

flowPloidy: Getting Started

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Overview

This document provides a walk-through of the normal workflow for analyzing flow cytometry (FCM) histograms with flowPloidy. See the vignette “flowPloidy: Flow Cytometry Histograms” (aka “histogram-tour”) for a general overview of FCM histograms.

Installation

Stable Release

flowPloidy is part of the BioConductor repository. You can install the stable version of the package directly from there. First, install bioconductor in your R session:

```
if (!requireNamespace("BiocManager", quietly = TRUE))  
  install.packages("BiocManager")
```

Then install flowPloidy:

```
BiocManager::install("flowPloidy")
```

The examples in this vignette, and the flowPloidy help files, use data in the flowPloidyData package, which is also on BioConductor. (You don't need flowPloidyData to use flowPloidy on your own data, only for the examples).

```
BiocManager::install("flowPloidyData")
```

Development Release

The Bioconductor system emphasizes stable packages. As a consequence, updates are only released twice a year. If you need the latest version of flowPloidy, you may prefer to use the developmental version. This is available directly from the GitHub repository, but requires a few more steps to install.

First, you need to install bioconductor, if you haven't already:

```
if (!requireNamespace("BiocManager", quietly=TRUE))  
  install.packages("BiocManager")  
BiocManager::install()
```

Next, you'll need to install two dependencies from bioconductor:

```
BiocManager::install("flowCore")  
BiocManager::install("flowPloidyData")
```

You also need the devtools package, if you don't have it already:

```
install.packages("devtools", dependencies = TRUE)
```

Now you can use devtools to import the latest version of flowPloidy direct from the repository:

```
library("devtools")
install_github("plantarum/flowPloidy", dependencies = TRUE,
              build_vignettes = TRUE)
```

This *should* install flowPloidy along with all its dependencies. However, sometimes R gets confused and will complain about missing packages, even though we've asked it to automatically install everything that flowPloidy needs (i.e., dependencies = TRUE). If you see messages in the terminal indicating a package name that isn't found, try installing that package directly and then re-run the install_github line above. You may need to repeat this process several times to get all the dependencies installed.

Loading the Package and Importing Data

You only need to install the package once (other than updating the package to get new versions as they are released). Once that's done, you can load it from your R session:

```
library(flowPloidy)
```

Once you have the package loaded, you're ready to process your files. You can load single files, but we usually process a directory-full of FCM files at once. For the purposes of this tutorial, we'll use the sample files provided in the flowPloidyData package, which we installed in the previous section.

```
library(flowPloidyData)
```

After loading this package, you'll have access to the function flowPloidyFiles(). This function returns the full paths to the sample data files on your system. Whenever we use it in the examples below, you can substitute a vector containing the paths to your FCM data files. Subsetting output (i.e., flowPloidyFiles()[1]) produces a single file name.

NOTE

To generate a list of your own files, use the R function list.files(). For example, if all your files are the directory ~/flow/data/, you can create a vector containing your files with:

```
my.files <- list.files("~/flow/data", full.names = TRUE)
```

If the directory contains additional files, you can provide a pattern to match only your FCM files. For example, if your FCM files have the suffix .LMD, you could use this:

```
my.files <- list.files("~/flow/data", full.names = TRUE, pattern = ".LMD")
```

Once you've generated a list of files, you can pass it to batchFlowHist as we do with flowPloidyFiles() in the following example.

Before we can load our flow data, we need to know which channel to use for our histogram. We can see a list of all channel names with the function viewFlowChannels:

```
viewFlowChannels(flowPloidyFiles()[1])
```

```
## [1] "FS.INT.LIN"   "SS.INT.LIN"   "TIME"         "FL3.INT.LIN"  "FL3.PEAK.LIN"
## [6] "FS.PEAK.LIN"  "SS.PEAK.LIN"  "FS.TOF.LIN"   "SS.TOF.LIN"
```

```
## or viewFlowChannels("my-flow-file.LMD")
```

For our flow cytometer, the primary data channel is called “FL3.INT.LIN”. Knowing this, we can now import all the files in a directory with the function `batchFlowHist()`:

```
batch1 <- batchFlowHist(flowPloidyFiles(), channel = "FL3.INT.LIN")
## 1: processing /Library/Frameworks/R.framework/Versions/4.5-x86_64/Resources/library/flow
## analyzing 188-15.LMD
##
## 2: processing /Library/Frameworks/R.framework/Versions/4.5-x86_64/Resources/library/flow
## analyzing 222.LMD
##      *** Model Fit Needs Attention: 222.LMD ***
##
## 3: processing /Library/Frameworks/R.framework/Versions/4.5-x86_64/Resources/library/flow
## analyzing 240+S.LMD
##      *** Model Fit Needs Attention: 240+S.LMD ***
```

[... output truncated to save space ...]

The output indicates that there were problems with some of the histograms (i.e., `*** Model Fit Needs Attention ***`). This is expected, and we’ll correct the problems in the next step.

Reviewing and Correcting Histogram Analyses

The function `browseFlowHist()` provides an interactive way to view and correct our analyses.

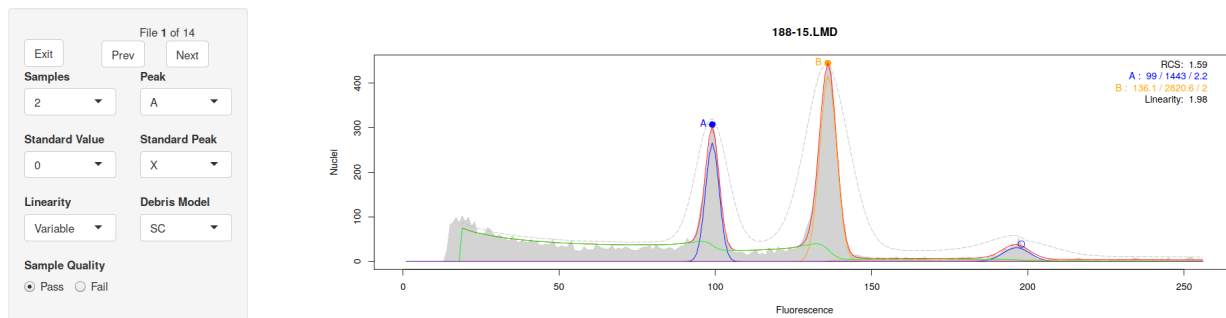
IMPORTANT

To save your corrections, you need to store the output of this function in a variable. For example `myresults <- browseFlowHist(batch1)` will store the updated results in the variable `myresults`. It’s often easiest to update the analyses ‘in place’, assigning the output of `browseFlowHist()` to the variable you pass to it as an argument, as in the following example.

```
batch1 <- browseFlowHist(batch1) ## update the histograms in batch1
```

Histogram presentation

Calling `browseFlowHist()` opens a window in your internet browser that will display your histograms. The first one looks like this:



On the right we see the histogram. The graphical elements are:

- **shaded area:** the raw data
- **dotted gray line:** the initial model estimate
- **red line:** the fitted model
- **green line:** the fitted debris component
- **blue line:** the fitted components for the G1 and G2 peaks of sample A
- **orange line:** the fitted components for the G1 and G2 peaks of sample B (the G2 peak is not visible on this plot)
- **magenta line:** the fitted s-phase component for sample A (too small to see in this plot)
- **turquoise line:** the fitted s-phase component for sample B (too small to see in this plot)
- **purple line:** the fitted aggregate component (too small to see in this plot)

You also see coloured circles. These are the initial peak estimates, blue for sample A, and orange for sample B. The solid circles are the G1 estimates, and there is a hollow circle to indicate the G2 peak (only visible for sample A in this plot).

The upper right corner of the plot contains some additional information regarding the model fit:

- **RCS:** the residual Chi-Square value. This is a rough goodness-of-fit value. Bagwell (1993) suggests that values between 0.7 and 4 indicate an acceptable model fit. See `?RCS` for more details in the online manual.
- **A and B:** the parameter estimates for samples A and B. The three numbers are the peak position, the number of nuclei counted, and the coefficient of variation.
- **Linearity:** The linearity parameter. This is the ratio between G1 and G2 peaks. By default, we fit it as a model parameter bounded between 1.5 and 2.5¹.

For this example, the default settings have worked perfectly. The model fit is acceptable (RCS 1.59), and the CVs for the peaks are well below the usual 5% threshold for acceptable.

One thing we don't see is an indication of which peak is the standard, and which is our unknown sample. That is because `flowPloidy` doesn't know, and has no way to infer this automatically. You will need to indicate this yourself, based on your understanding of the expected peak sizes and ratios, and perhaps running the standard on its own without an unknown. You can tell `flowPloidy` which peak is the standard by selecting the value in the "Standard Peak" drop-down selector, on the left side of the plot. By default this is "X", indicating the standard has not been identified. If you set it to "A" or "B", the value will be stored in the results. If you don't set the value, you will still be able to recover the peak ratio from your results, but will have to calculate the pg values yourself. We'll return to this below.

¹Linearity should be 2, but local environmental conditions may cause your flow cytometer to drift, and it may vary between days, or even from the beginning to the end of a run. If you don't have G2 peaks in your data, it won't matter at all. In general, you can ignore this, unless you are interested in developing new model components (in which case, see `?ModelComponent`).

We'll ignore the other options in the navigation panel for now (and the gating interface below), but will discuss them further below. For now, click the “Next” button to move on to the next histogram.

Correcting a Failed Model Fit

The second histogram didn't work at all:



Since the model fitting failed, the plot shows only the raw data and the initial estimates. The fitting parameters are also absent.

Looking at the initial estimates, it's easy to see what went wrong - a false peak in the noise near position 60 was incorrectly identified as a G1 peak. We can correct this by re-positioning the peak estimates. The “Peak” drop-down list at the left allows you to select which peak to move, and left-clicking on the plot will set the new position for that peak.

Moving Peak B to the second big peak, and Peak A to the first peak, the analysis works as expected:



In most cases, the exact placement of the initial peak isn't critical. As long as you put it somewhere near the top of the appropriate peak, flowPloidy will be able to find the true value. You can confirm this by clicking around within the peaks: you end up with the same result (within a small rounding error) for quite a range of initial peak positions.

Note that the A peak must have a lower mean (i.e., be further left on the histogram) than the B peak. If you try to put the A peak above the B peak, flowPloidy will swap them around for you, to make the lower peak A and the upper peak B. This is necessary for some of the internal model-fitting code to work properly.

Changing Model Components: Debris Model

Moving on to histogram 6, “248+r.LMD”, we see that the model fit succeeded, and the fitted components are added to our plot:

File 6 of 14

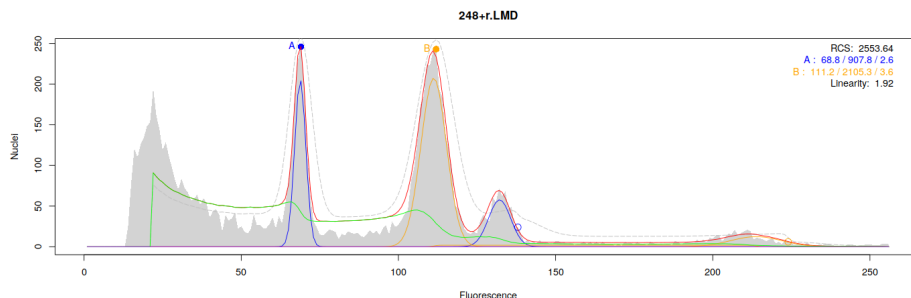
Exit Prev Next

Samples: 2 Peak: B

Standard Value: 0 Standard Peak: X

Linearity: Variable Debris Model: SC

Sample Quality: ☒ Pass ☐ Fail



However, the RCS value is very high - 2553! Looking at the model components, notice the gap between the raw data and the fitted between the G1 peaks for A and B (between 75 and 100). The largest fitted component in this region is the green debris curve. By default, it uses the Single-Cut model. We can change to the Multi-Cut model by selecting “MC” from the drop-down list under “Debris Model” in the control panel at the left.

File 6 of 14

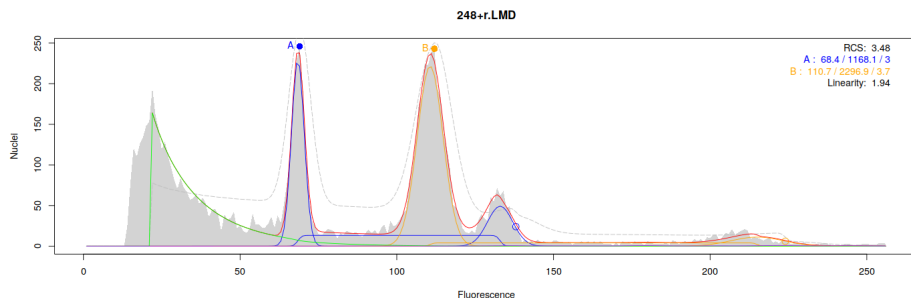
Exit Prev Next

Samples: 2 Peak: B

Standard Value: 0 Standard Peak: X

Linearity: Variable Debris Model: MC

Sample Quality: ☒ Pass ☐ Fail



With the Multi-Cut debris model, we have a much more sensible RCS value. You can also see the improved fit visually - note the top of the fitted model is much closer to the raw data with the “MC” debris model, especially in the region between 80 and 140.

The most appropriate way to model histogram debris varies for different samples. As botanists, we will analyze samples from many different species, tissue types, and using different preparation protocols. In some cases, the Single Cut debris model will be most effective; in others, the Multi-Cut model works better. When your analysis produces high RCS values, as in this case, it may be a clue that the other debris model is more appropriate.

For the nitty-gritty details of the debris models, see ?DebrisModels. In practice, use whichever one gives you a lower RCS value. Also keep in mind that there is usually only a very small difference between the two options when it comes to peak means and CVs, even if the difference in RCS can be very large. Note also that RCS is sensitive to a number of factors, not all of which are related to model fit (see ?RCS). So getting a very high RCS value doesn’t necessarily mean you can’t use your data; use your judgement, and if in doubt, re-run the sample.

Local Minima Traps

Despite its power, non-linear regressions can get stuck in ‘local minima’. Unlike linear regression, there is no single unique solution to a non-linear regression. Fitting a model requires testing out different parameters before determining which combination is best. Sometimes, the algorithm gets stuck on incorrect parameters. Luckily, this is usually easily detected by humans.

An example is file 11, “734.LMD” in our sample data:

File 11 of 14

Exit Prev Next

Samples: 2 Peak: B

Standard Value: 0 Standard Peak: X

Linearity: Variable Debris Model: SC

Sample Quality: ☒ Pass ☐ Fail



The real G1 Peak for sample B has been ignored, but the model ‘succeeded’ anyways. The RCS value is Inf (infinity), which is another clue that something has gone wrong here.

Once again, we need to correct our peak estimates. If we move the B peak over we get the following fit:

File 11 of 14

Exit Prev Next

Samples: 2 Peak: A

Standard Value: 0 Standard Peak: X

Linearity: Variable Debris Model: SC

Sample Quality: ☒ Pass ☐ Fail



That’s better - the model has fit all the peaks in our data, and the RCS value has come back down to an acceptable value. You need to be aware of these problems, and visually confirm the model fit for each of your histograms.

Annotating Histograms and Excluding Results

You may encounter a histogram that is successfully fit by flowPloidy, that you want to exclude from your data. Maybe it had extra peaks (such as from contaminants), or flowPloidy has fit peaks that you suspect are just noise in the debris. In cases like this, you can select the Fail button under the Sample Quality option on the control panel. This option instructs flowPloidy to exclude model fit data for this sample when exporting the results.

When you do this, you may want to leave yourself a note. That’s what the Annotation box at the bottom of the histogram is for. You can enter any text you like in this box, and after you press the **Annotation** button, it will be stored with that histogram, and included when you export the results (next section).

Exporting Results

After you’ve reviewed your analyses, you’ll want to export the results. To do this, you need to return to the R console. Click the Exit button in the upper-left corner of the navigation panel.

This will close the interactive GUI - you can now close that browser tab. **Do not close the browser tab without clicking exit!** If you do so, you’ll lose all your corrections.

You may also have noticed that while you’re reviewing your histograms in the browser, your R console is busy. You can’t interact with the R console until you’ve exited from the histogram browser. Should you accidentally close your browser tab without exiting, entering C-c in the R console should restore the console (without saving the edits you made!). Note this is what I do on Linux, for Windows and Mac you



Figure 1: Annotating a Failed Histogram

may need to use a different key to cancel the browser loop. If you are using RStudio, there's a little red "stop sign" button at the top of the console window you can click.

Assuming we've made all of our edits and exited cleanly from the browser window, the updated histogram data is now stored in our variable. We can retrieve a summary table with the function `tabulateFlowHist()`:

```
tabulateFlowHist(batch1)[1:4, ]
```

##	RCS	a_mean	a_stddev	linearity	b_mean	b_stddev
## 188-15.LMD	1.588616	99.04269	2.165241	1.982773	136.1169	2.701666
## 222.LMD	NA	NA	NA	NA	NA	NA
## 240+S.LMD	NA	NA	NA	NA	NA	NA
## 240-4-2+rad.LMD	1.960359	64.59736	1.815124	2.072776	108.2810	3.823363
##	a1_count	a2_count	a_s_count	b1_count	b_s_count	b2_count
## 188-15.LMD	1442.9880	336.5806	0.00000	2820.638	405.8704	NA
## 222.LMD	NA	NA	NA	NA	NA	NA
## 240+S.LMD	NA	NA	NA	NA	NA	NA
## 240-4-2+rad.LMD	451.6515	639.2178	95.85512	690.657	0.0000	100.4062
##	a_CV	b_CV				
## 188-15.LMD	0.02186169	0.01984814				
## 222.LMD	NA	NA				
## 240+S.LMD	NA	NA				
## 240-4-2+rad.LMD	0.02809905	0.03530964				

To save space, I've limited the output to the first four samples. Also, due to the way vignettes are generated, these are the uncorrected model fits. Your output may differ depending on the changes you made in the interactive browser.

The columns in this table may include:

- **StdPeak:** which peak (A or B) was the standard (only present if you indicated which peak was the standard using the Standard Peak dropdown, see below)
- **ratio:** the ratio of the sample size/standard size (also not always present, see below)
- **StdSize:** the size of the standard, if known (discussed below)
- **pg:** the estimated genome content for the unknown sample. Requires that the standard peak is identified, and the standard size has been set (see below)
- **RCS:** the Residual Chi-Square value
- ****_mean:**** the peak size for each sample

- ****_stddev:**** the standard deviation for the G1 peak of sample
- ****_count:**** the number of nuclei modeled for the G peak of sample . e.g., the number of nuclei in the G2 peak of sample B is b2_count and B (and C, in cases where three samples are co-chopped together)
- ****_s_count:**** the number of nuclei modeled for the s-phase of sample
- ****_CV:**** the coefficient of variation for the G1 peak of sample
- **linearity:** the linearity parameter (see below)

Note that the actual columns present will depend on your analysis. You can't count on the columns being in a particular order, so reference them by name, not number.

Printing this table to your console isn't very useful. You'll probably want to store it in a variable, so you can extract the values for further analysis:

```
results <- tabulateFlowHist(batch1)
```

Additionally, if you include a file argument, the table will be saved in csv format to that file:

```
results <- tabulateFlowHist(batch1, file = "FCMresults.csv")
```

At this point, you have your raw FCM data files, as produced by your flow cytometer, and you have the results of your histogram analyses. You don't have a permanent record of the actual model fits. If you want that, you'll need to save the object returned from `browseFlowHist()`.

```
save(batch1, file = "my-FCM-analyses.Rdata")
```

Note that this is a binary file format - you'll need to open it in R to access the data. We don't normally do this, as the FlowHist objects can be quite large, and if we need to we can quickly reload and review any histogram from the raw data files.

Size Standards

Up to this point, we've largely ignored size standards. There are three ways to incorporate size standards into your flowPloidy analysis.

You could ignore them entirely, as we have so far. If you do, the data returned by `tabulateFlowHist()` will include only the relative G1 peak positions for your samples, from which you can determine which one is the standard, based on the expected ratios for your study. This is a bit tedious.

A better option is telling flowPloidy the size of your standard when you load your files. You do this by providing the appropriate value of the standard as the standards argument to `batchFlowHist()`. For example, if we used *Raphananus sativus* Saxa, (genome size 1.11pg) as our standard, we'd call `batchFlowHist()` as:

```
batch1 <- batchFlowHist(flowPloidyFiles(), channel = "FL3.INT.LIN",
                        standards = 1.11)
```

This will set the standard size to 1.11pg for all samples. If there are any samples in the batch that use a different standard, or that you wish to exclude from automatic generation of the pg value for any reason, you can manually set the standard size to 0 to indicate this. You will still need to identify which peak is the standard. Once you've done that, the pg value will be calculated for you when you run `tabulateFlowHist()`.

If you have used more than one standard for different samples in batch, you can pass a vector of all the possible standard values to `batchFlowHist()`. For example, if some of your samples used *Raphananus sativus* Saxa, and others used *Pisum sativum* Ctirad (9.09pg), we'd call `batchFlowHist()` as:

```
batch1 <- batchFlowHist(flowPloidyFiles(), channel = "FL3.INT.LIN",
                        standards = c(1.11, 9.09))
```

Called like this, when you use `browseFlowHist()` the Standard Value dropdown list will have three options: 0 (for samples that you don't wish to have the pg value calculated), 1.11, and 9.09. Once again, any samples for which you have set the Standard Value and identified the Standard Peak will have the pg values calculated when you call `tabulateFlowHist()`.

Gating

One of our main motivations in writing `flowPloidy` was to minimize the need for gating data prior to analysis. Gating is unavoidably subjective, particularly as the approach to gating used in a particular study is rarely described in much (or any) detail.

That said, for some species it is unavoidable. The following example illustrates one such case, *Vaccinium oxycoccos*. As you'll see, the G1 peaks in this species are typically obscured by large amounts of debris. With some relatively straightforward gating, however, we can still use the model-fitting approach to determining genome size.

We'll use a single file for this example: `fpVac()`. Again, this is provided by the `flowPloidyData` package. As before, we start by determining the correct data channel to use:

```
viewFlowChannels(fpVac())
```

```
## [1] "FSC.H" "SSC.H" "FL1.H" "FL2.H" "FL3.H" "FL2.A" "FL2.W" "Time"
```

This data came from a different flow cytometer, so here we'll use the "FL2.A" channel:

```
vac <- batchFlowHist(fpVac(), channel = "FL2.A")
```

```
## 1: processing /Library/Frameworks/R.framework/Versions/4.5-x86_64/Resources/library/flowPloidy
```

```
## analyzing vac
```

```
##
```

Now we can take a look at the histogram:

```
vac <- browseFlowHist(vac)
```



That's terrible. The debris curve has completely obscured the histogram. To correct this we'll need to apply a gate, using the interface we've ignored up to now.

The Gating Interface

At the bottom of the browser window, click the Gating tab, and you'll see this:



At the left side is a simple control panel. We can select the X and Y variables using the dropdown menus. All of the data channels in your raw data are available. The zoom slider allows you to zoom in on the lower portion of the plot.

The Y axis dropdown allows you to toggle between two plotting relationships. Y/X plots your chosen X channel on the X axis, and the ratio of the Y and X channels on the Y axis. This is often useful in ‘straightening out’ the plot, making it easier to see the boundaries between cell populations. If you prefer, you can set the Y axis drop down to Y instead, which will give you the usual $Y \sim X$ plot.

Using the Y/X ratio is also useful in this context, because the interface we’re using (built with the R Shiny package) only allows for rectangular selections. So ‘straightening out’ our plot simplifies fitting our target points into a rectangular space.

The final element on the control panel is the Set Gate button. Clicking that will apply the current gate to our data (or remove an existing gate if none is selected).

Interpreting the Gate Plot

On the lower right we have three plot windows. The left-most is the actual gate window, which displays the raw data. The middle window is a preview of the raw data after the gate is applied. The right-most window shows the gate ‘residuals’, the complement of the middle window - it shows a histogram of all the observations that are *excluded* by the gate.

The plot we’re most interested in is the one on the left. For most of our gating, we set the X-axis to the fluorescence data channel; in this case that’s FL2.A. We plot side scatter (actually, the ratio side scatter/fluorescence) on the Y-axis.

The amount of fluorescence emitted by each particle tells us how much dye they have incorporated; we expect clusters corresponding to the G1 nuclei of our sample and the standard. There may also be clusters for the G2 nuclei. This is exactly what we saw in the previous examples where we didn’t need gating: the unaltered histograms were clear enough to fit our model and extract the parameters we need to determine genome size.

In this case, we have a lot of debris particles that are emitting the same fluorescence intensities as our intact nuclei, so we need another parameter to separate them. That’s where side scatter comes in. Side scatter is a measure of ‘optical complexity’ or ‘granularity’. Simply put, it tells us something about the shape of the particles. And our nuclei will have a characteristic shape. Typically, this shape has lower ‘optical complexity’ than the irregular debris particles.

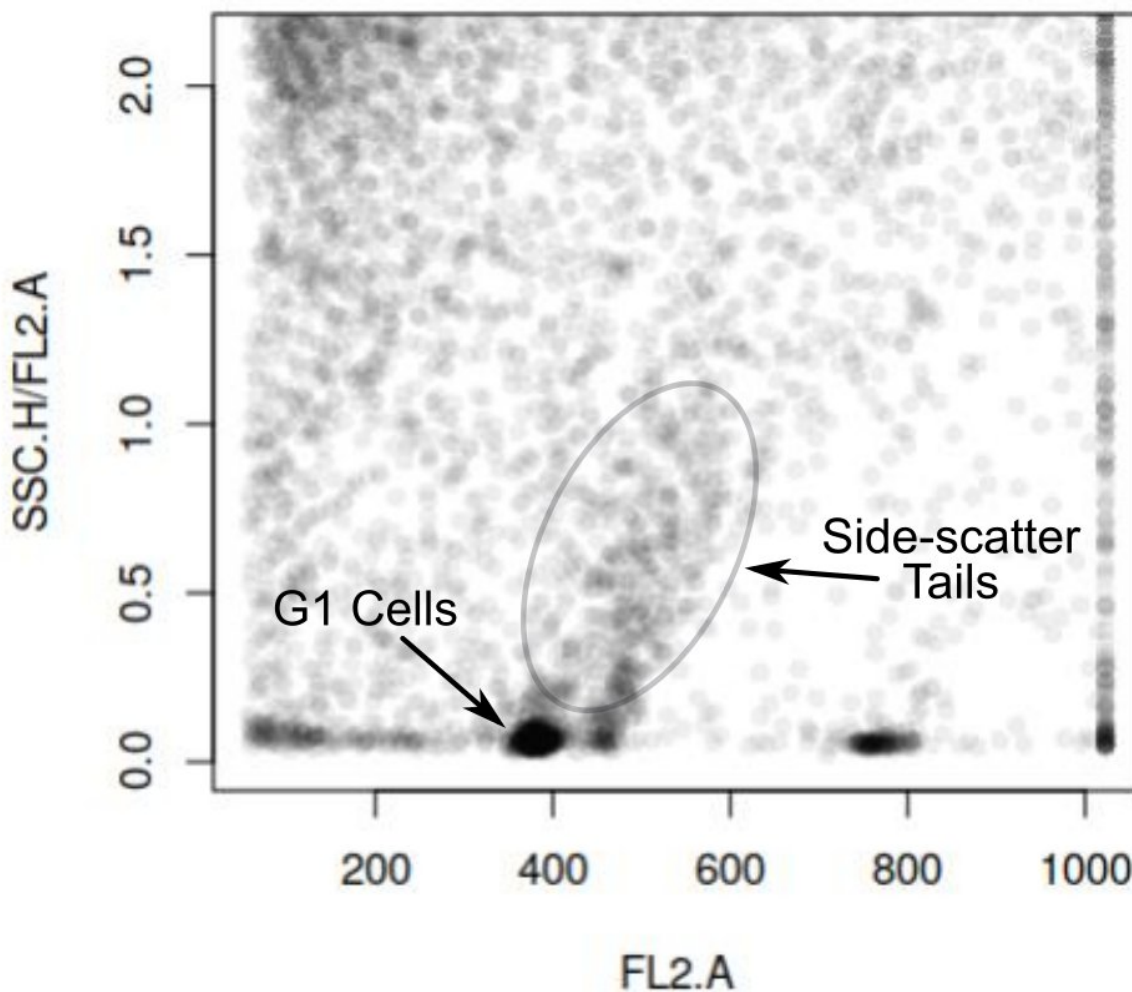
In order to see this, we’ll need to zoom in on the gate plot. Dragging the zoom slider about halfway across² we see this:

²The zoom slider numbers aren’t particularly meaningful in themselves; higher numbers mean a higher zoom, measured on a log scale.



Notice the dense cloud of points on the upper left. These particles have low fluorescence and high granularity. They could be bits of damaged nuclei, or irregularly-shaped cell components that are weakly fluorescent in the FCM laser. This is the debris we need to exclude.

When we do, we'll uncover our intact nuclei, which are visible along the bottom of the plot in three discrete clusters. The two near the center of the X-axis are the G1 peaks for the sample and standard. The smaller cluster further right is the G2 peak for the standard. Zooming in further we can see these more clearly:



Here, notice that the nuclei form tight, dark clusters, with particles trailing away upwards. Those upward 'side-scatter tails' are probably intact nuclei with bits of debris or fluorescent compounds stuck to them. This makes their shapes more irregular, increasing their side scatter values. Unfortunately, there is no clear dividing line between 'clean nuclei' and 'goopy nuclei'.

In practice, we place our gates high enough to retain all the particles in the densest section of the clusters, and extend them upwards to include the bottom portion of the tails. There is an element of subjectivity here: we are balancing biasing our results with goopy nuclei by including too much of the tail, and reducing the amount of precious data in the analysis by being too aggressive with our gate. Including more of the goopy nuclei increases our CV, and excluding them lowers our cell count.

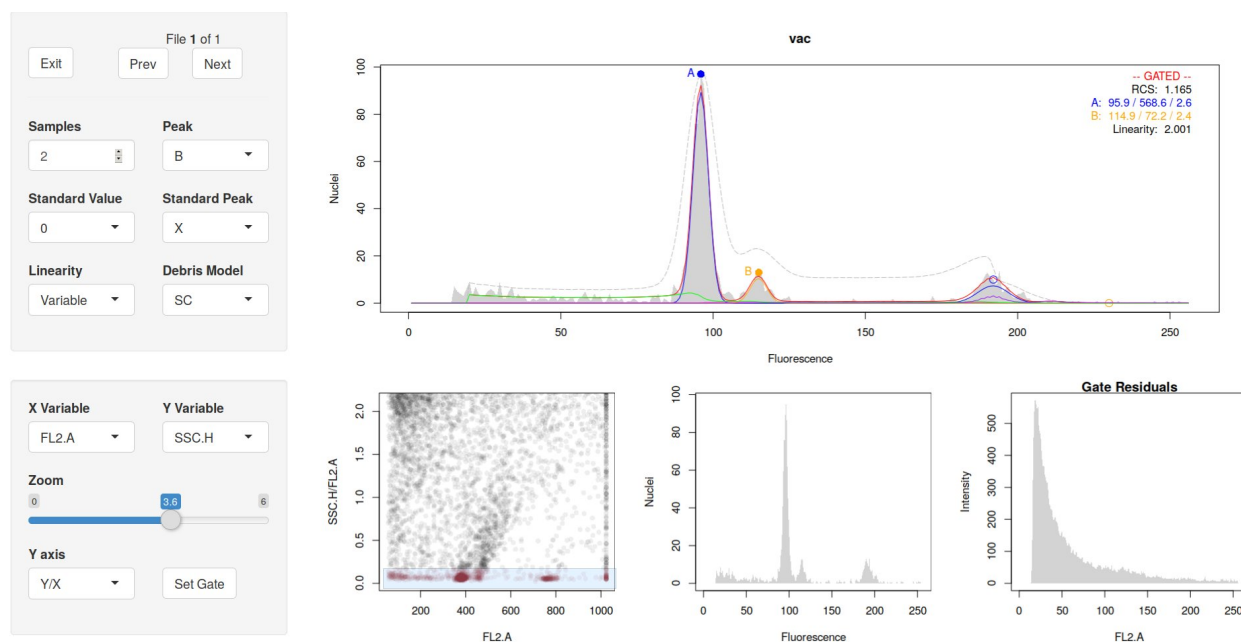
Applying a Gate

Use the mouse to left-click and drag a box around the cell clouds:



The upper boundary of the gate is important - it will have a direct influence on both our cell counts and CVs. However, the left and right boundaries have very little impact. The model-fitting process does a very nice job of determining where our peaks start and stop, so we don't need to worry about drawing 'correct' boundaries on the left and right. Consequently, we set our gates to include the full width of the plot.

We can see the effect of the gate in the middle window, and the events that we're excluding in the right window. At this point, we can see three peaks in the histogram preview. To apply this gate to the analysis, click the Set Gate button. The result should look something like this:



The histogram plot now includes the **-GATED-** flag. This is a caution to remind us that applying a gate may influence the model-fitting process in a number of ways, including how effective the debris model components (SC and MC) capture the remaining debris, and the absolute value of RCS. In general, these issues are outweighed by the improved fit for high-debris samples. See ?DebrisModels for further discussion.

The final outcome here is we have a usable result. Sadly for those of us working with *Vaccinium*, we're usually limited to very low cell counts. But given the challenges presented by this difficult tissue, we have succeeded in isolating the nuclei necessary to get a GC estimate.

Further Details

Additional documentation is provided in the built-in R manuals. `?flowPloidy` provides links to all the help files.

References

Bagwell, C. Bruce. 1993. “Theoretical Aspects of Flow Cytometry Data Analysis.” In *Clinical Flow Cytometry: Principles and Applications*, edited by Kenneth D. Bauer, Ricardo E. Duque, and T. Vincent Shankey, 41–61. Williams & Wilkins.