Robust Model-based Clustering of Flow Cytometry Data
The flowClust package

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1 Licensing

Under the Artistic License, you are free to use and redistribute this software. However, we ask you to cite the following paper if you use this software for publication.


2 Overview

We apply a robust model-based clustering approach proposed by Lo et al. (2008) to identify cell populations in flow cytometry data. The proposed approach is based on multivariate $t$ mixture models with the Box-Cox transformation. This approach generalizes Gaussian mixture models by modeling outliers using $t$ distributions and allowing for clusters taking non-ellipsoidal shapes upon proper data transformation. Parameter estimation is carried out using an Expectation-Maximization (EM) algorithm which simultaneously handles outlier identification and transformation selection. Please refer to Lo et al. (2008) for more details.

This *flowClust* package consists of a core function to implement the aforementioned clustering methodology. Its source code is built in C for optimal utilization of system resources. Graphical functionalities are available to users for visualizing a wealth of features of the clustering results, including the cluster assignment, outliers, and the size and shape of the clusters. The fitted mixture model may be projected onto any one/two dimensions and displayed by means of a contour or image plot. Currently, *flowClust* provides two options for estimating the number of clusters when it is unknown, namely, the Bayesian Information Criterion (BIC) and the Integrated Completed Likelihood (ICL).

*flowClust* is built in a way such that it is highly integrated with *flowCore*, the core package for flow cytometry that provides data structures and basic manipulation of flow cytometry data. Please read Section 4.3 for details about actual implementation.

3 Installation

3.1 Unix/Linux/Mac Users

To build the *flowClust* package from source, make sure that the following is present on your system:
- a C compiler
- GNU Scientific Library (GSL)
- Basic Linear Algebra Subprograms (BLAS)

A C compiler is needed to build the package as the core function is coded in C. GSL can be downloaded at [http://www.gnu.org/software/gsl/](http://www.gnu.org/software/gsl/). In addition, the package uses BLAS to perform basic vector and matrix operations. Please go to [http://www.netlib.org/blas/faq.html#5](http://www.netlib.org/blas/faq.html#5) for a list of optimized BLAS libraries for a variety of computer architectures. For instance, Mac users may use the built-in vecLib framework, while users of Intel machines may use the Math Kernel Library (MKL).

For the package to be installed properly you may have to type the following command before installation:

```
export LD_LIBRARY_PATH="/path/to/GSL/:/path/to/BLAS/":$LD_LIBRARY_PATH
```

which will tell R where your GSL and BLAS libraries are. Note that this may have already been configured on your system, so you may not have to do so. In case you need to do it, you may consider including this line in your `.bashrc` such that you do not have to type it every time.

If GSL is installed to some non-standard location such that it cannot be found when installing `flowClust`, you may set the environment variable `GSL_CONFIG` to point to the correct copy of `gsl-config`, for example,

```
export GSL_CONFIG="/global/home/username/gsl-1.12/bin/gsl-config"
```

For convenience sake, this line may also be added to `.bashrc`.

Now you are ready to install the package:

```
R CMD INSTALL flowClust_x.y.z.tar.gz
```

The package will look for a BLAS library on your system, and by default it will choose gslcblas, which is not optimized for your system. To use an optimized BLAS library, you can use the `--with-blas` argument which will be passed to the `configure.ac` file. For example, on a Mac with vecLib pre-installed the package may be installed via:

```
R CMD INSTALL flowClust_x.y.z.tar.gz --configure-args="--with-blas=-framework vecLib"
```

On a 64-bit Intel machine which has MKL as the optimized BLAS library, the command may look like:

```
R CMD INSTALL flowClust_x.y.z.tar.gz --configure-args="--with-blas='-L/usr/local/mkl/lib/em64t/ -lmkl -lguide -lpthread'"
```

where `/usr/local/mkl/lib/em64t/` is the path to MKL.

If you prefer to install a prebuilt binary, you need GSL for successful installation.
3.2 Windows Users

You need the GNU Scientific Library (GSL) for the flowClust package. GSL is freely available at http://gnuwin32.sourceforge.net/packages/gsl.htm for Windows distributions.

To install a prebuilt binary of flowClust and to load the package successfully you need to tell R where to link GSL. You can do that by adding /path/to/libgsl.dll to the Path environment variable. To add this you may right click on “My Computer”, choose “Properties”, select the “Advanced” tab, and click the button “Environment Variables”. In the dialog box that opens, click “Path” in the variable list, and then click “Edit”. Add /path/to/libgsl.dll to the “Variable value” field. It is important that the file path does not contain any space characters; to avoid this you may simply use the short forms (8.3 DOS file names) found by typing dir /x at the Windows command line. For example, the following may be added to the Path environment variable:

C:\PROGRA~1/GNUWIN32/bin

and the symbol ; is used to separate it from existing paths.

To build flowClust from source (using Rtools), in addition to adding /path/to/libgsl.dll to Path, you need to tell flowClust where your GSL library and header files are. You can do that by setting up two environment variables GSL_LIB and GSL_INC with the correct path to the library files and header files respectively. You can do this by going to the “Environment Variables” dialog box as instructed above and then clicking the “New” button. Enter GSL_LIB in the “Variable name” field, and /path/to/your/gsl/lib/directory in the “Variable value” field. Likewise, do this for GSL_INC and /path/to/your/gsl/include/directory. Remember to use “/” instead of “\” as the directory delimiter.

You can download Rtools at http://www.murdoch-sutherland.com/Rtools/ which provides the resources for building R and R packages. You should add to the Path variable the paths to the various components of Rtools. Please read the “Windows Toolset” appendix at http://cran.r-project.org/doc/manuals/R-admin.html#The-Windows-toolset for more details.

4 Example: Clustering of the Rituximab Dataset

4.1 The Core Function

To demonstrate the functionality we use a flow cytometry dataset from a drug-screening project to identify agents that would enhance the anti-lymphoma activity of Rituximab, a therapeutic monoclonal antibody. The dataset is an object of class flowFrame; it consists of eight parameters, among them only the two scattering parameters (FSC.H, SSC.H) and two fluorescence parameters (FL1.H, FL3.H) are of interest in this experiment. Note that, apart from a typical matrix or data.frame object, flowClust may directly take a flowFrame, the standard R implementation of an FCS file, which may be returned from the
read.FCS function in the flowCore package, as data input. The following code performs an analysis with one cluster using the two scattering parameters:

```r
> library(flowCore)
> library(flowClust)
> data(rituximab)
> summary(rituximab)
```

<table>
<thead>
<tr>
<th></th>
<th>Min.</th>
<th>1st Qu.</th>
<th>Median</th>
<th>Mean</th>
<th>3rd Qu.</th>
<th>Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSC.H</td>
<td>59.0</td>
<td>178.0</td>
<td>249.0</td>
<td>287.1</td>
<td>331.0</td>
<td>1023.0</td>
</tr>
<tr>
<td>SSC.H</td>
<td>11.0</td>
<td>130.0</td>
<td>199.0</td>
<td>251.8</td>
<td>307.0</td>
<td>1023.0</td>
</tr>
<tr>
<td>FL1.H</td>
<td>0.0</td>
<td>55.0</td>
<td>116.0</td>
<td>126.4</td>
<td>185.0</td>
<td>705.0</td>
</tr>
<tr>
<td>FL2.H</td>
<td>0.0</td>
<td>150.0</td>
<td>203.0</td>
<td>258.3</td>
<td>315.0</td>
<td>1023.0</td>
</tr>
<tr>
<td>FL3.H</td>
<td>1.0</td>
<td>0.00</td>
<td>0.00</td>
<td>8.00</td>
<td>17.6</td>
<td>444.0</td>
</tr>
<tr>
<td>FL1.A</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>444.0</td>
</tr>
<tr>
<td>FL1.W</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>598.0</td>
</tr>
<tr>
<td>Time</td>
<td>2</td>
<td>140</td>
<td>285</td>
<td>294</td>
<td>451</td>
<td>598</td>
</tr>
</tbody>
</table>

```r
> res1 <- flowClust(rituximab, varNames=c("FSC.H", "SSC.H"), K=1, B=100)
```

B is the maximum number of EM iterations; for demonstration purpose here we set a small value for B. The main purpose of performing an analysis with one cluster here is to identify outliers, which will be removed from subsequent analysis.

Next, we would like to proceed with an analysis using the two fluorescence parameters on cells selected from the first stage. The following code performs the analysis with the number of clusters being fixed from one to six in turn:

```r
> rituximab2 <- rituximab[rituximab %in% res1,]
> res2 <- flowClust(rituximab2, varNames=c("FL1.H", "FL3.H"), K=1:6, B=100)
```

We select the best model based on the BIC. Values of the BIC can be retrieved through the criterion method. By inspection, the BIC values stay relatively constant beyond three clusters. We therefore choose the model with three clusters and print a summary of the corresponding clustering result:

```r
> criterion(res2, "BIC")
```

```
[1] -34167.12 -33103.77 -33065.41 -32998.05 -32993.74 -32942.54
> summary(res2[[3]])
```

** Experiment Information **
Experiment name: Flow Experiment
Variables used: FL1.H FL3.H
** Clustering Summary **
Number of clusters: 3
Proportions: 0.1784141 0.5975411 0.2240448
** Transformation Parameter **
lambda: 0.472139
** Information Criteria **
Log likelihood: -16467.68
BIC: -33065.41
ICL: -33795.22
** Data Quality **
Number of points filtered from above: 0 (0%)
Number of points filtered from below: 0 (0%)
Rule of identifying outliers: 90% quantile
Number of outliers: 102 (7.42%)
Uncertainty summary:

The summary states that the rule used to identify outliers is 90% quantile, which means that a point outside the 90% quantile region of the cluster to which it is assigned will be called an outlier. To specify a different rule, we make use of the ruleOutliers replacement method. The example below applies the more conservative 95% quantile rule to identify outliers:

> ruleOutliers(res2[[3]]) <- list(level=0.95)

Rule of identifying outliers: 95% quantile

> summary(res2[[3]])

** Experiment Information **
Experiment name: Flow Experiment
Variables used: FL1.H FL3.H
** Clustering Summary **
Number of clusters: 3
Proportions: 0.1784141 0.5975411 0.2240448
** Transformation Parameter **
lambda: 0.472139
** Information Criteria **
Log likelihood: -16467.68
BIC: -33065.41
ICL: -33795.22
** Data Quality **
Number of points filtered from above: 0 (0%)
Number of points filtered from below: 0 (0%)
Rule of identifying outliers: 95% quantile
Number of outliers: 38 (2.77%)
Uncertainty summary:

We can also combine the rule set by the z.cutoff argument to identify outliers. Suppose we would like to assign an observation to a cluster only if the associated posterior probability is greater than 0.6. We can add this rule with the following command:

> summary(res2[[3]])
Rule of identifying outliers: 95% quantile, probability of assignment < 0.6

> summary(res2[[3]])

** Experiment Information **
Experiment name: Flow Experiment
Variables used: FL1.H FL3.H

** Clustering Summary **
Number of clusters: 3
Proportions: 0.1784141 0.5975411 0.2240448

** Transformation Parameter **
lambda: 0.472139

** Information Criteria **
Log likelihood: -16467.68
BIC: -33065.41
ICL: -33795.22

** Data Quality **
Number of points filtered from above: 0 (0%)
Number of points filtered from below: 0 (0%)
Rule of identifying outliers: 95% quantile, probability of assignment < 0.6

Number of outliers: 94 (6.84%)
Uncertainty summary:

This time more points are called outliers. Note that such a change of the rule will not incur a change of the model-fitting process. The information about which points are called outliers is conveyed through the flagOutliers slot, a logical vector in which the positions of TRUE correspond to points being called outliers.

By default, when 10 or more points accumulate on the upper or lower boundary of any parameter, the flowClust function will filter those points. To change the threshold count from the default, users may specify max.count and min.count when running flowClust. To suppress filtering at the upper and/or the lower boundaries, set max.count and/or min.count as -1. We can also use the max and min arguments to control filtering of points, but from a different perspective. For instance, if we are only interested in cells which have a FL1.H measurement within (0, 400) and FL3.H within (0, 800), we may use the following code to perform the cluster analysis:

> flowClust(rituximab2, varNames=c("FL1.H", "FL3.H"), K=2, B=100, min=c(0,0), max=c(400,800))

4.2 Visualization of Clustering Results

Information such as the cluster assignment, cluster shape and outliers may be visualized by calling the plot method to make a scatterplot:
The `level` and/or `z.cutoff` arguments are needed when we want to apply a rule different from that stored in the `ruleOutliers` slot of the `flowClust` object to identify outliers.

To look for densely populated regions, a contour/image plot can be made:

```R
> plot(res2[[3]], data=rituximab2, level=0.8, z.cutoff=0)
```

Rule of identifying outliers: 80% quantile
When we want to examine how the fitted model and/or the data are distributed along one chosen dimension, we can use the `hist` method:
Likewise, the effort has been made to integrate the package with flowCore.

> hist(res2[[3]], data=rituximab2, subset="FL1.H")

The subset argument may also take a numeric value:

> hist(res2[[3]], data=rituximab2, subset=1)

Since FL1.H is the first element of res2[[3]]@varNames, this line produces exactly the same histogram as the one generated by the line taking subset="FL1.H". Likewise, the subset argument of both plot methods accepts either a numeric or a character vector to specify which two variables are to be shown on the plot.

### 4.3 Integration with flowCore

As mentioned in Overview, effort has been made to integrate flowClust with the flowCore package. Users will find that most methods defined in flowCore also work in the context of flowClust.
The very first step of integration is to replace the core function `flowClust` with a call to the constructor `tmixFilter` followed by the `filter` method. The aim is to wrap clustering in a filtering operation like those found in `flowCore`. The `tmixFilter` function creates a `filter` object to store all settings required for the filtering operation. The object created is then passed to the actual filtering operation implemented by the `filter` method. The use of a dedicated `tmixFilter`-class object separates the task of specifying the settings (tmixFilter) from the actual filtering operation (`filter`), facilitating the common scenario in FCM gating analysis that filtering with the same settings is performed upon a set of data files.

As an example, the filtering operation that resembles the second-stage clustering using FL1.H and FL3.H with three clusters (see Section 4.1) is implemented by the following code:

```r
> s2filter <- tmixFilter("s2filter", c("FL1.H", "FL3.H"), K=3, B=100)
> res2f <- filter(rituximab2, s2filter)
```

The object `res2f` is of class `tmixFilterResult`, which extends the `multipleFilterResult` class defined in `flowCore`. Users may apply various subsetting operations defined for the `multipleFilterResult` class in a similar fashion on a `tmixFilterResult` object:

```r
> Subset(rituximab2, res2f)
```

`flowFrame` object 'A02' with 1272 cells and 8 observables:

```
name desc range maxRange minRange
$P1 FSC.H FSC-Height 1024 1023 0
$P2 SSC.H Side Scatter 1024 1023 0
$P3 FL1.H Anti-BrdU FITC 1024 1023 0
$P4 FL2.H <NA> 1024 1023 0
$P5 FL3.H 7 AAD 1024 1023 0
$P6 FL1.A <NA> 1024 1023 0
$P7 FL1.W <NA> 1024 1023 0
$P8 Time Time (204.80 sec.) 1024 1023 0
```

135 keywords are stored in the 'description' slot

```r
> split(rituximab2, res2f, population=list(sc1=1:2, sc2=3))
```

`$sc1`

`flowFrame` object 'A02 (1,2)' with 1064 cells and 8 observables:

```
name desc range maxRange minRange
$P1 FSC.H FSC-Height 1024 1023 0
$P2 SSC.H Side Scatter 1024 1023 0
$P3 FL1.H Anti-BrdU FITC 1024 1023 0
$P4 FL2.H <NA> 1024 1023 0
$P5 FL3.H 7 AAD 1024 1023 0
```

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The Subset method above outputs a flowFrame consisting of observations within the data-driven filter constructed. The split method separates the data into two populations upon the removal of outliers: the first population is formed by observations assigned to clusters 1 and 2 constructed by the filter, and the other population consists of observations assigned to cluster 3. The two populations are returned as two separate flowFrame’s, which are stored inside a list and labelled with sc1 and sc2 respectively.

The %in% operator from flowCore is also defined for a tmixFilterResult object. A logical vector will be returned in which a TRUE value means that the corresponding observation is accepted by the filter. In fact, the implementation of the Subset method needs to call %in%.

The object returned by tmixFilter is of class tmixFilter, which extends the filter class in flowCore. Various operators, namely, &, |, ! and %subset%, which have been constructed for filter objects in flowCore, also produce similar outcomes when applied to a tmixFilter object. For example, to perform clustering on a subset of data enclosed by a rectangle gate, we may apply the following code:

```r
> rectGate <- rectangleGate(filterId="rectRegion", "FL1.H"=c(0, 400), "FL3.H"=c(0, 800))
> MBCfilter <- tmixFilter("MBCfilter", c("FL1.H", "FL3.H"), K=2, B=100)
> filter(rituximab2, MBCfilter %subset% rectGate)
```

A filterResult produced by the filter named 'MBCfilter in rectRegion' resulting in multiple populations:

```plaintext
2
1
```
4.4 Using Bayesian Priors

The latest version of flowClust now supports priors through a Multivariate Normal-Inverse Wishart model.

One way to construct a prior is to use an existing flowClust model fit. For example, we can construct a prior from the rituximab fit. Then we can adjust the prior so that the FL1-/FL3- population is centered at 200,200 \textit{a priori}, and both populations have an \textit{a priori} smaller variance.

The parameter kappa acts as a weighting factor for the prior, while Nt controls the number of equivalent observations for the prior.

```r
> prior<-flowClust2Prior(res2[[2]], kappa=1, Nt=5000)
> prior2<-prior
> prior2$Mu0[1,]<-rep(box(200,prior2$lambda),2)
> prior2$Lambda0<-prior2$Lambda0/2
> pfit2<-flowClust(rituximab2, varNames=c("FL1.H", "FL3.H"), K=2, prior=prior2, usePrior="yes")
> par(mfrow=c(1,2))
> plot(res2[[2]], data=rituximab2)
> plot(pfit2, data=rituximab2)

Rule of identifying outliers: 90% quantile

> plot(pfit2, data=rituximab2)

Rule of identifying outliers: 90% quantile
```