An Introduction to MAST

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1 Philosophy

`MAST`is an R/Bioconductor package for managing and analyzing qPCR and sequencing-based single–cell gene expression data, as well as data from other types of single–cell assays. Our goal is to support assays that have multiple *features* (genes, markers, etc) per *well* (cell, etc) in a flexible manner. Assays are assumed to be mostly *complete* in the sense that most *wells* contain measurements for all features.

1.1 Internals

A `SingleCellAssay` object can be manipulated as a matrix, with rows giving features and columns giving cells. It derives from `SummarizedExperiment`.

1.2 Statistical Testing

Apart from reading and storing single–cell assay data, the package also provides functionality for significance testing of differential expression using a Hurdle model, gene set enrichment, facilities for visualizing patterns in residuals indicative of differential expression, and power calculations (soon).

There is also some facilities for inferring background thresholds, and filtering of individual outlier wells/libraries. These methods are described our papers.

2 Examples

With the cursory background out of the way, we'll proceed with some examples to help understand how the package is used.

2.1 Reading Data

Data can be imported in a Fluidigm instrument-specific format (the details of which are undocumented, and likely subject-to-change) or some derived, annotated format, or in "long" (melted) format, in which each row is a measurement, so if there are N wells and M cells, then the `data.frame` should contain $N \times M$ rows.

For example, the following data set was provided in as a comma-separated value file. It has the cycle threshold (ct) recorded. Non-detected genes are recorded as NAs. For the Fluidigm/qPCR single cell expression functions to work as expected, we must use the *expression threshold*, defined as $et = c_{\max} - ct$, which is proportional to the log-expression.

Below, we load the package and the data, then compute the expression threshold from the ct, and construct a `FluidigmAssay`.

```
library(MAST)
library(data.table)
data(vbeta)
colnames(vbeta)
```

```
##  [1] "Sample.ID"         "Subject.ID"        "Experiment.Number"
##  [4] "Chip.Number"       "Stim.Condition"    "Time"
##  [7] "Population"        "Number.of.Cells"   "Well"
## [10] "Gene"              "Ct"
```

```
vbeta <- computeEtFromCt(vbeta)
vbeta.fa <- FromFlatDF(vbeta, idvars=c("Subject.ID", "Chip.Number", "Well"),
                          primerid='Gene', measurement='Et', ncells='Number.of.Cells',
                          geneid="Gene",  cellvars=c('Number.of.Cells', 'Population'),
                          phenovars=c('Stim.Condition','Time'), id='vbeta all', class='FluidigmAssay')
show(vbeta.fa)
```

```
## class: FluidigmAssay
## dim: 75 456
## metadata(0):
## assays(1): Et
## rownames(75): B3GAT1 BAX ... TNFRSF9 TNFSF10
## rowData names(2): Gene primerid
## colnames(456): Sub01 1 A01 Sub01 1 A02 ... Sub02 3 H10 Sub02 3 H11
## colData names(9): Number.of.Cells Population ... Time wellKey
```

We see that the variable `vbeta` is a `data.frame` from which we construct the `FluidigmAssay` object. The `idvars` is the set of column(s) in `vbeta` that uniquely identify a well (globally), the `primerid` is a column(s) that specify the feature measured at this well. The `measurement` gives the column name containing the log-expression measurement, `ncells` contains the number of cells (or other normalizing factor) for the well. `geneid`, `cellvars`, `phenovars` all specify additional columns to be included in the `featureData`, `phenoData` and `cellData` (TODO: wellData). The output is a `FluidigmAssay` object with 456 wells and 75 features.

We can access the feature–level metadata and the cell–level metadata using the `mcols` and `colData` accessors.

```
head(mcols(vbeta.fa),3)
```

```
## DataFrame with 3 rows and 2 columns
##          Gene    primerid
##   <character> <character>
## 1      B3GAT1      B3GAT1
## 2         BAX         BAX
## 3        BCL2        BCL2
```

```
head(colData(vbeta.fa),3)
```

```
## DataFrame with 3 rows and 9 columns
##             Number.of.Cells            Population    ncells Subject.ID
##                   <integer>           <character> <integer>   <factor>
## Sub01 1 A01               1 CD154+VbetaResponsive         1      Sub01
## Sub01 1 A02               1 CD154+VbetaResponsive         1      Sub01
## Sub01 1 A03               1 CD154+VbetaResponsive         1      Sub01
##             Chip.Number        Well Stim.Condition     Time     wellKey
##               <integer> <character>    <character> <factor> <character>
## Sub01 1 A01           1         A01      Stim(SEB)       12 Sub01 1 A01
## Sub01 1 A02           1         A02      Stim(SEB)       12 Sub01 1 A02
## Sub01 1 A03           1         A03      Stim(SEB)       12 Sub01 1 A03
```

We see this gives us the set of genes measured in the assay, or the cell-level metadata (i.e. the number of cells measured in the well, the population this cell belongs to, the subject it came from, the chip it was run on, the well id, the stimulation it was subjected to, and the timepoint for the experiment this cell was part of). The wellKey are concatenated idvars columns, helping to ensure consistency when splitting and merging `SingleCellAssay`objects.

2.2 Importing Matrix Data

Data can also be imported in matrix format using command `FromMatrix`, and passing a matrix of expression values and `DataFrame` coercible cell and feature data.

2.3 Subsetting, splitting, combining, melting

It's possible to subset `SingleCellAssay`objects by wells and features. Square brackets ("[") will index on the first index (features) and by features on the second index (cells). Integer and boolean and indices may be used, as well as character vectors naming the wellKey or the feature (via the primerid). There is also a `subset` method, which will evaluate its argument in the frame of the `colData`, hence will subset by wells.

```
sub1 <- vbeta.fa[,1:10]
show(sub1)

## class: FluidigmAssay
## dim: 75 10
## metadata(0):
## assays(1): Et
## rownames(75): B3GAT1 BAX ... TNFRSF9 TNFSF10
## rowData names(2): Gene primerid
## colnames(10): Sub01 1 A01 Sub01 1 A02 ... Sub01 1 A09 Sub01 1 A10
## colData names(9): Number.of.Cells Population ... Time wellKey

sub2 <- subset(vbeta.fa, Well=='A01')
show(sub2)

## class: FluidigmAssay
## dim: 75 5
## metadata(0):
## assays(1): Et
## rownames(75): B3GAT1 BAX ... TNFRSF9 TNFSF10
## rowData names(2): Gene primerid
## colnames(5): Sub01 1 A01 Sub01 2 A01 Sub02 1 A01 Sub02 2 A01 Sub02
##   3 A01
## colData names(9): Number.of.Cells Population ... Time wellKey

sub3 <- vbeta.fa[6:10, 1:10]
show(sub3)

## class: FluidigmAssay
## dim: 5 10
## metadata(0):
## assays(1): Et
## rownames(5): CCL4 CCL5 CCR2 CCR4 CCR5
## rowData names(2): Gene primerid
## colnames(10): Sub01 1 A01 Sub01 1 A02 ... Sub01 1 A09 Sub01 1 A10
## colData names(9): Number.of.Cells Population ... Time wellKey

colData(sub3)

## DataFrame with 10 rows and 9 columns
##             Number.of.Cells            Population    ncells Subject.ID
##                   <integer>           <character> <integer>   <factor>
## Sub01 1 A01               1 CD154+VbetaResponsive         1      Sub01
## Sub01 1 A02               1 CD154+VbetaResponsive         1      Sub01
## Sub01 1 A03               1 CD154+VbetaResponsive         1      Sub01
## Sub01 1 A04               1 CD154+VbetaResponsive         1      Sub01
## Sub01 1 A05               1 CD154+VbetaResponsive         1      Sub01
## Sub01 1 A06               1 CD154+VbetaResponsive         1      Sub01
## Sub01 1 A07               1 CD154+VbetaResponsive         1      Sub01
## Sub01 1 A08               1 CD154+VbetaResponsive         1      Sub01
## Sub01 1 A09               1 CD154+VbetaResponsive         1      Sub01
```

```
## Sub01 1 A10               1 CD154+VbetaResponsive         1      Sub01
##             Chip.Number        Well Stim.Condition     Time     wellKey
##               <integer> <character>    <character> <factor> <character>
## Sub01 1 A01           1         A01      Stim(SEB)       12 Sub01 1 A01
## Sub01 1 A02           1         A02      Stim(SEB)       12 Sub01 1 A02
## Sub01 1 A03           1         A03      Stim(SEB)       12 Sub01 1 A03
## Sub01 1 A04           1         A04      Stim(SEB)       12 Sub01 1 A04
## Sub01 1 A05           1         A05      Stim(SEB)       12 Sub01 1 A05
## Sub01 1 A06           1         A06      Stim(SEB)       12 Sub01 1 A06
## Sub01 1 A07           1         A07      Stim(SEB)       12 Sub01 1 A07
## Sub01 1 A08           1         A08      Stim(SEB)       12 Sub01 1 A08
## Sub01 1 A09           1         A09      Stim(SEB)       12 Sub01 1 A09
## Sub01 1 A10           1         A10      Stim(SEB)       12 Sub01 1 A10

mcols(sub3)

## DataFrame with 5 rows and 2 columns
##          Gene    primerid
##   <character> <character>
## 1        CCL4        CCL4
## 2        CCL5        CCL5
## 3        CCR2        CCR2
## 4        CCR4        CCR4
## 5        CCR5        CCR5
```

The cellData and featureData `AnnotatedDataFrames` are subset accordingly as well.

A `SingleCellAssay`may be split into a list of `SingleCellAssay`. The split method takes an argument which names the column (factor) on which to split the data. Each level of the factor will be placed in its own `SingleCellAssay`within the list.

```
sp1 <- split(vbeta.fa, 'Subject.ID')
show(sp1)

## $Sub01
## class: FluidigmAssay
## dim: 75 177
## metadata(0):
## assays(1): Et
## rownames(75): B3GAT1 BAX ... TNFRSF9 TNFSF10
## rowData names(2): Gene primerid
## colnames(177): Sub01 1 A01 Sub01 1 A02 ... Sub01 2 H09 Sub01 2 H10
## colData names(9): Number.of.Cells Population ... Time wellKey
##
## $Sub02
## class: FluidigmAssay
## dim: 75 279
## metadata(0):
## assays(1): Et
## rownames(75): B3GAT1 BAX ... TNFRSF9 TNFSF10
## rowData names(2): Gene primerid
## colnames(279): Sub02 1 A01 Sub02 1 A02 ... Sub02 3 H10 Sub02 3 H11
## colData names(9): Number.of.Cells Population ... Time wellKey
```

The splitting variable can either be a character vector naming column(s) of the `SingleCellAssay`, or may be a `factor` or `list` of `factor`s.

It's possible to combine `SingleCellAssay`objects with the `cbind` method.

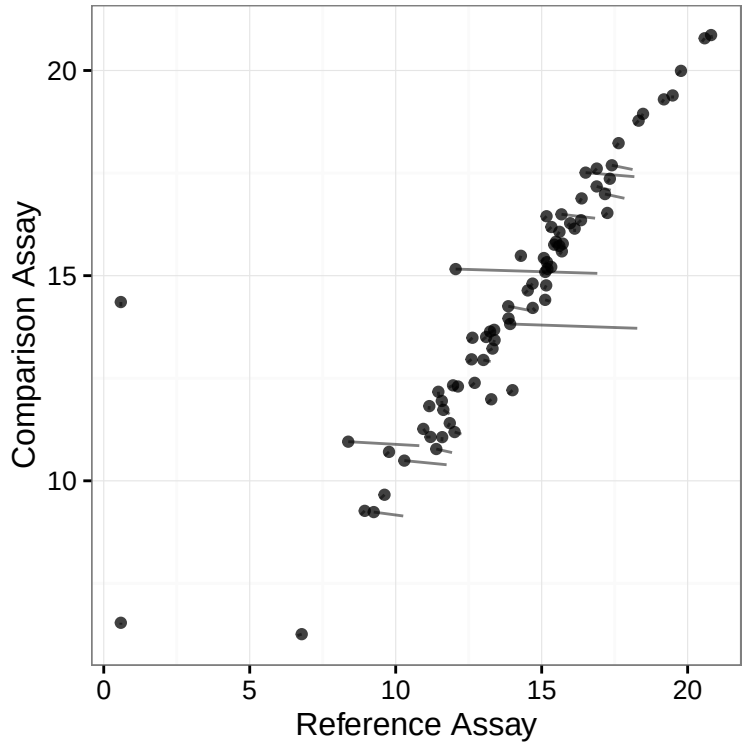
```
## class: FluidigmAssay
## dim: 75 456
## metadata(0):
## assays(1): Et
## rownames(75): B3GAT1 BAX ... TNFRSF9 TNFSF10
## rowData names(2): Gene primerid
## colnames(456): Sub01 1 A01 Sub01 1 A02 ... Sub02 3 H10 Sub02 3 H11
## colData names(9): Number.of.Cells Population ... Time wellKey
```

2.4 Filtering

We can filter and perform some significance tests on the `SingleCellAssay`. We may want to filter any wells with at least two outlier cells where the discrete and continuous parts of the signal are at least 9 standard deviations from the mean. This is a very conservative filtering criteria. We'll group the filtering by the number of cells.

We'll split the assay by the number of cells and look at the concordance plot after filtering.

```
vbeta.split<-split(vbeta.fa,"Number.of.Cells")
#see default parameters for plotSCAConcordance
plotSCAConcordance(vbeta.split[[1]],vbeta.split[[2]],
                   filterCriteria=list(nOutlier = 1, sigmaContinuous = 9,
                       sigmaProportion = 9))

## Using primerid as id variables
## Using primerid as id variables
## Using primerid as id variables
## Using primerid as id variables
## Sum of Squares before Filtering:  14.89
##  After filtering:  12.41
##  Difference:  2.48
```



The filtering function has several other options, including whether the filter shuld be applied (thus returning a new `SingleCellAssay`object) or returned as a matrix of boolean values.

```
vbeta.fa
```

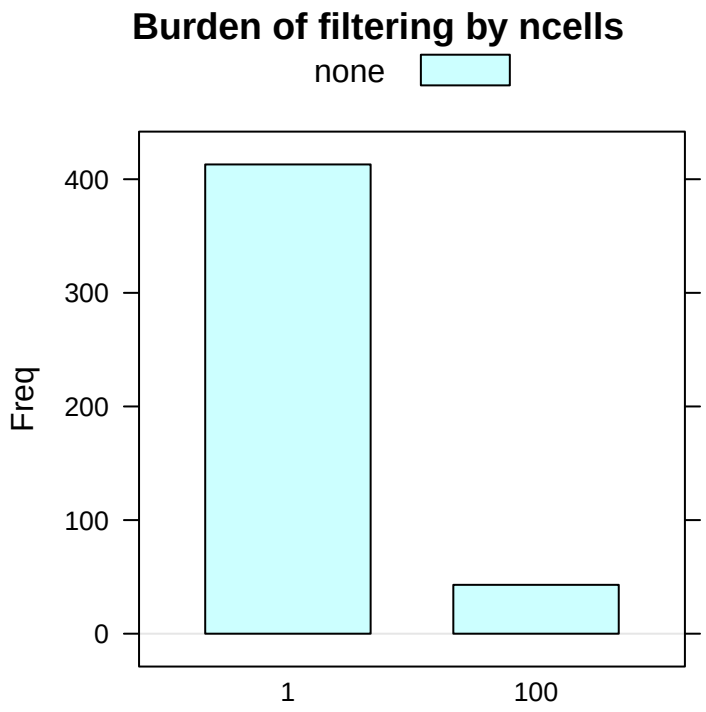
```
## class: FluidigmAssay
## dim: 75 456
## metadata(0):
## assays(1): Et
## rownames(75): B3GAT1 BAX ... TNFRSF9 TNFSF10
## rowData names(2): Gene primerid
## colnames(456): Sub01 1 A01 Sub01 1 A02 ... Sub02 3 H10 Sub02 3 H11
## colData names(9): Number.of.Cells Population ... Time wellKey
```

```
## Split by 'ncells', apply to each component, then recombine
vbeta.filtered <- filter(vbeta.fa, groups='ncells')
## Returned as boolean matrix
was.filtered <- filter(vbeta.fa, apply_filter=FALSE)
## Wells filtered for being discrete outliers
head(subset(was.filtered, pctout))
```

```
##             intout null pctout
## Sub01 1 D05  FALSE TRUE   TRUE
## Sub01 1 D06  FALSE TRUE   TRUE
## Sub01 1 D07  FALSE TRUE   TRUE
## Sub01 1 D08  FALSE TRUE   TRUE
## Sub01 1 D10  FALSE TRUE   TRUE
## Sub01 1 D11  FALSE TRUE   TRUE
```

There's also some functionality for visualizing the filtering.

```
burdenOfFiltering(vbeta.fa, 'ncells', byGroup=TRUE)
```



3 Significance testing under the Hurdle model

There are two frameworks available in the package. The first framework `zlm` offers a full linear model to allow arbitrary comparisons and adjustment for covariates. The second framework `LRT` can be considered essentially performing t-tests (respecting the discrete/continuous nature of the data) between pairs of groups. `LRT` is subsumed by the first framework, but might be simpler for some users, so has been kept in the package.

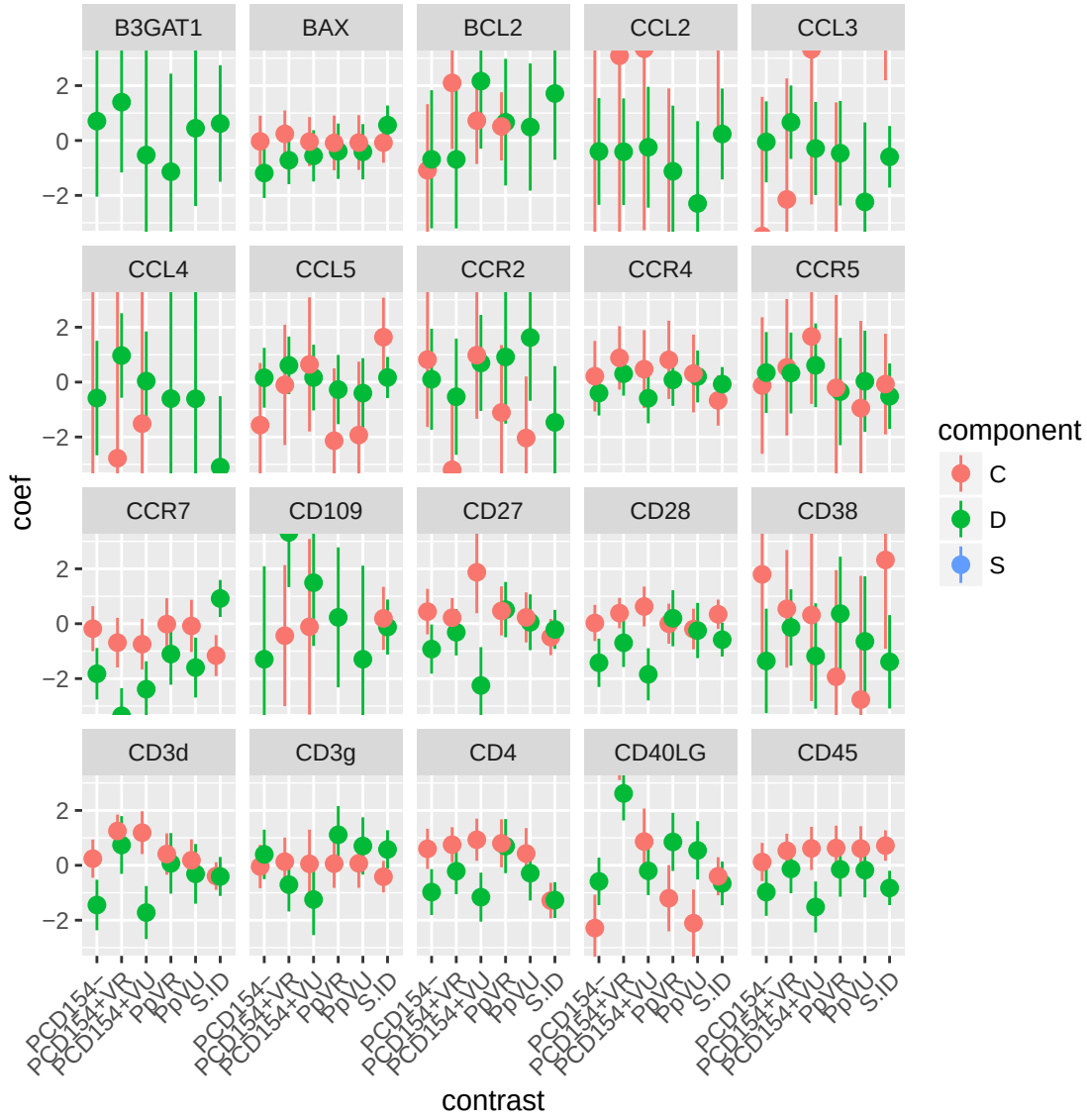
We'll describe `zlm`. Models are specified in terms of the variable used as the measure and covariates present in the `cellData` using symbolic notation, just as the `lm` function in R.

```
vbeta.1 <- subset(vbeta.fa, ncells==1)
## Consider the first 20 genes
vbeta.1 <- vbeta.1[1:20,]
head(colData(vbeta.1))

## DataFrame with 6 rows and 9 columns
##             Number.of.Cells            Population    ncells Subject.ID
##                   <integer>           <character> <integer>   <factor>
## Sub01 1 A01               1 CD154+VbetaResponsive         1      Sub01
## Sub01 1 A02               1 CD154+VbetaResponsive         1      Sub01
## Sub01 1 A03               1 CD154+VbetaResponsive         1      Sub01
## Sub01 1 A04               1 CD154+VbetaResponsive         1      Sub01
## Sub01 1 A05               1 CD154+VbetaResponsive         1      Sub01
## Sub01 1 A06               1 CD154+VbetaResponsive         1      Sub01
##             Chip.Number        Well Stim.Condition     Time     wellKey
##               <integer> <character>    <character> <factor> <character>
## Sub01 1 A01           1         A01      Stim(SEB)       12 Sub01 1 A01
## Sub01 1 A02           1         A02      Stim(SEB)       12 Sub01 1 A02
## Sub01 1 A03           1         A03      Stim(SEB)       12 Sub01 1 A03
## Sub01 1 A04           1         A04      Stim(SEB)       12 Sub01 1 A04
## Sub01 1 A05           1         A05      Stim(SEB)       12 Sub01 1 A05
## Sub01 1 A06           1         A06      Stim(SEB)       12 Sub01 1 A06
```

Now, for each gene, we can regress on `Et` the factors `Population` and `Subject.ID`.

In each gene, we'll fit a Hurdle model with a separate intercept for each population and subject. A an S4 object of class "ZlmFit" is returned, containing slots with the genewise coefficients, variance-covariance matrices, etc.

```
library(ggplot2)
zlm.output <- zlm(~ Population + Subject.ID, vbeta.1,)
show(zlm.output)

## Fitted zlm on 20 genes and 413 cells.
##  Using BayesGLMlike ~ Population + Subject.ID

## returns a data.table with a summary of the fit
coefAndCI <- summary(zlm.output, logFC=FALSE)$datatable
coefAndCI <- coefAndCI[contrast != '(Intercept)',]
coefAndCI[,contrast:=abbreviate(contrast)]
ggplot(coefAndCI, aes(x=contrast, y=coef, ymin=ci.lo, ymax=ci.hi, col=component))+
    geom_pointrange(position=position_dodge(width=.5)) +facet_wrap(~primerid) +
    theme(axis.text.x=element_text(angle=45, hjust=1)) + coord_cartesian(ylim=c(-3, 3))
```

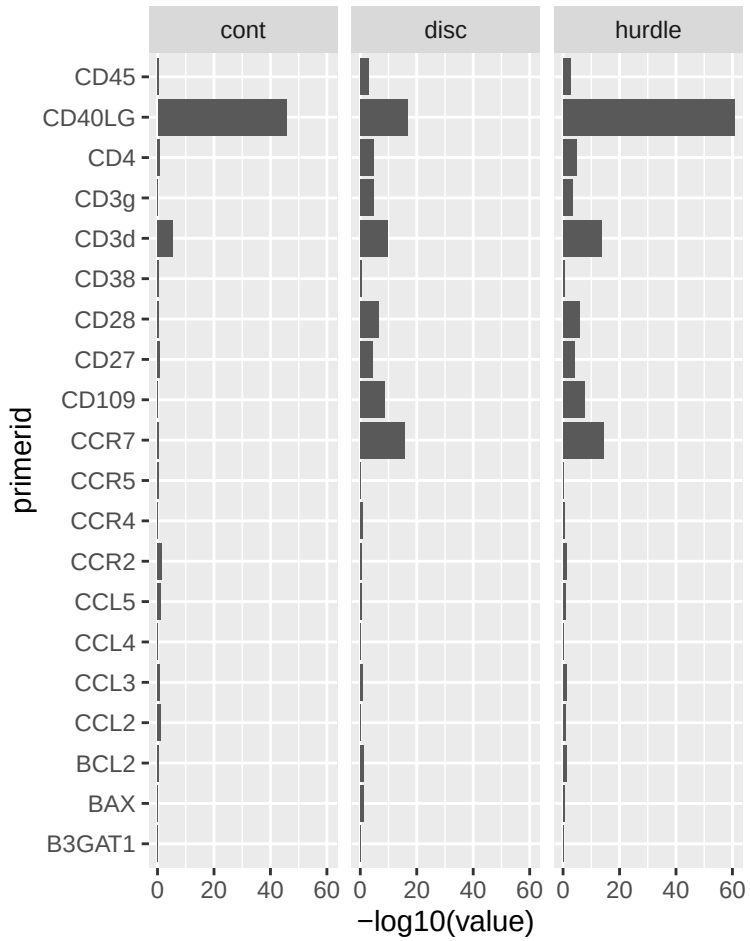


Try `?ZlmFit-class` or `showMethods(classes='ZlmFit')` to see a full list of methods. Multicore support is offered by setting `options(mc.cores=4)`, or however many cores your system has.

The combined test for differences in proportion expression/average expression is found by calling a likelihood ratio test on the fitted object. An array of genes, metrics and test types is returned. We'll plot the -log10 P values by gene and test type.

```
zlm.lr <- lrTest(zlm.output, 'Population')

## Refitting on reduced model...
## .
##
## Done!

dimnames(zlm.lr)

## $primerid
##  [1] "B3GAT1" "BAX"    "BCL2"   "CCL2"   "CCL3"   "CCL4"   "CCL5"
##  [8] "CCR2"   "CCR4"   "CCR5"   "CCR7"   "CD109"  "CD27"   "CD28"
## [15] "CD38"   "CD3d"   "CD3g"   "CD4"    "CD40LG" "CD45"
##
## $test.type
```

```
## [1] "cont"   "disc"   "hurdle"
##
## $metric
## [1] "lambda"     "df"         "Pr(>Chisq)"

pvalue <- ggplot(melt(zlm.lr[,,'Pr(>Chisq)']), aes(x=primerid, y=-log10(value)))+
    geom_bar(stat='identity')+facet_wrap(~test.type) + coord_flip()
print(pvalue)
```



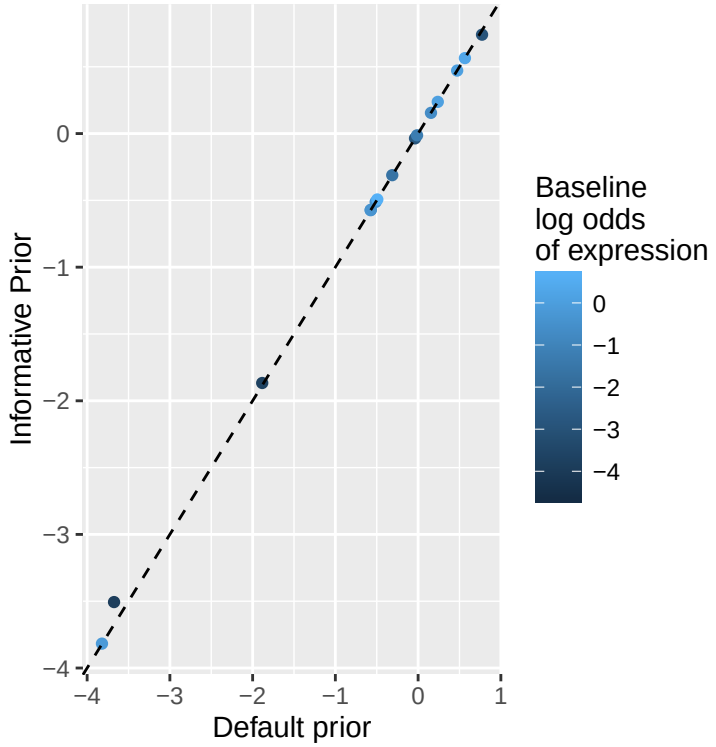
In fact, the `zlm` framework is quite general, and has wrappers for a variety of modeling functions that accept `glm`-like arguments to be used, such as mixed models (using `lme4`).

```
library(lme4)
lmer.output <- zlm(~ Stim.Condition +(1|Subject.ID), vbeta.1, method='glmer', ebayes=FALSE)
```

By default, we employ Bayesian logistic regression, which imposes a Cauchy prior of the regression coefficients, for the discrete component. This provides reasonable inference under linear separation. We default to regular least squares for the continuous component with an empirical Bayes' adjustment for the dispersion (variance) estimate. However, the prior can be adjusted (see `defaultPrior`) or eliminated entirely by setting `method='glm'` in `zlm`. It is also possible to use Bayesian linear regression for the continuous component by setting `useContinuousBayes=TRUE` in `zlm`. For example:

```
orig_results <- zlm(~Stim.Condition, vbeta.1)
dp <- defaultPrior('Stim.ConditionUnstim')
new_results <-  zlm(~Stim.Condition, vbeta.1, useContinuousBayes=TRUE,coefPrior=dp)
qplot(x=coef(orig_results, 'C')[, 'Stim.ConditionUnstim'],
```

```
       y=coef(new_results, 'C')[, 'Stim.ConditionUnstim'],
       color=coef(new_results, 'D')[,'(Intercept)']) +
     xlab('Default prior') + ylab('Informative Prior') +
     geom_abline(slope=1, lty=2) + scale_color_continuous('Baseline\nlog odds\nof expression')
```



After applying a prior to the continuous component, its estimates are shrunken towards zero, with the amount of shrinkage inversely depending on the number of expressing cells in the gene.

3.1 Two-sample Likelihood Ratio Test

Another way to test for differential expression is available through the `LRT` function, which is analogous to two-sample T tests.

```
two.sample <- LRT(vbeta.1, 'Population', referent='CD154+VbetaResponsive')
head(two.sample)
```

```
##              Population test.type primerid    lrstat direction   p.value
## 1 CD154-VbetaResponsive      comb   B3GAT1 1.5722257        -1 0.4556124
## 2 CD154-VbetaResponsive      comb      BAX 1.5847859         1 0.4527601
## 3 CD154-VbetaResponsive      comb     BCL2 0.7821003        -1 0.6763462
## 4 CD154-VbetaResponsive      comb     CCL2 3.3431031        -1 0.1879552
## 5 CD154-VbetaResponsive      comb     CCL3 0.1401862        -1 0.9323070
## 6 CD154-VbetaResponsive      comb     CCL4 0.6305951         1 0.7295718
```

Here we compare each population (`CD154-VbetaResponsive, CD154-VbetaUnresponsive CD154+VbetaUnresponsive, VbetaResponsive, VbetaUnresponsive`) to the `CD154+VbetaResponsive` population. The `Population` column shows which population is being compared, while `test.type` is `comb` for the combined normal theory/binomial test. Column `primerid` gives the gene being tested, `direction` shows if the comparison group mean is greater (1) or less (-1) than the referent group, and `lrtstat` and `p.value` give the test statistic and χ^2 p-value (two degrees of freedom). Other options are whether additional information about the tests are returned (`returnall=TRUE`) and if the testing should be stratified by a character vector naming columns in `colData` containing grouping variables (`groups`).

These tests have been subsumed by `zlm` but remain in the package for user convenience.

4 Use with single cell RNA-sequencing data

In RNA-sequencing data is essentially no different than qPCR-based single cell gene expression, once it has been aligned and mapped, if one is willing to reduce the experiment to counts or count-like data for a fixed set of genes/features. We assume that suitable tools (eg, RSEM, Kallisto or TopHat) have been applied to do this.

An example of this use is provided in a vignette. Type `vignette('MAITAnalysis')` to view.