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 $et = c_{\text{max}} - ct$, 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.

```r
library(MAST)
library(data.table)
data(vbeta)
colnames(vbeta)
## [1] "Sample.ID"    "Subject.ID"    "Experiment.Number"
## [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 \texttt{vbeta} is a \texttt{data.frame} from which we construct the \texttt{FluidigmAssay} object. The \texttt{idvars} is the set of column(s) in \texttt{vbeta} that uniquely identify a well (globally), the \texttt{primerid} is a column(s) that specify the feature measured at this well. The \texttt{measurement} gives the column name containing the log-expression measurement, \texttt{ncells} contains the number of cells (or other normalizing factor) for the well. \texttt{geneid}, \texttt{cellvars}, \texttt{phenovars} all specify additional columns to be included in the \texttt{featureData}, \texttt{phenoData} and \texttt{cellData} (TODO: \texttt{wellData}). The output is a \texttt{FluidigmAssay} object with 456 wells and 75 features.

We can access the feature–level metadata and the cell–level metadata using the \texttt{mcols} and \texttt{colData} accessors.

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 \texttt{wellKey} are concatenated idvars columns, helping to ensure consistency when splitting and merging \texttt{SingleCellAssay} objects.

### 2.2 Importing Matrix Data

Data can also be imported in matrix format using command \texttt{FromMatrix}, and passing a matrix of expression values and \texttt{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.

```r
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
```

```r
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
```

```r
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
```

```r
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
```
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.

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

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

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

```r
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))
```

The filtering function has several other options, including whether the filter should 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)`

---

**Burden of filtering by ncells**

![Bar chart showing the burden of filtering by ncells with three bins: none, 1 to 100, and 101 or more. The majority of values fall within the none bin.]
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.

```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

<table>
<thead>
<tr>
<th>#</th>
<th>Number.of.Cells</th>
<th>Population</th>
<th>ncells</th>
<th>Subject.ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sub01 A01</td>
<td>CD154+VbetaResponsive</td>
<td>1</td>
<td>Sub01</td>
</tr>
<tr>
<td>2</td>
<td>Sub01 A02</td>
<td>CD154+VbetaResponsive</td>
<td>1</td>
<td>Sub01</td>
</tr>
<tr>
<td>3</td>
<td>Sub01 A03</td>
<td>CD154+VbetaResponsive</td>
<td>1</td>
<td>Sub01</td>
</tr>
<tr>
<td>4</td>
<td>Sub01 A04</td>
<td>CD154+VbetaResponsive</td>
<td>1</td>
<td>Sub01</td>
</tr>
<tr>
<td>5</td>
<td>Sub01 A05</td>
<td>CD154+VbetaResponsive</td>
<td>1</td>
<td>Sub01</td>
</tr>
<tr>
<td>6</td>
<td>Sub01 A06</td>
<td>CD154+VbetaResponsive</td>
<td>1</td>
<td>Sub01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>#</th>
<th>Chip.Number</th>
<th>Well</th>
<th>Stim.Condition</th>
<th>Time</th>
<th>wellKey</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sub01 A01</td>
<td>A01</td>
<td>Stim(SEB)</td>
<td>12</td>
<td>Sub01</td>
</tr>
<tr>
<td>2</td>
<td>Sub01 A02</td>
<td>A02</td>
<td>Stim(SEB)</td>
<td>12</td>
<td>Sub01</td>
</tr>
<tr>
<td>3</td>
<td>Sub01 A03</td>
<td>A03</td>
<td>Stim(SEB)</td>
<td>12</td>
<td>Sub01</td>
</tr>
<tr>
<td>4</td>
<td>Sub01 A04</td>
<td>A04</td>
<td>Stim(SEB)</td>
<td>12</td>
<td>Sub01</td>
</tr>
<tr>
<td>5</td>
<td>Sub01 A05</td>
<td>A05</td>
<td>Stim(SEB)</td>
<td>12</td>
<td>Sub01</td>
</tr>
<tr>
<td>6</td>
<td>Sub01 A06</td>
<td>A06</td>
<td>Stim(SEB)</td>
<td>12</td>
<td>Sub01</td>
</tr>
</tbody>
</table>

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.

```r
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
coeffAndCI <- summary(zlm.output, logFC=FALSE)$datatable
coeffAndCI <- coeffAndCI[,contrast != '(Intercept)',]
coeffAndCI[,contrast:=abbreviate(contrast)]

```r
ggplot(coeffAndCI, 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.

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

\begin{verbatim}
library(lme4)
lmer.output <- zlm(formula(~ Stim.Condition +(1|Subject.ID), vbeta.1, method='glmer', ebayes=FALSE)
\end{verbatim}

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 \texttt{defaultPrior}) or eliminated entirely by setting \texttt{method='glm'} in \texttt{zlm}. It is also possible to use Bayesian linear regression for the continuous component by setting \texttt{useContinuousBayes=TRUE} in \texttt{zlm}. For example:

\begin{verbatim}
orig_results <- zlm(formula(~Stim.Condition, vbeta.1)
dp <- defaultPrior('Stim.ConditionUnstim')
new_results <- zlm(formula(~Stim.Condition, vbeta.1, useContinuousBayes=TRUE, coefPrior=dp)
qplot(x=coef(orig_results, 'C')[, 'Stim.ConditionUnstim'],
\end{verbatim}
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.

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

<table>
<thead>
<tr>
<th>Population</th>
<th>test.type</th>
<th>primerid</th>
<th>lrstat</th>
<th>direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD154+VbetaUnresponsive</td>
<td>comb</td>
<td>B3GAT1</td>
<td>1.6054216</td>
<td>-1</td>
</tr>
<tr>
<td>CD154+VbetaUnresponsive</td>
<td>comb</td>
<td>BAX</td>
<td>0.3889518</td>
<td>-1</td>
</tr>
<tr>
<td>CD154+VbetaUnresponsive</td>
<td>comb</td>
<td>BCL2</td>
<td>10.6098463</td>
<td>-1</td>
</tr>
<tr>
<td>CD154+VbetaUnresponsive</td>
<td>comb</td>
<td>CCL2</td>
<td>0.7687432</td>
<td>-1</td>
</tr>
<tr>
<td>CD154+VbetaUnresponsive</td>
<td>comb</td>
<td>CCL3</td>
<td>1.9129480</td>
<td>1</td>
</tr>
<tr>
<td>CD154+VbetaUnresponsive</td>
<td>comb</td>
<td>CCL4</td>
<td>0.1862808</td>
<td>1</td>
</tr>
<tr>
<td>p.value</td>
<td></td>
<td></td>
<td>0.44811256</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.8232660</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.00496708</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.68087836</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.38424535</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.91106559</td>
<td></td>
</tr>
</tbody>
</table>

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 $\chi^2$ 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.