`methyvim`: Targeted and Model-free Analysis of Differential Methylation in R

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Abstract We present `methyvim`, a general algorithm for the nonparametric estimation of treatment/exposure effects on DNA methylation at CpG sites, complete with straightforward statistical inference for such estimates. This approach leverages variable importance measures, a class of parameters that arise in causal inference and are defined in such a manner that they provide targeted estimates of the relative importance of individual CpG site with respect to a binary exposure/treatment assigned at the level of subjects. Additionally, this procedure is computationally efficient; incorporating a preliminary screening step to isolate a subset of sites for which there is cursory evidence of differential methylation and a multiple testing correction step to control the False Discovery Rate as if all sites were tested. This technique for analysis of differentially methylated positions provides an avenue to move beyond linear models without the loss of interpretable statistical inference.

Keywords

DNA methylation, differential methylation, epigenetics, causal inference, statistical variable importance, targeted minimum loss-based estimation, machine learning

Introduction

DNA methylation is a fundamental epigenetic process known to play an important role in the regulation of gene expression. DNA CpG methylation involves the addition of a methyl group (CH_3) to the fifth carbon of the cytosine ring structure to form 5-methylcytosine. Numerous biological and medical studies have implicated DNA CpG methylation as playing a role in disease and development (Robertson 2005). Perhaps unsurprisingly then, biotechnologies have been developed to study the molecular mechanisms of this epigenetic process. Modern assays, like the Illumina *Infinium* Methylation assay, allow for quantitative interrogation of DNA methylation of CpG sites scattered across the genome at single-nucleotide resolution; moreover, much effort has been invested, by the bioinformatics community, in the development of tools for properly removing technological effects that may contaminate biological signatures measured by such assays. Despite these advances in both biological and bioninformatical techniques, most statistical methods available for differential analysis of data produced by such assays rely on over-simplified (often generalized linear) models. This is because typical statistical estimation approaches are not achievable for such high-dimensional data without restrictive modeling assumptions. When these assumptions are violated, estimates of the population-level effect of an exposure or treatment may have large bias. Data-adaptive estimation procedures that utilize machine learning can control for high-dimensional confounders when estimating treatment/exposure effects. However, interpretable statistical inference (i.e. confidence intervals, hypothesis tests) resulting from these data-adaptive estimates is challenging (Libbrecht and Noble 2015).

In this paper, we present an alternative such statistical analysis approaches in the form of nonparametric estimation procedures that provide simple and well interpretable statistical inference. Inspired by concepts of counterfactual effects from the causal inference literature and machine learning algorithms, we provide a computationally efficient technique for obtaining targeted estimates of nonparametric *variable importance measures* (**VIM**) for a pre-screened set of CpGs, controlling for the False Discovery Rate (FDR) as if all sites were tested. Under counterfactual assumptions, these estimators have asymptotic normal sampling distributions and thus provide reliable inference. Counterfactuals are “counter”-fact, defined in terms of an unrealistic yet ideal experiment. In the context of DNA methylation and for a binary treatment, we define the counterfactual outcomes as the observed methylation value (whether Beta- or M-values) a CpG site would have if all subjects were treated and the methylation value a CpG site would have if all subjects were not treated. Of course, these counterfactual outcomes are impossible to observe but they are very helpful for us to asses the importance of a CpG site relative to a subjects treatment/exposure and they can be statistically estimated (M. J. van der Laan 2006). Our VIM definition uses the concepts of counterfactuals and the framework for estimation is built on *targeted maximum likelihood estimation* or *targeted minimum loss-based estimation* (**TMLE**) (M. J. van der Laan and Rubin 2006). This methodology targets the individual importance of each CpG site by taking advantage of the prediction power of machine learning algorithms. The mathematical properties of this approach have been explored in (Tuglus and van der Laan 2008) and we focus on our contribution to it’s application, `methyvim`, an R package that make this technique for differential methylation analysis easily accessible.

For a general discussion of the framework of targeted minimum loss-based estimation and the role this approach plays in statistical causal inference, the interested reader is invited to consult M. J. van der Laan and Rose (2011) and M. J. van der Laan and Rose (2017). For a more general introduction to (statistical) causal inference, Pearl (2009) and Hernan and Robins (2018, forthcoming) may be of interest.

Methods

Implementation

The core functionality of this package is made available via the eponymous `methyvim` function, which implements a statistical algorithm designed to compute targeted estimates of VIMs, defined in such a way that the VIMs represent parameters of scientific interest in computational biology experiments; moreover, these VIMs are defined such that they may be estimated in a manner that is very nearly assumption-free, that is, within a *fully nonparametric statistical model*. **The statistical algorithm consists in several major steps**:

1. Pre-screening of genomic sites is used to isolate a subset of sites for which there is cursory evidence of differential methylation. For the sake of computational feasibility, targeted minimum loss-based estimates of VIMs are computed only for this subset of sites. Currently, the available screening approach adapts core routines from the `limma` R package. Future releases will support functionality from other packages (e.g., `randomForest`, `tmle.npvi`). Following the style of the function for performing screening via `limma`, users may write their own screening functions and are invited to contribute such functions to the core software package by opening pull requests at the GitHub repository.

2. Nonparametric estimates of VIMs, for the specified target parameter, are computed at each of the CpG sites passing the screening step. The VIMs are defined in such a way that the estimated effects is of an exposure/treatment on the methylation status of a target CpG site, controlling for the observed methylation status of the neighbors of that site. Currently, routines are adapted from the `tmle` R package. Future releases will support doubly-robust estimates of these VIMs (via the `drtmle` package) and add parameters for continuous treatments/exposures (via the `tmle.npvi` package).

3. Since pre-screening is performed prior to estimating VIMs, we make use of a multiple testing correction uniquely suited to such settings. Due to the multiple testing nature of the estimation problem, a vari-

ant of the Benjamini & Hochberg procedure for controlling the False Discovery Rate (FDR) is applied (Benjamini and Hochberg 1995). Specifically, we apply the "modified marginal Benjamini & Hochberg step-up False Discovery Rate controlling procedure for multi-stage analyses" (FDR-MSA), which is guaranteed to control the FDR as if all sites were tested (i.e., without screening) (Tuglus and van der Laan 2009).

Parameters of Interest For the CpG sites that passed the pre-screening step, the user chooses the parameter of interest that will be computed at each site. *In all cases, an estimator of the parameter of interest is constructed via targeted minimum loss-based estimation.*
For discrete-valued treatments or exposures:

- The average treatment effect (ATE): The effect of a binary exposure or treatment on the observed methylation at a target CpG site is estimated, controlling for the observed methylation at all other CpG sites in the same neighborhood as the target site, based on an additive form. Often denoted $\psi_0 = \psi_0(1) - \psi_0(0)$, the parameter estimate represents the additive difference in methylation that would have been observed at the target site had all observations received the treatment versus the counterfactual under which none received the treatment.

- The relative risk (RR): The effect of a binary exposure or treatment on the observed methylation at a target CpG site is estimated, controlling for the observed methylation at all other CpG sites in the same neighborhood as the target site, based on a geometric form. Often denoted, $\psi_0 = \frac{\psi_0(1)}{\psi_0(0)}$, the parameter estimate represents the multiplicative difference in methylation that would have been observed at the target site had all observations received the treatment versus the counterfactual under which none received the treatment.

Estimating the VIM corresponding to the parameters above, for discrete-valued treatments or exposures, requires two separate regression steps: one for the treatment mechanism (propensity score) and one for the outcome regression. Technical details on the nature of these regressions are discussed in Hernan and Robins (2018, forthcoming), and details for estimating these regressions in the framework of targeted minimum loss-based estimation are discussed in M. J. van der Laan and Rose (2011).
Support for continuous-valued treatments or exposures is *planned but not yet available*, though work is underway to incorporate into our methodology the following

- A nonparametric variable importance measure (NPVI) (Chambaz, Neuvial, and van der Laan 2012): The effect of continuous-valued exposure or treatment (the observed methylation at a target CpG site) on an outcome of interest is estimated, controlling for the observed methylation at all other CpG sites in the same neighborhood as the target (treatment) site, based on a parameter that compares values of the treatment against a reference value taken to be the null. In particular, the implementation provided is designed to assess the effect of differential methylation at the target CpG site on a (typically) phenotype-level outcome of interest (e.g., survival), in effect providing an nonparametric evaluation of the impact of methylation at the target site on said outcome.

Class `methytmle` We have adopted a class `methytmle` to help organize the functionality within this package. The `methytmle` class builds upon the `GenomicRatioSet` class provided by the `minfi` package so all of the slots of `GenomicRatioSet` are contained in a `methytmle` object. The new class introduced in the `methyvim` package includes several new slots:

- `call` - the form of the original call to the `methyvim` function.

- `screen_ind` - indices identifying CpG sites that pass the screening process.

- `clusters` - non-unique IDs corresponding to the manner in wich sites are treated as neighbors. These are assigned by genomic distance (bp) and respect chromosome boundaries (produced via a call to `bumphunter::clusterMaker`).

- `var_int` - the treatment/exposure status for each subject. Currently, these must be binary, due to the definition of the supported targeted parameters.

- `param` - the name of the target parameter from which the estimated VIMs are defined.

- `vim` - a table of statistical results obtained from estimating VIMs for each of the CpG sites that pass the screening procedure.

- `ic` - the measured array values for each of the CpG sites passing the screening, transformed into influence curve space based on the chosen target parameter.

We refer the reader to the package vignette, "`methyvim`: Targeted Learning for Differential Methylation Analysis," included in any distribution of the software package, for further details.

Operation

A standard computer with the latest version of R and Bioconductor 3.6 installed will handle applications of the `methyvim` package.

Use Cases

To examine the practical applications and the full set of utilities of the `methyvim` package, we will use a publicly available example data set produced by the Illumina 450K array, from the `minfiData` R package.

Preliminaries: Setting up the Data We begin by loading the package and the data set. After loading the data, which comes in the form of a raw `MethylSet` object, we perform some further processing by mapping to the genome (with `mapToGenome`) and converting the values from the methylated and unmethylated channels to Beta-values (via `ratioConvert`). These two steps together produce an object of class `GenomicRatioSet`, provided by the `minfi` package.

```
suppressMessages(library(minfiData))
data(MsetEx)
mset <- mapToGenome(MsetEx)
grs <- ratioConvert(mset)
grs
```

```
## class: GenomicRatioSet
## dim: 485512 6
## metadata(0):
## assays(2): Beta CN
## rownames(485512): cg13869341 cg14008030 ... cg08265308 cg14273923
## rowData names(0):
## colnames(6): 5723646052_R02C02 5723646052_R04C01 ...
##   5723646053_R05C02 5723646053_R06C02
## colData names(13): Sample_Name Sample_Well ... Basename filenames
## Annotation
##   array: IlluminaHumanMethylation450k
##   annotation: ilmn12.hg19
## Preprocessing
##   Method: Raw (no normalization or bg correction)
##   minfi version: 1.21.2
##   Manifest version: 0.4.0
```

We can create an object of class `methytmle` from any `GenomicRatioSet` object simply invoking the S4 class constructor:

```
library(methyvim)
```

```
## methyvim: Targeted Learning for Differential Methylation Analysis
```

```
## Version: 0.99.8
```

```
mtmle <- .methytmle(grs)
```

Additionally, a `GenomicRatioSet` can be created from a matrix with the function `makeGenomicRatioSetFromMatrix` provided by the `minfi` package.

Differential Methylation Analysis For this example analysis, we'll treat the condition of the patients as the exposure/treatment variable of interest. The `methyvim` function requires that this variable either be `numeric` or easily coercible to `numeric`. To facilitate this, we'll simply convert the covariate (currently a `character`):

```
var_int <- (as.numeric(as.factor(colData(grs)$status)) - 1)
```

n.b., the re-coding process results in "normal" patients being assigned a value of 1 and cancer patients a 0. Now, we are ready to analyze the effects of cancer status on DNA methylation using this data set. We proceed as follows with a targeted minimum loss-based estimate of the Average Treatment Effect.

```
suppressMessages(
  methyvim_cancer_ate <- methyvim(data_grs = grs, var_int = var_int,
                                  vim = "ate", type = "Beta", filter = "limma",
                                  filter_cutoff = 0.20, obs_per_covar = 2,
                                  parallel = FALSE, sites_comp = 125,
                                  tmle_type = "glm"
                                 )
)
```

```
## Warning in set_parallel(parallel = parallel, future_param = future_param, : Sequential evaluatio
##  Proceed with caution.
```

Note that we set the obs_per_covar argument to a relatively low value (2, where the recommended default is 20) for the purposes of this example as the sample size is only 10. We do this only to exemplify the estimation procedure and it is important to point out that such low values for obs_per_covar will compromise the quality of inference obtained because this setting directly affects the definition of the target parameter.
Further, note that here we apply the glm flavor of the tmle_type argument, which produces faster results by fitting models for the propensity score and outcome regressions using a limited number of parametric models. By contrast, the sl (for “Super Learning”) flavor fits these two regressions using highly nonparametric and data-adaptive procedures (i.e., via machine learning). Obtaining the estimates via GLMs results in each of the regression steps being less robust than if nonparametric regressions were used.
We can view a table of results by examining the vim slot of the produced methytmle object.

```
head(slot(methyvim_cancer_ate, "vim"))
```

```
##            lowerCI_ATE      est_ATE   upperCI_ATE      Var_ATE
## cg14008030 -0.11956597 -0.031415962  0.0567340489 2.022705e-03
## cg20253340 -0.08850637 -0.058866142 -0.0292259165 2.286919e-04
## cg21870274 -0.09499057 -0.029118982  0.0367526091 1.129495e-03
## cg17308840 -0.04626018 -0.007152452  0.0319552773 3.981191e-04
## cg00645010 -0.02677328 -0.013465554 -0.0001578256 4.609945e-05
## cg27534567  0.06745648  0.115711854  0.1639672225 6.061486e-04
##                    pval n_neighbors_all n_neighbors_w max_corr_w
## cg14008030 4.848468e-01               0             0         NA
## cg20253340 9.917426e-05               0             0         NA
## cg21870274 3.862537e-01               2             1  0.9443580
## cg17308840 7.199943e-01               2             1  0.9443580
## cg00645010 4.734007e-02               2             2  0.5236810
## cg27534567 2.602936e-06               1             0  0.9362968
```

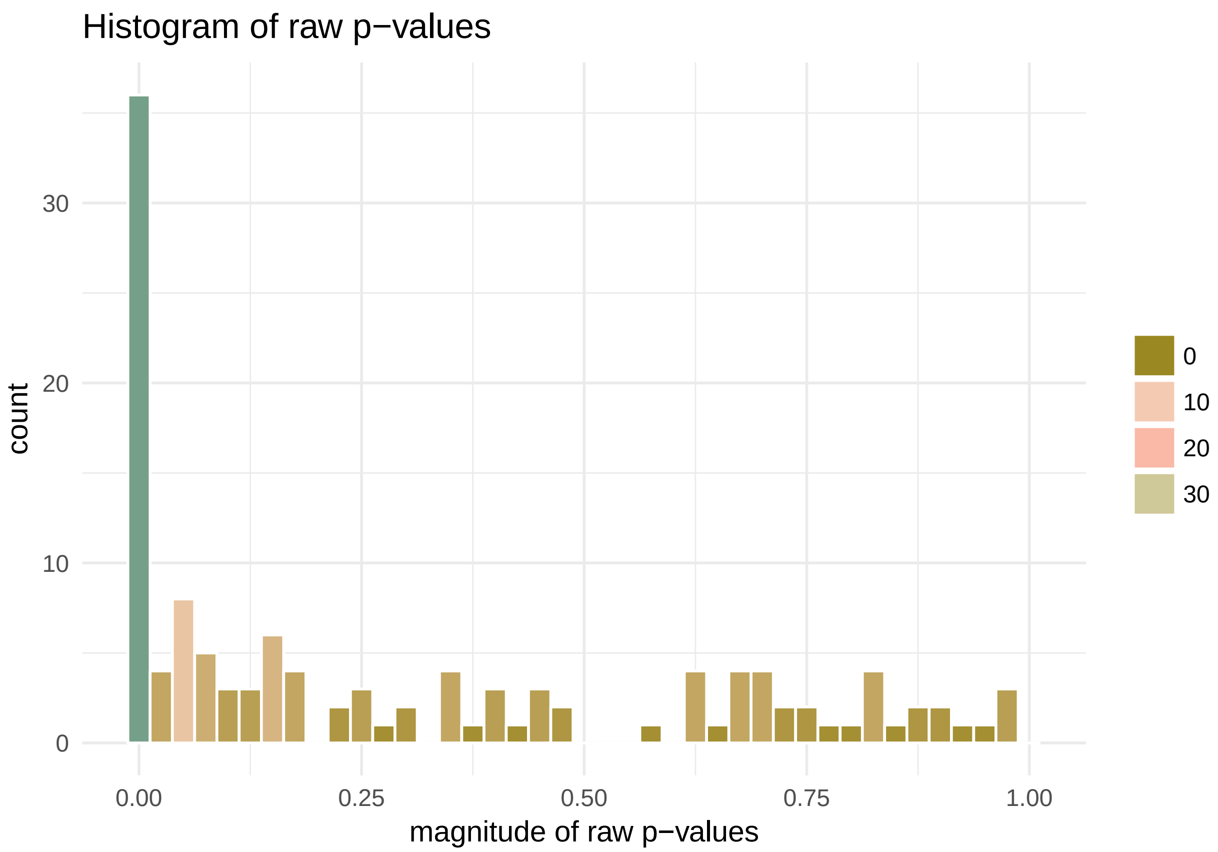
Finally, we may compute FDR-corrected p-values, by applying a modified procedure for controlling the False Discovery Rate for multi-stage analyses (FDR-MSA) (Tuglus and van der Laan 2009). We do this by simply applying the fdr_msa function.

```
fdr_p <- fdr_msa(pvals = slot(methyvim_cancer_ate, "vim")$pval,
                 total_obs = nrow(methyvim_cancer_ate))
```

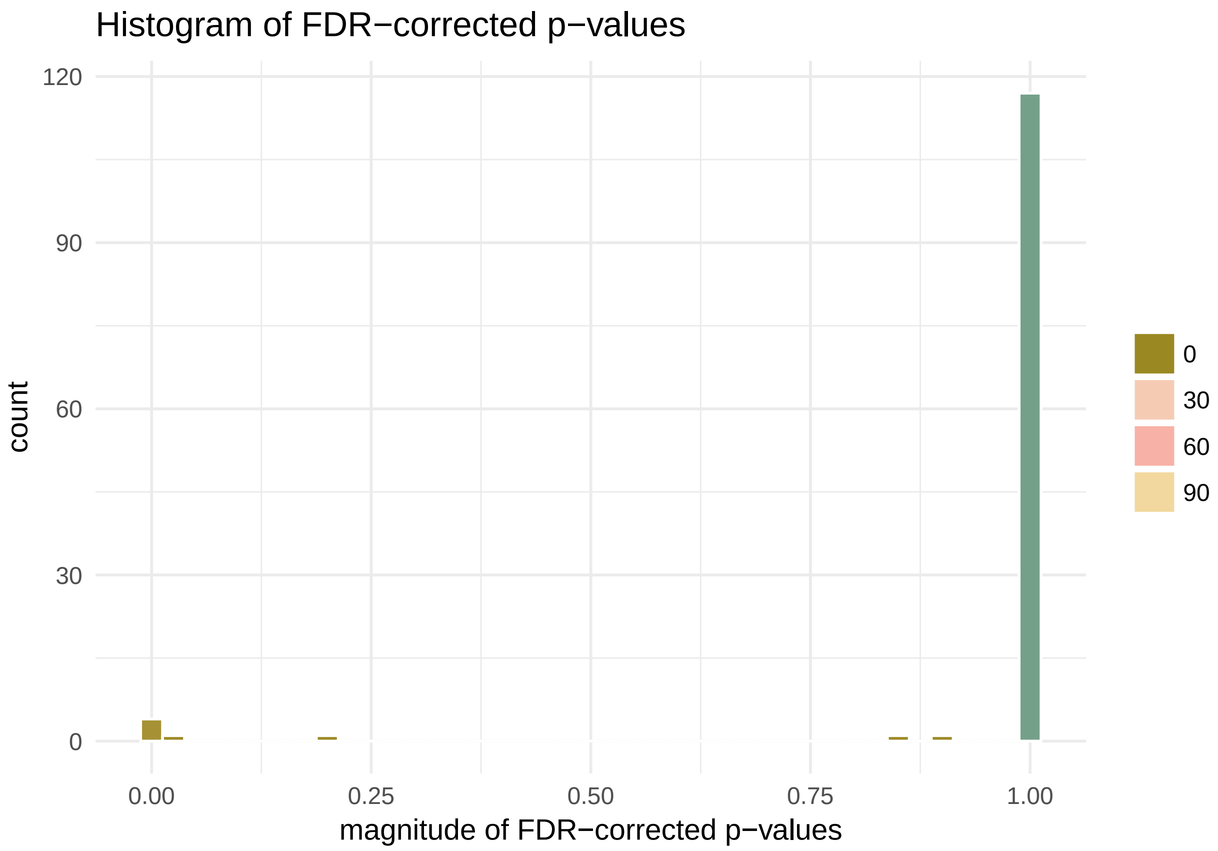
Having explored the results of our analysis numerically, we now proceed to use the visualization tools provided with the methyvim R package to further enhance our understanding of the results.

Visualization of Results While making allowance for users to explore the full set of results produced by the estimation procedure (by way of exposing these directly to the user), the methyvim package also provides *three* (3) visualization utilities that produce plots commonly used in examining the results of differential methylation analyses.
A simple call to plot produces side-by-side histograms of the raw p-values computed as part of the estimation process and the corrected p-values obtained from using the FDR-MSA procedure.

```
plot(methyvim_cancer_ate, type = "raw_pvals")
```



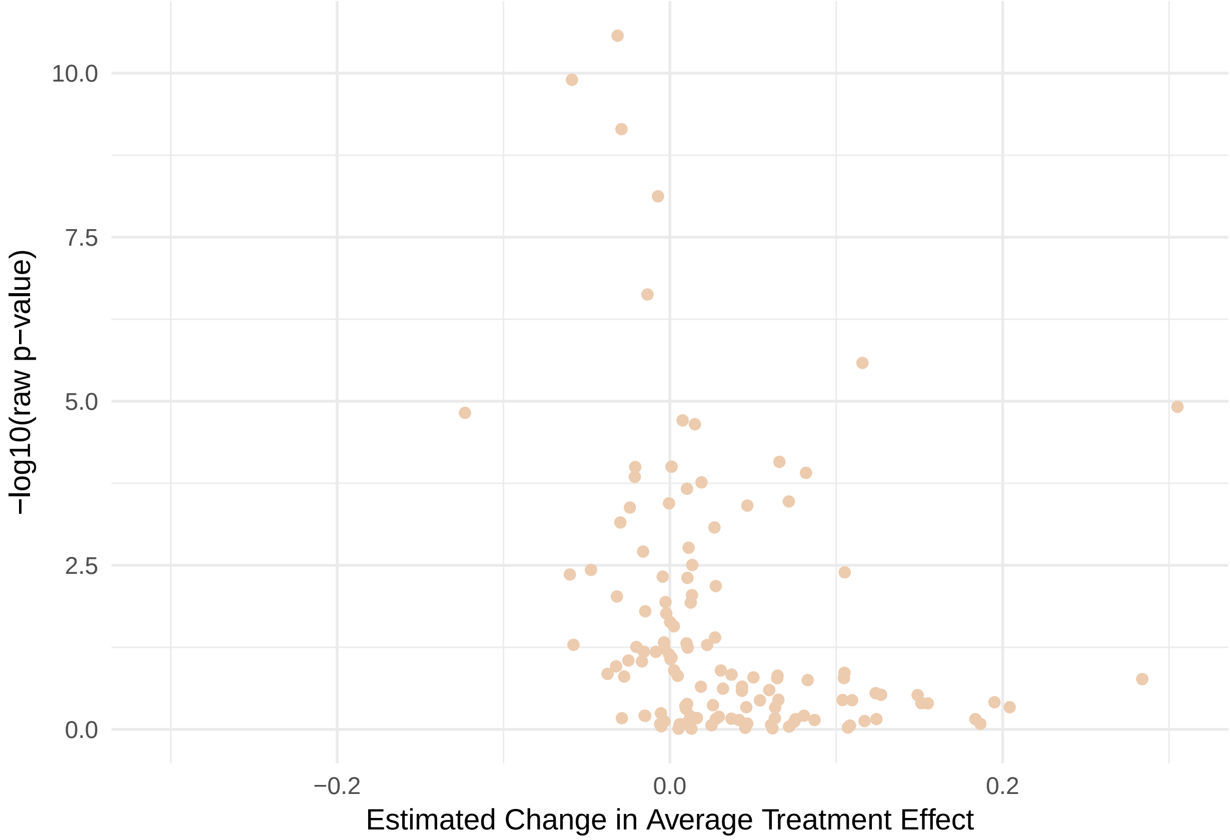
```r
plot(methyvim_cancer_ate, type = "fdr_pvals")
```



Remark: The plots displayed above may also be generated as side-by-side histograms in a single plot object. This is the default for the `plot` method and may easily be invoked by specifying no additional arguments to the `plot` function, unlike in the above.

While histograms of the p-values may be generally useful in inspecting the results of the estimation procedure, a more common plot used in examining the results of differential methylation procedures is the volcano plot, which plots the parameter estimate along the x-axis and $-\log_{10}$(p-value) along the y-axis. We implement such a plot in the `methyvolc` function:

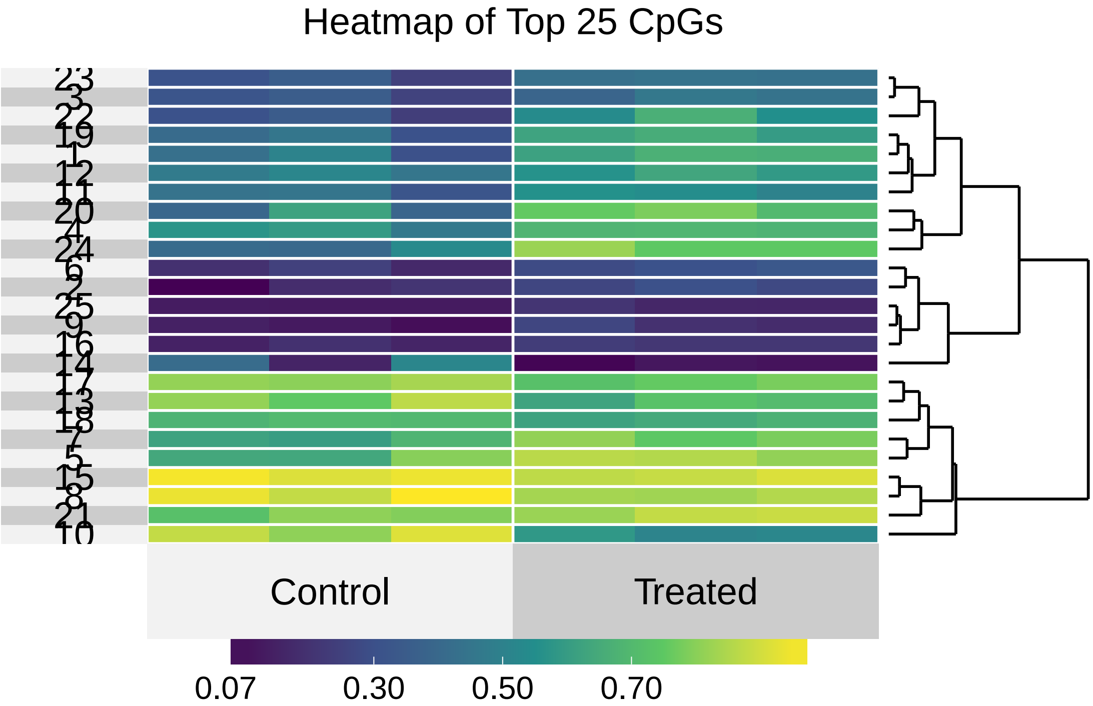
```
methyvolc(methyvim_cancer_ate)
```



The purpose of such a plot is to ensure that very low (possibly statistically significant) p-values do not arise from cases of low variance. This appears to be the case in the plot above (notice that most parameter estimates are near zero, even in cases where the raw p-values are quite low).

Yet another popular plot for visualizing effects in such settings is the heatmap, which plots estimates of the raw methylation effects (as measured by the assay) across subjects using a heat gradient. We implement this in the `methyheat` function:

```
methyheat(methyvim_cancer_ate)
```



Invoking `methyheat` in this manner produces a plot of the top sites (25, by default) based on the raw p-value, using the raw methylation measures in the plot. This uses the exceptional `superheat` R package (**???**).

Summary

Here we introduce the R package `methyvim`, an algorithm for differential methylation analysis that moves beyond linear models. Additionally, this estimation procedure produces straightforward statistical inference. Inspired by concepts of counterfactual effects from the causal inference literature and machine learning algorithms, we provide a computationally efficient technique for obtaining targeted estimates of nonparametric variable importance measures for a pre-screened set of CpGs, controlling for the False Discovery Rate as if all sites were tested, and the estimation framework is built on targeted minimum loss-based estimation. By taking advantage of the prediction power of machine learning algorithms, this methodology targets the individual importance of each CpG site, providing a fully nonparametric statistical model for the analysis of differential methylation.

Software availability

Latest source code (development version): `https://github.com/nhejazi/methyvim`
Bioconductor (stable release): `https://bioconductor.org/packages/methyvim`
Archived source code as at time of publication: `https://github.com/nhejazi/methyvim`
Software license: The MIT License, copyright Nima S. Hejazi

Author contributions

NH designed and implemented the software package, applied the tool to the use cases, and co-drafted the present article. RP helped in designing the software and co-drafted the present article. AH and ML serve as the advisors for the development of this software and general statistical algorithm.

Competing interests

No competing interests were disclosed.

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