-based inference for DESeq2 is also found to exceed the nominal level possibly due to misspecification of the parametric model.

In terms of the detection power, strictly speaking, it is only fair to compare methods that maintain the type-I error guarantee. The subject-level Mann–Whitney test hardly has any power. In contrast, BSDE seems to be able to detect differential expression with excellent power in all cases. In particular, we find that the differential expression in variance seems challenging to most of the methods—those based on bulk or summary ‘pseudo-bulk’ counts are unable to detect these signals. The only other method that seems powerful is MAST (mixed effect), which unfortunately does not tightly control the type-I error.

3.3 Analysis of pulmonary fibrosis and multiple sclerosis
To demonstrate the use of BSDE on real data, we take two public, case-control study datasets from single-nucleus sequencing: the pulmonary fibrosis (PF) dataset (Habermann et al., 2020, GSE135893) and the multiple sclerosis (MS) dataset (Schirmer et al., 2019, PRJN544731). The PF dataset contains 20 cases and 10 controls; the MS dataset contains 12 cases and 9 controls. The data were collected with 10× Genomics Single-Cell 3’ system and were preprocessed with software CellRanger. The cell types and meta-information were annotated. We imported data from the matrices with Unique Molecular Identifier counts, with additional normalization and log transformation.

3.3.1 Results on PF
We summarize the results in Figure 4. Figure 4A shows the subject-level distributions of the number of cells from case and control samples. There is no significant difference (\( P \)-value = 0.16,
two-sided Bonferroni-corrected t-test) in the numbers of cells between case and control. Figure 4B displays the differentially expressed genes (DEGs) in epithelial cells detected by BSDE. Most signals are found in epithelial cell types SCG3A2+, AT2, Basal, MUC5B+ and AT1. Among these cell types, we perform Gene Ontology (GO) enrichment analysis; the significant pathways are reported in Figure 4C. Further, within each of the aforementioned cell types, in Figure 4D, we contrast the Wasserstein barycenter...
distributions between case and control for the top four DEGs. Only those genes with a median expression level above four are ranked.

Support from the literature. In light of these findings, we review previous results from the literature on the identified cell types and DEGs related to PF; see Table 1 and Table S1 (Supplementary Materials). In particular, DEG-enriched cell types SCGB3A2 (Cai and Kimura, 2015; Parimon et al., 2020; Wu et al., 2020), MUC5B (Peljto et al., 2013; Seibold et al., 2011; Zuo et al., 2020) and Basal (Carraro et al., 2020; Richelh et al., 2017) have been previously reported.

3.3.2 Results on MS

The results are summarized in Figure 5. Figure 5A shows the subject-level distributions of the number of cells from case and control samples. Figure 5B displays the top eight cell types with the largest number of differentially expressed genes (DEGs) detected by BSDE. Figure 5C shows the total number of DEGs identified by each method. Right: the percentage of identified DEGs that overlap with the GWAS findings reported in Patsopoulos et al. (2019). Figure 5D, we contrast the case and the control Wasserstein barycenter distributions for the top four differentially expressed genes (log-transformed expression). Support from the literature. MS is one of the most common demyelinating diseases of the central nervous system. BSDE successfully detects cell-type-specific DEGs in L2-L3 EN, OL-A, IN-VIP, astrocytes, microglia and OPC, whose pathological roles have been established in the literature; see Table 2 and Supplementary Table S2 for more details. Additionally, we correlate the DEGs identified by BSDE and other methods with those reported by Patsopoulos et al. (2019) from a GWAS study. In that study, the International Multiple Sclerosis Genetics Consortium identified more than 233 MS risk loci from more than 47,000 cases and 68,000 controls; see Figure 5C. For the aforementioned cell types where BSDE finds the strongest signal, the findings seem to achieve a high percentage of overlap with the GWAS study.

4 Discussion

Traditionally, scRNAseq datasets are collected from many cells of different cell types, but from only a few individuals, due to the high cost of sequencing and technical limitations. Consequently, most methods developed for scRNAseq analysis are focused on differential expression across cell types, instead of that between case and control individuals. However, as single-cell sequencing becomes cheaper and easier to use, an increasing number of datasets from case-control studies, especially those related to complex human diseases such as autism (Velmeshev et al., 2019), PF (Habermann et al., 2020) and MS (Schirmer et al., 2019), are now available to us. Our
Microglia

Microglial activation is associated with tissue injury in the later stage of MS. They form a glial scar when the demyelination is completed at the later stage of MS.

Astrocytes

The astrocytes are active for the lesion formation at the early stage of MS. They play a role in the remyelination and disease remission.

OPCs

OPCs to mature oligodendrocytes, which are required for remyelination and disease remission.

Oligodendrocytes

Oligodendrocytes get the H₂O₂ produced from activated microglia through oxidative burst, and accumulate toxic Fe²⁺, which leads to the cell death and release of more Fe³⁺ to the environment.

IN-VIP

The experimental autoimmune encephalomyelitis (EAE) in mouse is an inflammatory autoimmune demyelinating disease of the central nervous system, which shares similarity to MS pathologically and clinically. Neuropeptide VIP protects against EAE by downregulating the inflammation and T-helper type-1 driven autoreactive response of MS.

Table 2. Previous reports of DEG-enriched cell types in MS identified by BSDE

| Cell type | Previous reports | References |
|-----------|------------------|------------|
| OL-A      | Oligodendrocytes get the H₂O₂ produced from activated microglia through oxidative burst, and accumulate toxic Fe²⁺, which leads to the cell death and release of more Fe³⁺ to the environment. | Lassmann et al. (2012) and Nikić et al. (2011) |
| Astrocytes| The astrocytes are active for the lesion formation at the early stage of MS. They form a glial scar when the demyelination is completed at the later stage of MS. | Ponath et al. (2018) |
| OPC       | MS is associated with the inhabitation of the differentiation of OPCs to mature oligodendrocytes, which are required for remyelination and disease remission. | Deshmukh et al. (2013) and Boyd et al. (2013) |
| Microglia | Microglial activation is associated with tissue injury in progressive MS. | Princeas et al. (2001) |
| IN-VIP    | The experimental autoimmune encephalomyelitis (EAE) in mouse is an inflammatory autoimmune demyelinating disease of the central nervous system, which shares similarity to MS pathologically and clinically. Neuropeptide VIP protects against EAE by downregulating the inflammation and T-helper type-1 driven autoreactive response of MS. | Miller et al. (2010) |

A major challenge of such analysis is to compare individual-level distributions between case and control. Traditionally, parametric models (e.g., log-normal, Poisson, zero-inflated negative binomial) are developed to fit these distributions, through which the comparison can be performed with parametric two-sample tests. However, the control of type-I error is not guaranteed if the model is misspecified, or when a naive likelihood ratio test is applied to mixed-effect models (see Section 3.2). In fact, misspecification is highly likely for real datasets, where batch effects, heterogeneity and dropouts are frequently observed. Through a fully nonparametric approach based on permutation tests and optimal transport, our method is free from these issues. Further, subject-level distributions within case/control are aggregated via Wasserstein barycenter, a type of distributional averaging that takes account of the metric information in data, i.e., the difference in expression levels. Compared to other types of averaging (e.g., arithmetic) that ignore the metric information, the resulting aggregated distributions are much more informative; see Figures 4D and 5D. Additionally, BSDE is computationally affordable thanks to recent developments of fast algorithms for (entropy-transport) optimal transport (Altschuler et al., 2017; Cuturi, 2013; Cuturi and Doucet, 2014).

Data-driven methods for identifying differential expression provide important guidelines by suggesting candidate genes for further experimental studies. To this end, a short list of key genes is more valuable than a long list of irrelevant genes. With its nonparametric flexibility, strict type-I error guarantee and excellent detection power, BSDE is applicable to a wide range of DE analyses for case–control studies.

Financial Support: none declared.

Conflict of Interest: none declared.

Data availability

The two single-cell sequencing datasets analyzed in the paper can be downloaded with UCSC cell browser from cells.ucsc.edu/?ds=ms and cells.ucsc.edu/?ds=lung-pf-control.

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