

Original Article

Data-Driven Programmatic Approach to Analysis of Basophil Activation Tests

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Background: Conventional data analysis of flow cytometry-based basophil activation testing requires repetitive, labor-intensive analysis that hampers efforts to standardize testing for clinical applications. Using an open-source platform, we developed and implemented a programmatic approach to the analysis of the basophil activation test (BAT) by flow cytometry.

Methods: Using the BÜHLMANN FlowCAST® assay, peripheral blood from peanut allergic patients undergoing oral immunotherapy was incubated with peanut allergens (Arah1, Arah2, Arah6, whole peanut extract) and stained with fluorescent antibodies to CCR3 and CD63 for the development of a data-driven programmatic analysis using Bioconductor and R. Basophil identification using clustering and classification was validated using manually gated comparisons in an experimental subset. Reproducibility of CD63 upregulation set on unstimulated or anti-FcεRI stimulated basophils was compared.

Results: BAT analysis of 294 experiments was successful in 91.5% using the above approach, with a total of 7,166 individual basophil activation tests from 269 experiments. We estimate this represents a net saving of 1340 min of labor by a skilled operator. Medium-based gating correlated to respective manual gating more closely than anti-FcεRI based gating ($R = 0.96$ vs. $R = 0.84$, $P < 0.001$). Only 2% of the basophil activation results were significantly different from manual gating. Quality measures of the experiments and other measures of basophil activation were also provided by the analysis.

Conclusions: We present a novel data-driven flow cytometric platform for the analysis of clinical basophil activation testing, providing a high throughput objective approach to basophil activation analysis. © 2017 International Clinical Cytometry Society

Key terms: computational analysis; basophil granulocytes; allergy

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INTRODUCTION

Reproducible research is increasingly important in this era of globalized science as a way for scientists to transparently share data analysis. Conventional data analysis of flow cytometry data is repetitive and labor-intensive, lacks transparency and is susceptible to

bias. Particularly in the context of large clinical trials, in which data acquisition can occur longitudinally over a long time period, sometimes on different instruments and at multiple centers (1,2), the ability to use a programmatic gating strategy not only is more transparent and reproducible but also facilitates

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better quality control that improve interpretation of the data (3–5).

Measurement of antigen-induced basophil activation *ex vivo* has been shown to be a promising diagnostic test in many allergic disorders (6–8). Suppression of basophil activation has been associated with immunotherapy to both environmental (9,10) and food allergens (1,11–14). Historically, identification of rare cells such as basophils has been difficult with flow cytometry due to their relative scarcity (15,16). However, wide-spread adoption of this flow cytometry-based test has been limited by the intensive nature of analysis and interpretation required for flow cytometry data by expert operators.

We therefore wrote a data-driven algorithm for flow cytometry data analysis of basophil activation testing (BAT) with the following objectives: transparency and reproducibility, efficiency, quality control, and multiple aggregated statistics of basophil activation. As a proof-of-principle, we applied this script to the analysis of BAT in patients undergoing peanut oral immunotherapy for the treatment of peanut allergy.

METHODS

Basophil Activation Assay

Peripheral blood was obtained at multiple time points from pediatric peanut allergic subjects (aged 7–12, positive specific IgE, skin prick testing, and history of

reaction, $n = 30$) who underwent a single-center peanut oral immunotherapy trial with peanut antigen (Golden peanut flour) (NCT01324401).

Using the FlowCAST® assay (BÜHLMANN Laboratories, Schönenbuch, Switzerland) and following the manufacturer's protocol, 0.05 mL of peripheral blood was incubated with peanut allergens: Ara h 1, Ara h 2, Ara h 6, whole peanut extracts (0503 or Golden peanut flour) for 15 min at 37°C along with anti-CCR3 PE and -CD63 FOTC antibodies. Control conditions included a medium-only negative control, a positive control involving the crosslinking of the high-affinity Fc epsilon receptor (anti-FcERI), and a positive control independent of FcERI signaling, involving stimulation with *N*-Formylmethionyl-leucyl-phenylalanine (fMLP). Flow cytometry data was collected on a BD LSRII or BD Fortessa as FCS data files (Supporting Information MiCyte Appendix).

Computational Analysis: Autogating

For development of this algorithm, we created the algorithm based on a subset of the BAT experiments from clinical trials, validated it with the standard manual analysis of the same data, and then applied the algorithm to a larger dataset (Fig. 1A).

Using Bioconductor tools in R (flowCore and associated packages) (17–23), we developed a data-driven, automated gating strategy in which basophils were

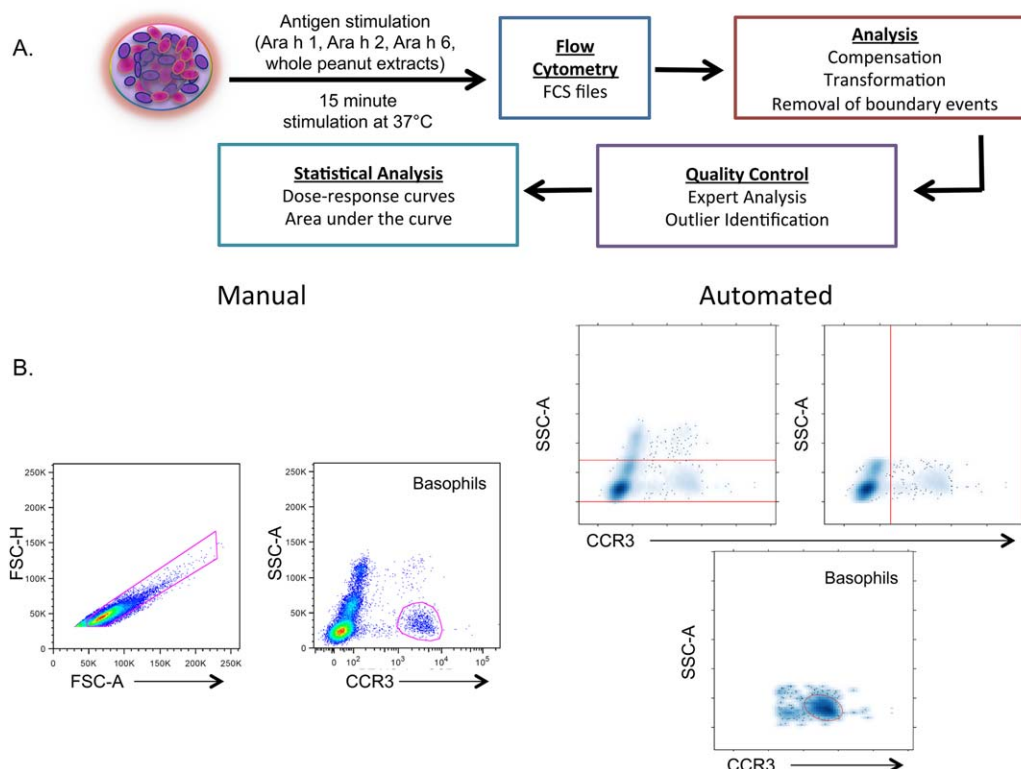


FIG. 1. Analysis of basophil activation tests (BAT). A. Flow cytometry of antigen-stimulated whole blood is analyzed using data-driven analysis. B. Basophils are identified as SSC^{lo}, CCR3^{hi} cells, with activated basophils as CD63^{hi} using manual versus autogating analysis. [Color figure can be viewed at wileyonlinelibrary.com]

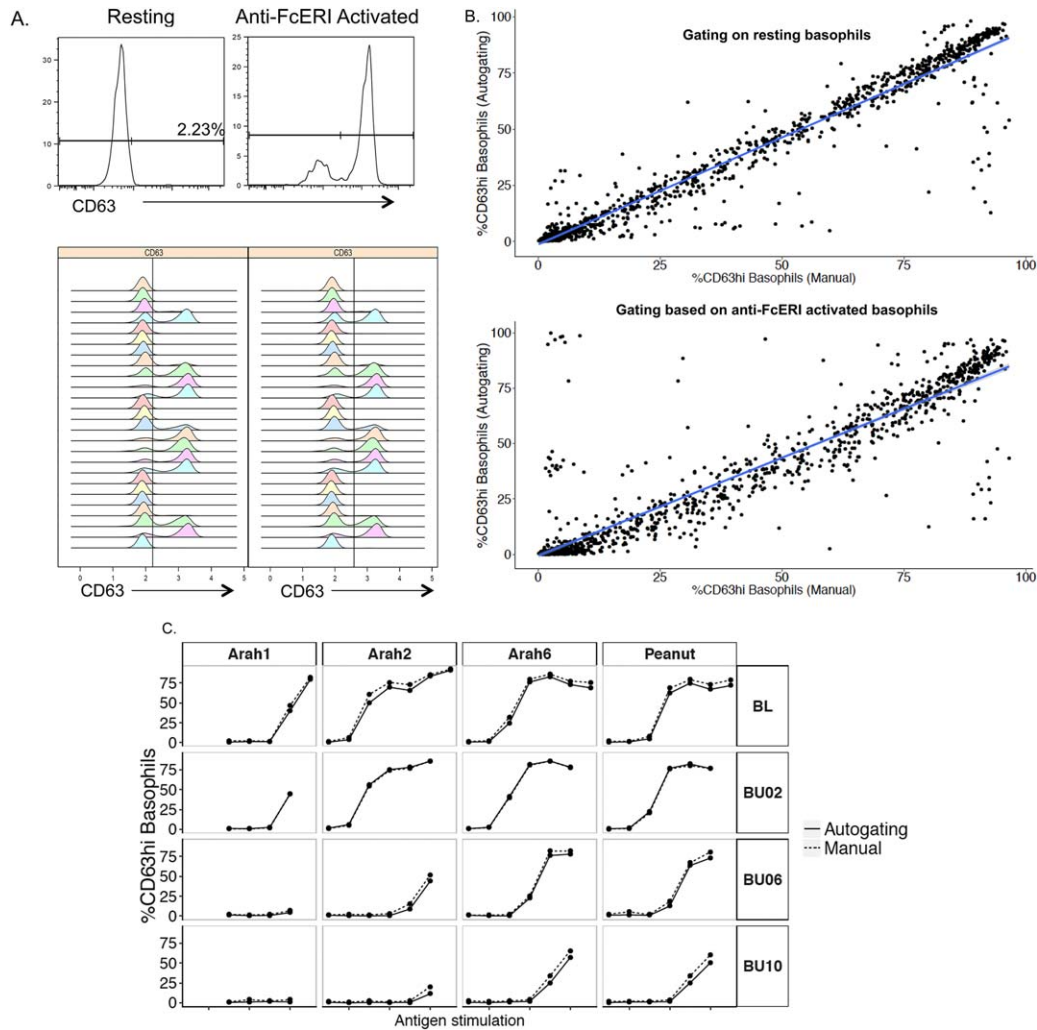


FIG. 2. Comparison of basophil gating strategies. A. The threshold for identification of CD63hi basophils was set either using resting or anti-FcERI activated basophils. B. Comparison with manually gated basophils found that gating based on resting basophils were more reliable ($p = 0.001$). C. Using this strategy, dose-response curves in a single patient over time are reliably identified and are similar across both the autogating (solid line) and manual gates (dashed line). [Color figure can be viewed at wileyonlinelibrary.com]

defined as bivariate normally distributed $SSC^{lo}CCR3^{+}$ cells after removal of SSC^{hi} and $CD63^{+}CCR3^{lo}$ populations using clustering approaches (kmeans and norm2-Filter). Comparable basophil populations were achieved, as shown above in the same sample. Gate visualization on plots of all stimulation conditions provided additional quality control (Fig. 1B, Supporting Information Figures) (<https://github.com/saritaupatil/AutoBAT>).

Similar to the manual gating, two gating strategies to determine CD63 upregulation were employed. Gating on resting basophils determines CD63 upregulation by setting the cutoff at the 97.5 percentile of CD63 expression of unstimulated basophils. Gating on activated basophils determines CD63 upregulation using k-means clustering to distinguish between bimodal CD63 populations in anti-FcERI treated basophils. The estimated time for implementation of this analysis is about 5 min of total screen time by a skilled operator.

Manual analysis

For comparison with the programmatic approach, a subset ($n = 52$) of the experiments were manually gated using FlowJo (Tree Star, USA) software. On manual gating using FlowJo templates, basophils were identified as single cells on FSC-H and FSC-A followed by gating on the $SSC^{lo}CCR3^{+}$ population. These were adjusted as needed after visual inspection of each sample in the experiment (Fig. 1B). CD63 upregulation was defined using two methods: either on resting basophils, where the cutoff is set at the 97.5 percentile of CD63 expression of unstimulated basophils, or on activated basophils where the cutoff is set at the midpoint of the bimodal populations of the CD63 expression in anti-FcERI stimulated basophils. The estimated time for analysis of each experiment was about 5 min. We also used manual gating for data from experiments with limited quality.

Statistical Analysis

Statistical comparison of the percentage of CD63hi basophils derived from similar manual and automated gating strategies was analyzed using the Spearman correlation coefficient. Dose-response curves were fitted using the R package drc (24) assuming a four-parameter log-logistic dose-response model and the effective dose ED50 was derived. The default, nonrobust least square unconstrained estimation fitting procedure (“mean”) was not always the optimal solution, so we also fitted the same dose-response using robust methods (25): median estimation (“median”), least median of squares (“lms”). Because the percentage of CD63hi basophils were bounded (they cannot be less than 0 or greater than 100) we also extended the mean fitting procedure by a “constrain” estimation (25) restricting the lower and upper bounds (so-called box constraints) to those allowed by the data. For each of these four estimating procedures we selected the dose-response curve with the smallest AIC (Akaike’s Information Criterion).

RESULTS

Autogating is Reproducible and Comparable to Manual Gating

During development of the autogating approach, we first tested a subset of the data, 48 experiments (16% of the total of 294 experiments) with both conventional gating and programmatic approach. CD63 upregulation using conventional gating and the programmatic approach are linearly correlated across all stimulation conditions.

Gating based on resting basophils correlated to respective manual gating more closely than gating on activated basophils ($R = 0.96$ vs. $R = 0.84$, $P < 0.001$) (Fig. 2). The correlation of automated and manual analysis significantly depends the activation status ($P = 0.025$) and number of basophils per analysis ($P < 0.0001$). This subgroup analysis highlights again the need for robust BAT data acquisition, which increases the probability of success with automated gating.

Analysis of residuals, defined as the difference between CD63 upregulation in the conventional and the programmatic approach, found that only 2.0% of the comparisons fell outside the standard error. Incongruence between manual and automated gating is primarily due to poor basophil identification by the autogating algorithm in one of the experiments used to test the accuracy of the gating algorithm.

Interoperator variability using conventional manual gating based on resting basophil CD63 fluorescence was performed on 250 samples, revealing a correlation of 0.98.

Data for analysis was collected over 4 years, by multiple operators, and two flow cytometers, of which one had a change in laser configuration.

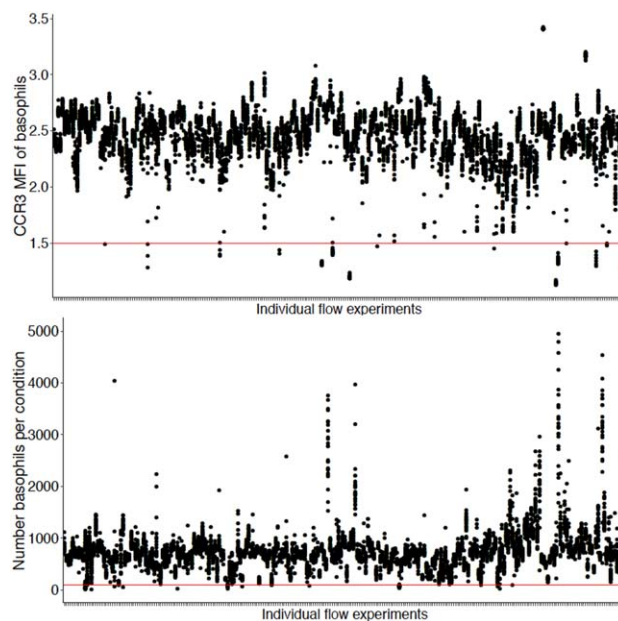


Fig. 3. Quality control measures in BAT. Longitudinal CCR3MFI and basophil counts in experiments throughout the protocol identifies outlier basophil activation tests. All together, 29 experiments (10.8%) were identified as having at least one stimulation condition that did not meet our quality threshold. [Color figure can be viewed at wileyonlinelibrary.com]

Autogating Improves Efficiency

In 269 experiments, with a total of 7,166 individual basophil activation samples from 26 subjects, we successfully implemented the above algorithm. We estimate this represents a net saving of 1340 minutes of labor by a skilled operator.

Of the 294 experiments, 25 (8.5%) failed automated gating required more permissive manual gating. Technical errors including experimental error and errors during flow acquisition, as well as poor sample quality contributed to failure of autogating.

Autogating Facilitates Transparency

Longitudinal analysis of automated basophil flow cytometric characteristics captures aberrations and outliers, which in conjunction with visual quality control, identifies experiments for which automated gating strategy is less robust, likely due to poor quality data. Our strategy also incorporates graphical output for each experiment for visual quality control analysis as desired (Supporting Information Appendix B).

By aggregating data across all of the experimental conditions, we used longitudinal measures of CCR3 MFI and basophil enumeration to identify experiments with limited quality, due to low or absent fluorescence signal or high parameter variance, likely reflecting technical problems with sample acquisition or preparation or low numbers of basophil events reflecting biological variation. Of the 264 experiments, 16 (5.9%) had identified basophils with abnormally low CCR3 expression and 16

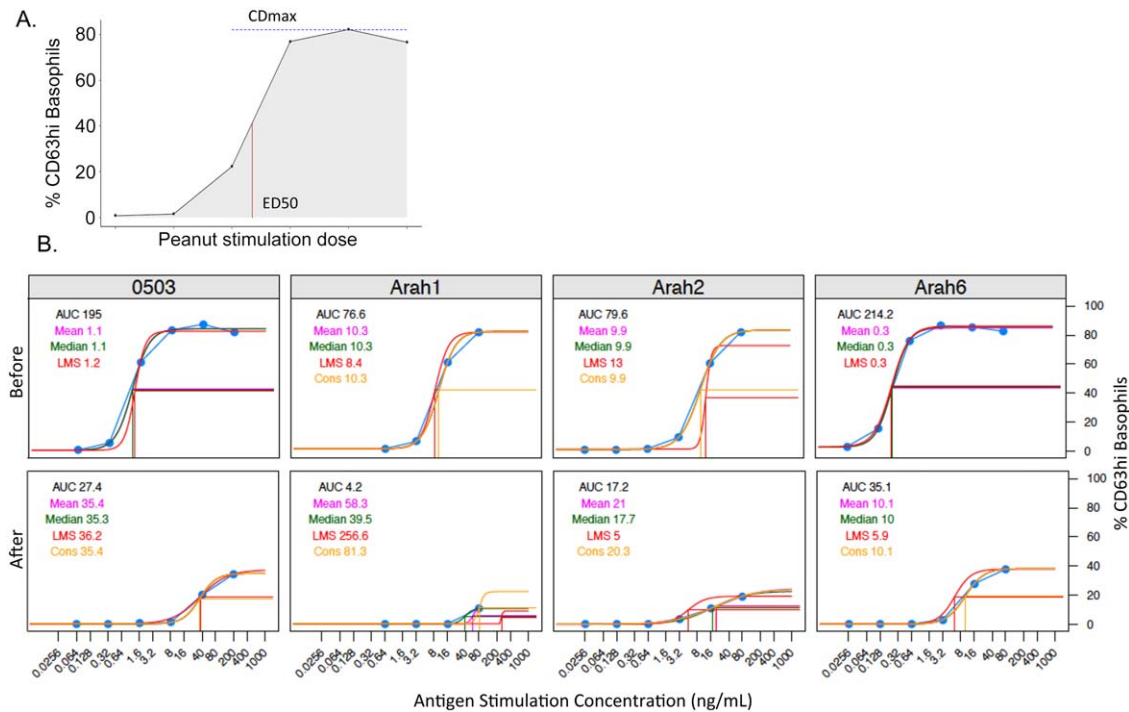


FIG. 4. Dose-response measures in BAT. A. Our analytical script supports quantification of additional measures of the dose-response curve in BAT. The CDmax (dashed line), or maximal stimulation, of the dose-response curve can be calculated, as can the ED50 (solid line), which is the antigen concentration at which one-half of the CDmax is achieved. The area-under-the-curve which can be an aggregate measure of the dose-response curve can also be calculated using the trapezoidal approach (gray shaded area). B. Analysis of the basophil dose-responses to peanut antigens using different model fitting for the calculation of AUC and ED50 shown in a single patient before and after therapy. The AIC describing the fit for each dose-response model is shown in the upper left corner of each graph, and the model with the smallest AIC was selected. [Color figure can be viewed at wileyonlinelibrary.com]

experiments (5.9%) had insufficient basophils. All together, 29 experiments (10.8%) were identified as having at least one stimulation condition, with either low CCR3 expression or insufficient basophil numbers that did not meet our quality threshold (Fig. 3). The workflow using this algorithm can also identify experiments which fail on quality control measures and which may require subsequent more permissive or manual gating.

Autogating Provides Additional Statistical Measures of Basophil Activation

Various measures of basophil activation, including ED50 (CDsens), area-under-the-curve (AUC), and maximum CD63hi (CDmax) (9,16,26) can be calculated using our tools (Fig. 4).

In the current BAT database, the AUC was calculated for 99.18% and an ED50 was calculated for 85.02% of the 1094 BAT dose-response curves.

DISCUSSION

In this paper, we adopted the R Bioconductor package, flowCore, to implement a k-means clustering-based gating strategy to analyze 269 basophil activation tests from a clinical trial. We found this strategy to be time-efficient and highly concordant with more conventional, manual gating. The application of a programmatic approach to the BAT provides a high-throughput,

reproducible, unbiased approach to flow cytometry data analysis.

By facilitating analysis of entire experiments or 'flow sets', programmatic approaches facilitate quality control and easy monitoring of flow cytometry parameters and assay variability over time, which is particularly useful in clinical and diagnostic applications, including multicenter studies. In our dataset, using CCR3 MFI clustering, we were able to readily identify experimental outliers which were the result of poor quality data, whether technical/experimental error or error during data collection. These outliers can be further analyzed using automated analysis, by changing and adapting the algorithm. As this particular basophil assay relies on side scatter as an important flow cytometry parameter, it may have been less robust to these types of technical error which could be a consideration for future studies.

The notable limitation of this analytical approach is that the data-driven programmatic analysis of basophil activation testing is dependent on the acquisition of high quality flow cytometry data, including the use of a well-designed antibody staining panel, setting of appropriate PMTs, lack of technical error, and reasonable basophil counts. In our analysis, the number of basophils acquired within each condition significantly influence the accuracy of the autogating algorithm. Using repeated sampling, basophil degranulation measured by

CD63 varies <10% at an N of 100 and <5% at an N of 400. Dose-response measures, such as AUC, vary <10% at an N of 500. Further validation of this tool would benefit from testing a wide range of antigens, instrumentation, and centers. The use of this algorithm, for example, in multicenter trials would rely on the implementation of flow cytometry parameters that allow for accurate discrimination of different cellular populations, and some real-time review of quality control parameters for the assays, such as a minimal number of basophil events acquired and fluorescence measurements falling within a prespecified range.

This particular algorithm depends heavily on control tubes, with medium-only and anti-FcERI stimulation to set the parameters for identifying basophil degranulation. During the development of this approach, we observed that setting a cutoff to resolve activated versus resting basophils was more reliable when using the unimodal distribution of the resting basophils compared to the bimodal distribution of the anti-FcERI activated basophils. Though gating on resting basophils will tend to derive a lower cutoff and may be more sensitive to 'drift' in absolute fluorescence of the non-degranulated basophils, gating on activated anti-FcERI requires bi-modal upregulation of CD63, if the basophils undergo piecemeal degranulation, a CD63-intermediate population complicates the identification of a reproducible cut off (27). Furthermore, the fact that the number of anti-FcERI activated basophils were consistently lower, made the midpoint determination unreliable in a small, but significant number of experiments. We speculate that the lower basophil event numbers in the anti-FcERI activation condition may be partly an artifact of them having increased side scatter characteristics and that together with simply acquiring more total events, the use of more than a single fluorescence channel, might overcome this limitation. Nevertheless, the use of a cutoff derived from the resting basophils correlated well with conventional gating. The presence of nonresponders, which can be seen in 10% of the population, is a known limitation of BAT, and the use of resting basophils to derive the cutoff for activation also has the advantage of reliable application in basophil activation nonresponders.

In our data, we recognized several sources of variability. Experimental variability in sample preparation and technique had a strong adverse impact on data quality and the study-wide data analysis and QC that is facilitated by an automated gating workflow helped quickly identify this (Fig. 3A, low CCR3 MFI events). Technical variation from instrument variation or parameter settings during data acquisition was another key source of variability, and these pieces of flow file metadata can be incorporated into a quality control workflow. Longitudinal studies with repeated measures can be effective in measuring this variability. These types of variability can be mitigated by training, delineating parameters for sample preparation and flow cytometry data acquisition, and utilization of frequent quality control measures during the clinical trial or period of experimentation. Other

sources of variability for this assay, including the stimulants and reagent (i.e., antibody) variability was minimized in this flow set by using a prestandardized and validated basophil activation kit. Finally, significant inter-subject variability in human subjects exists, both at baseline and with therapeutic intervention, as in this dataset, and should be captured for further analysis.

Integrating the derivation of summary statistics for the dose-response curve is another important component of this program. Previous studies have correlated various aspects of basophil activation, including the ED50 (CD-sens) and maximal response (CDmax) with distinct clinical outcomes (6,7,16,26). We have also included the AUC as a summary statistic of the dose-response, which can be particularly helpful when dose-responses do not follow the expected sigmoidal shape and curve fitting fails (9,28). In this data set, derived from a study of peanut oral immunotherapy, we found that accurate ED50 derivation from dose-response curves was computationally challenging when dose-response curves had very low variability, which occurs with strong basophil suppression during therapy. In an attempt to overcome the issue of atypical dose-response curves, we evaluated multiple models, including a least square unconstrained estimation fit, median estimation fit, and least median of squares fit to derive the most robust ED50. Despite this, calculating an ED50 is less computationally tractable than the maximal response or AUC as a measure of BAT.

As flow cytometry data files can be sizable, the computational burden of high-throughput data analysis of a large experiment will be best run on a high performance server, now readily available through cloud computing. Implementation requires basic knowledge of R and Bioconductor.

On the other hand, this tool is highly proficient at conducting high throughput analysis, can readily integrate clinical phenotypic data, allows for reproducible and easily shared data analysis between multiple groups. This approach can be further expanded to include high-dimensional, multicolor flow cytometry BAT experiments, which have the additional advantage of utilizing more sophisticated clustering algorithms to improve both the success rate of the automated gating approach as well as the accuracy (29).

In conclusion, this data-driven automated gating algorithm for basophil activation testing provides a data analysis platform to promote transparency, reproducibility, quality controls, and data sharing in clinical research using basophil activation testing.

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CONFLICT OF INTEREST

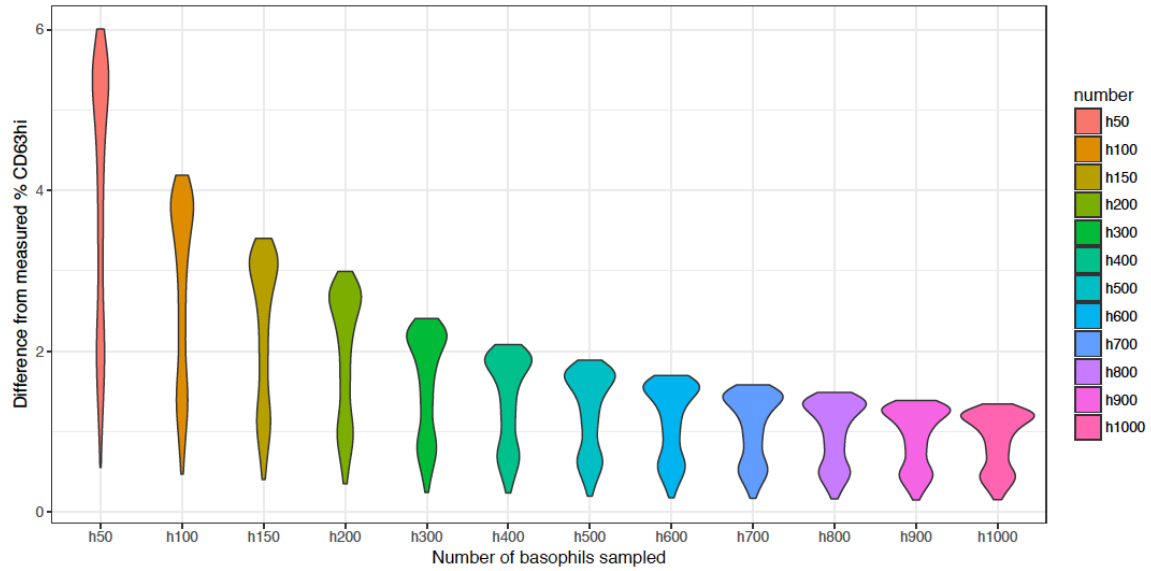
Experimental support and reagents provided by BÜHLMANN Laboratories.

LITERATURE CITED

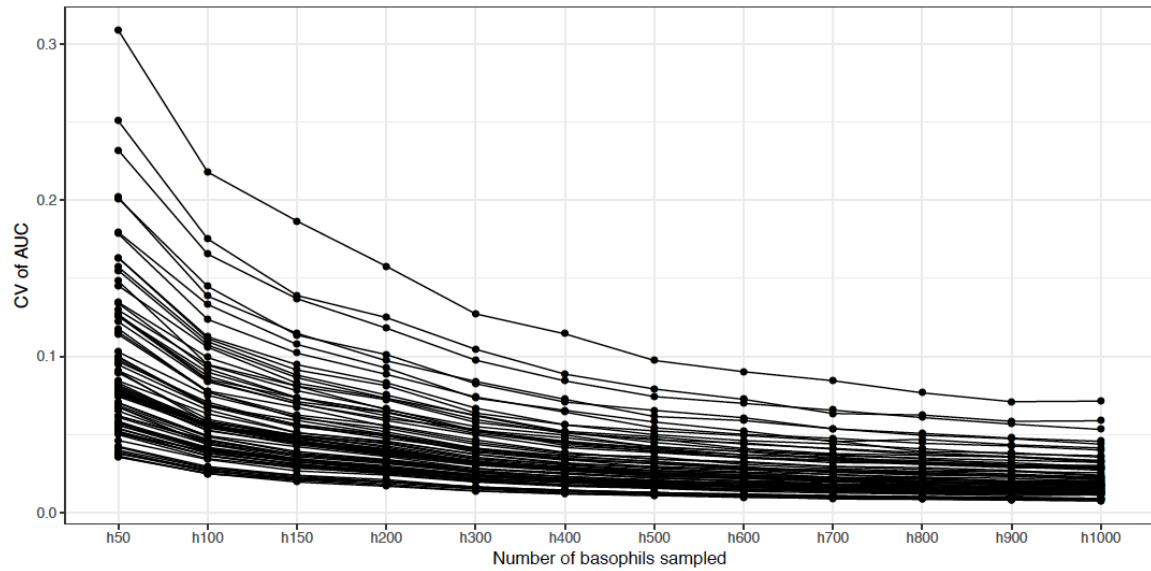
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Appendix A:

A.

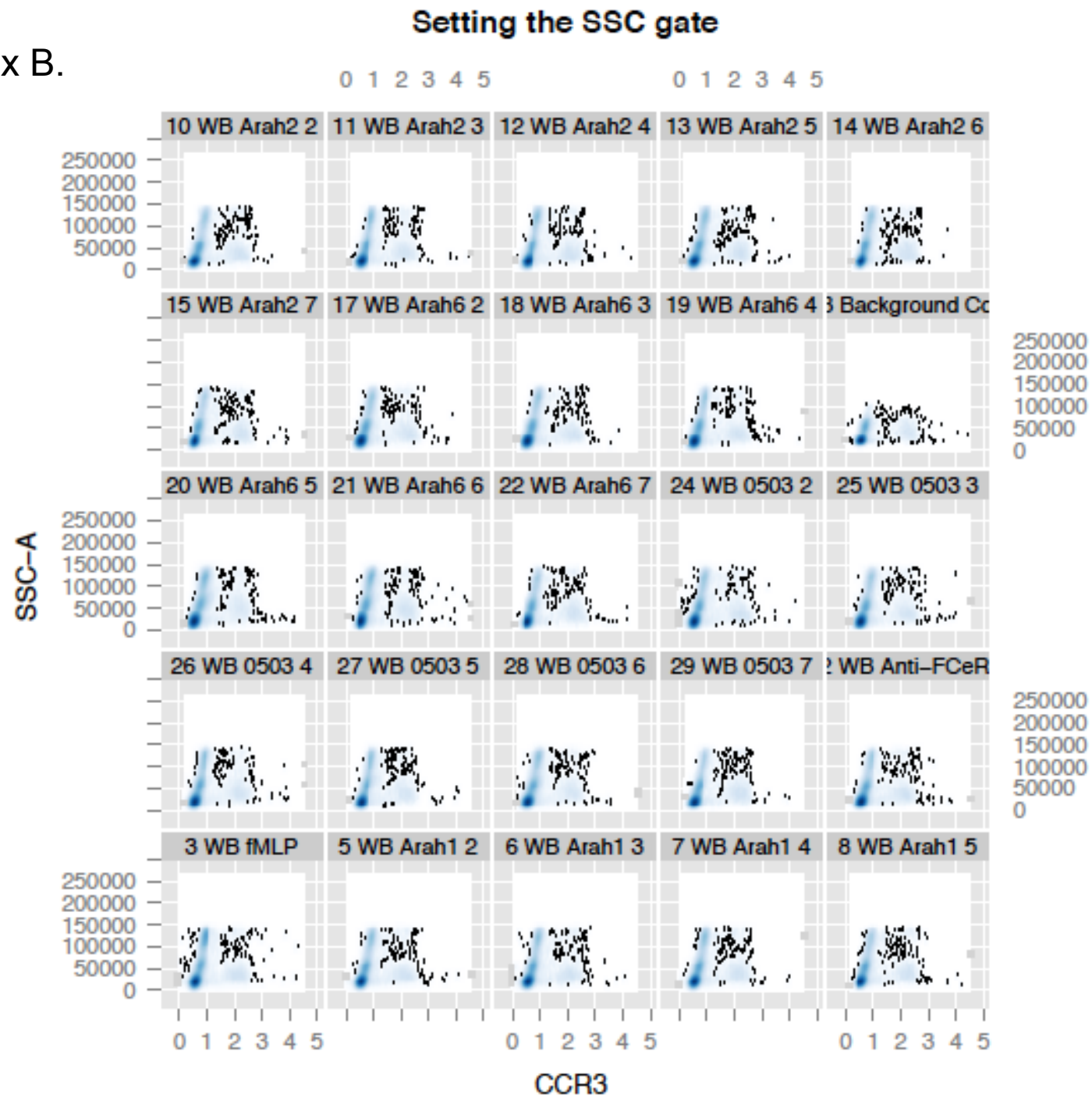


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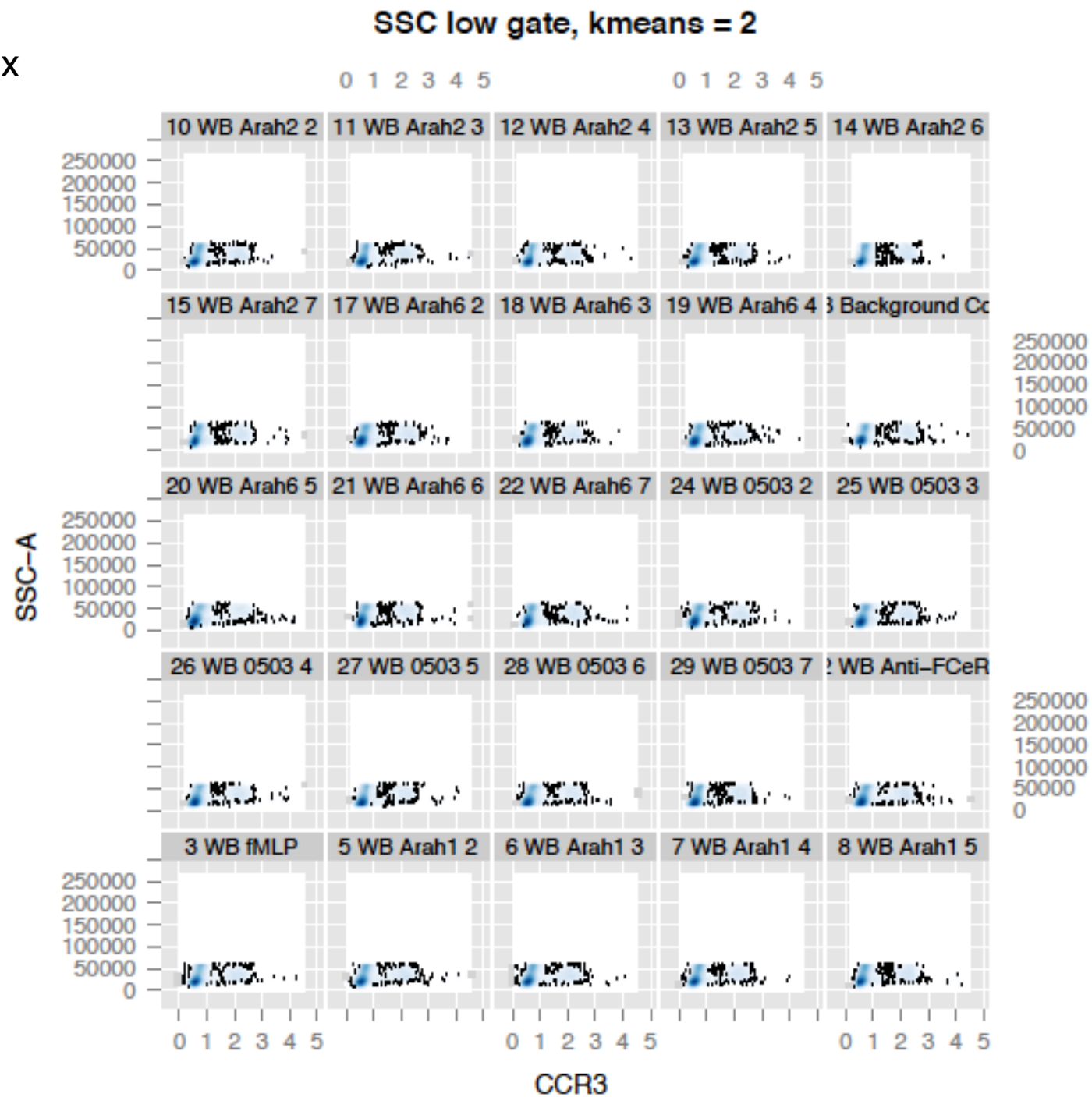


Basophil sample number derivation: Using repeated sampling of SSC^{lo}CCR3⁺ basophils from 22 experiments (~10% of the total experiment number), we randomly selected different sample numbers of basophils, ranging from 50 to 1000) 1000 times and calculated the percentage of CD63hi basophils (A) and the AUC of the antigen stimulation dose response curve (B). Variation from the measured value decreases with increased basophil sample numbers.

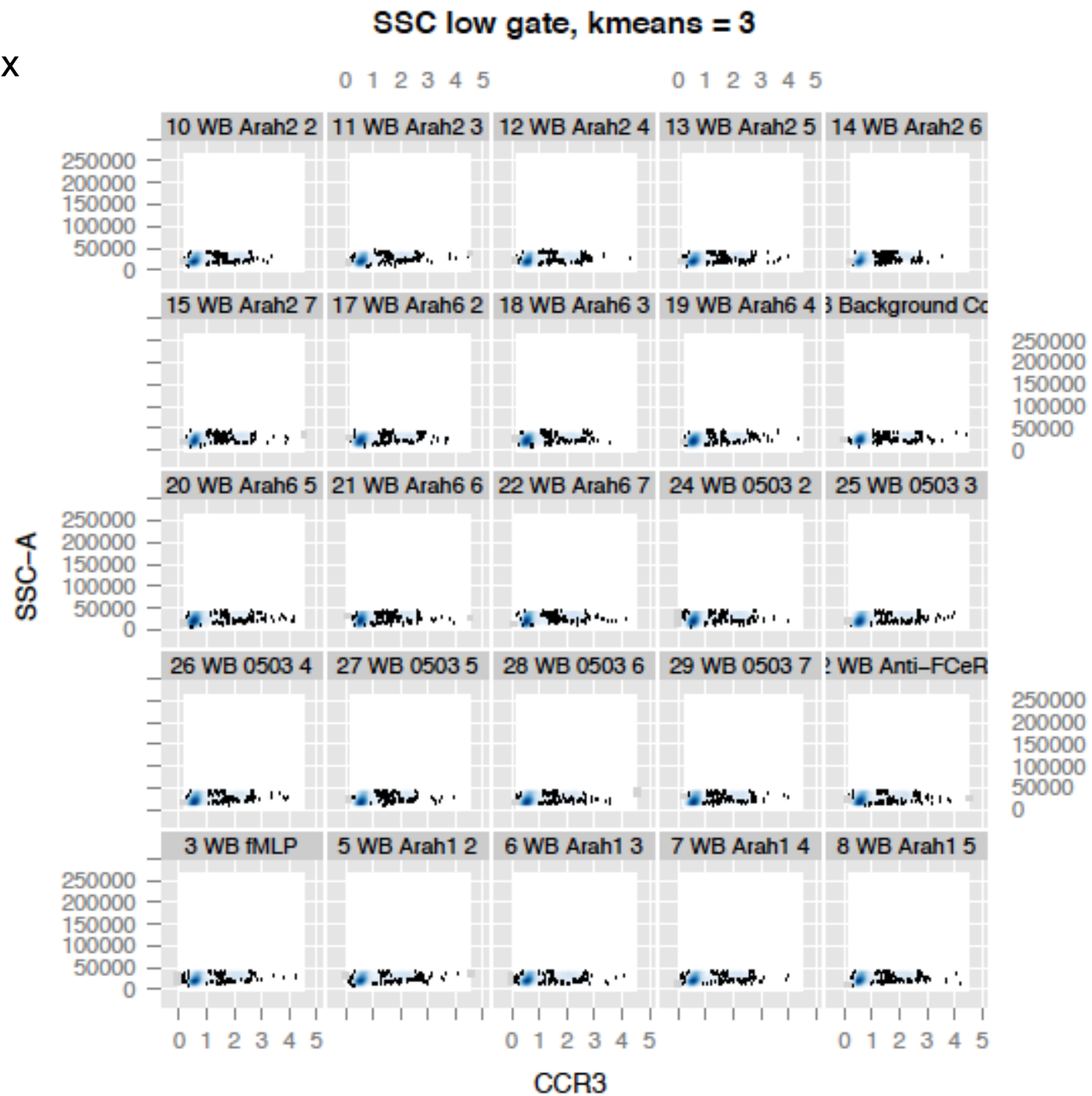
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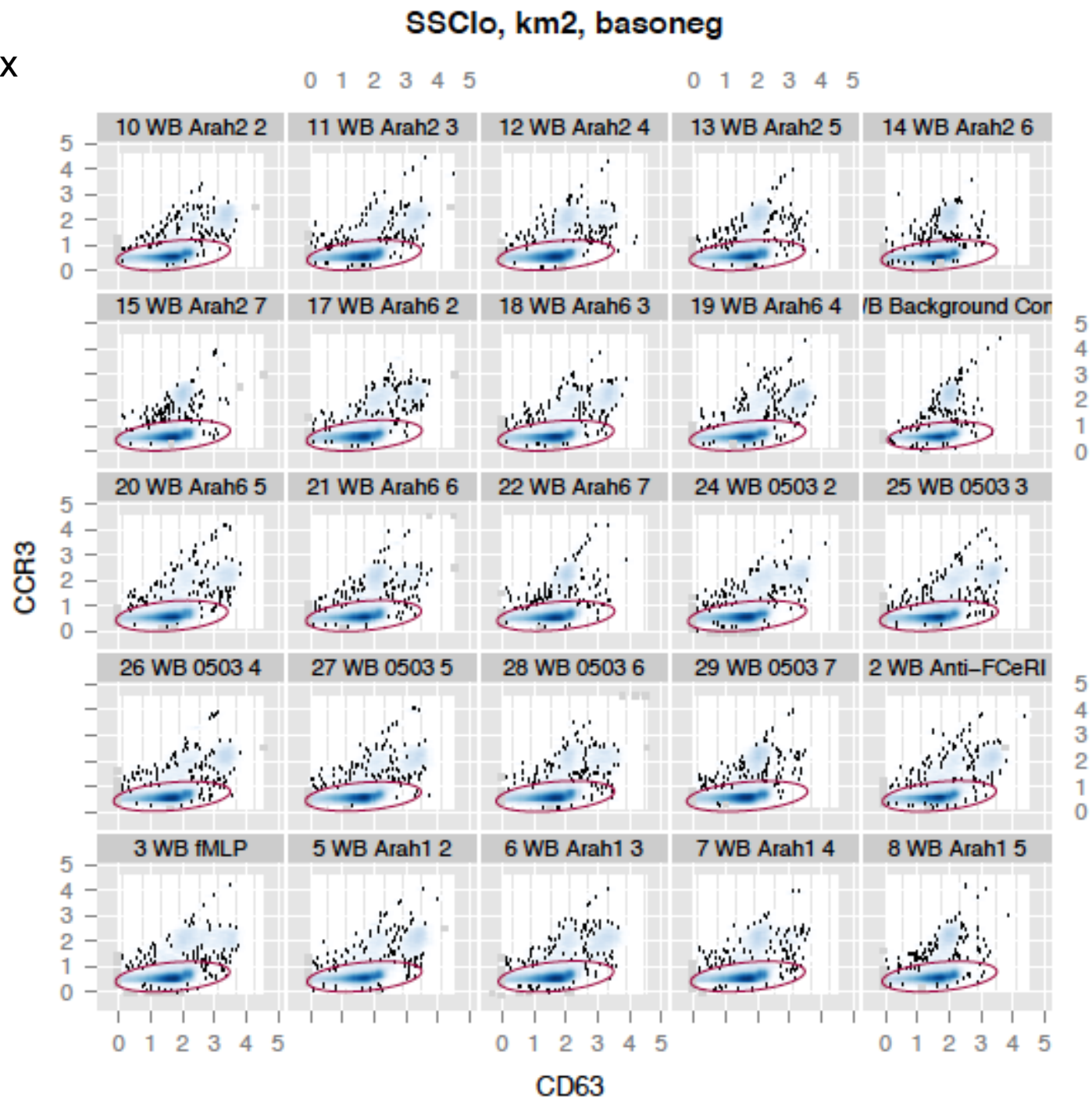
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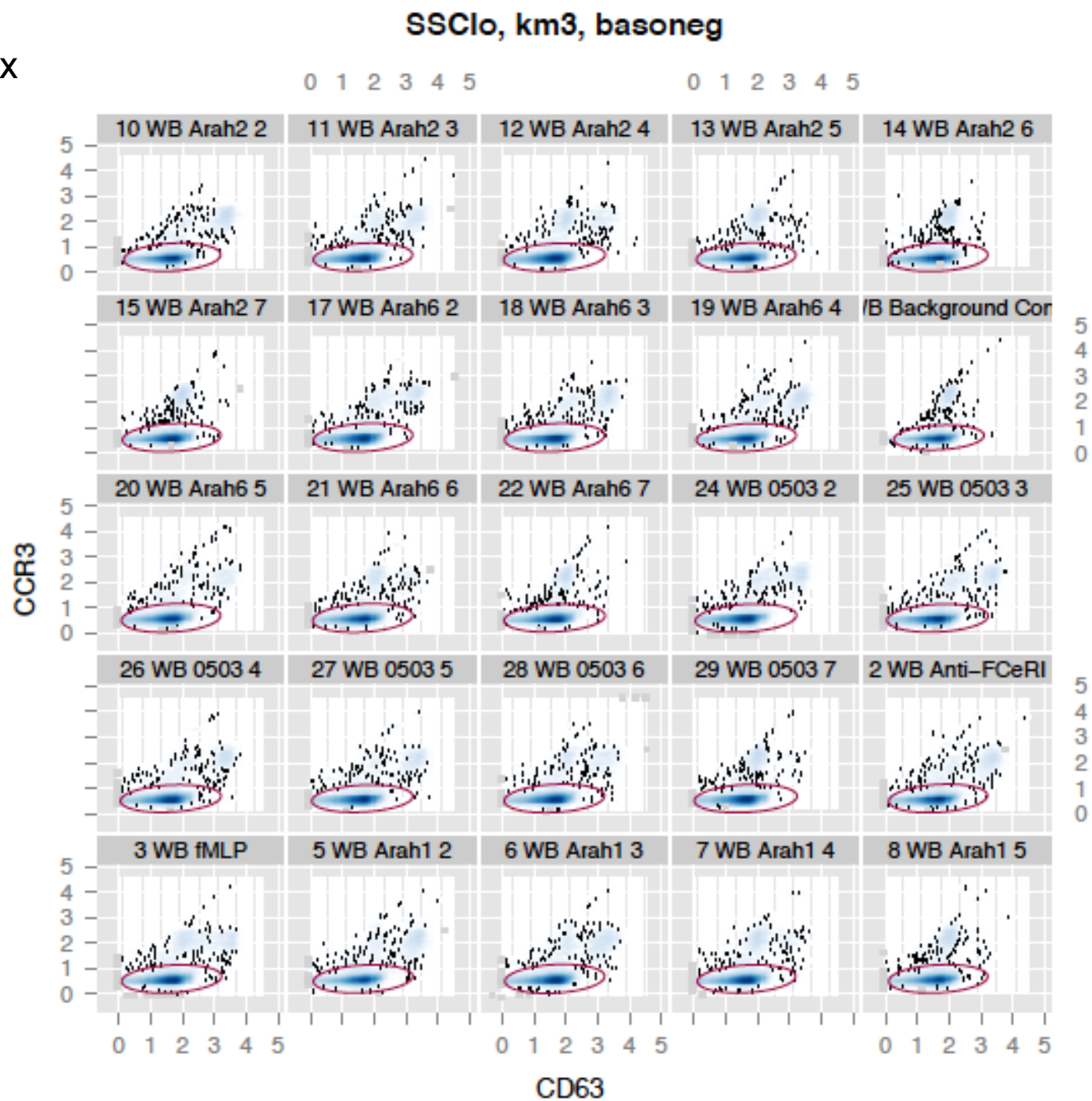
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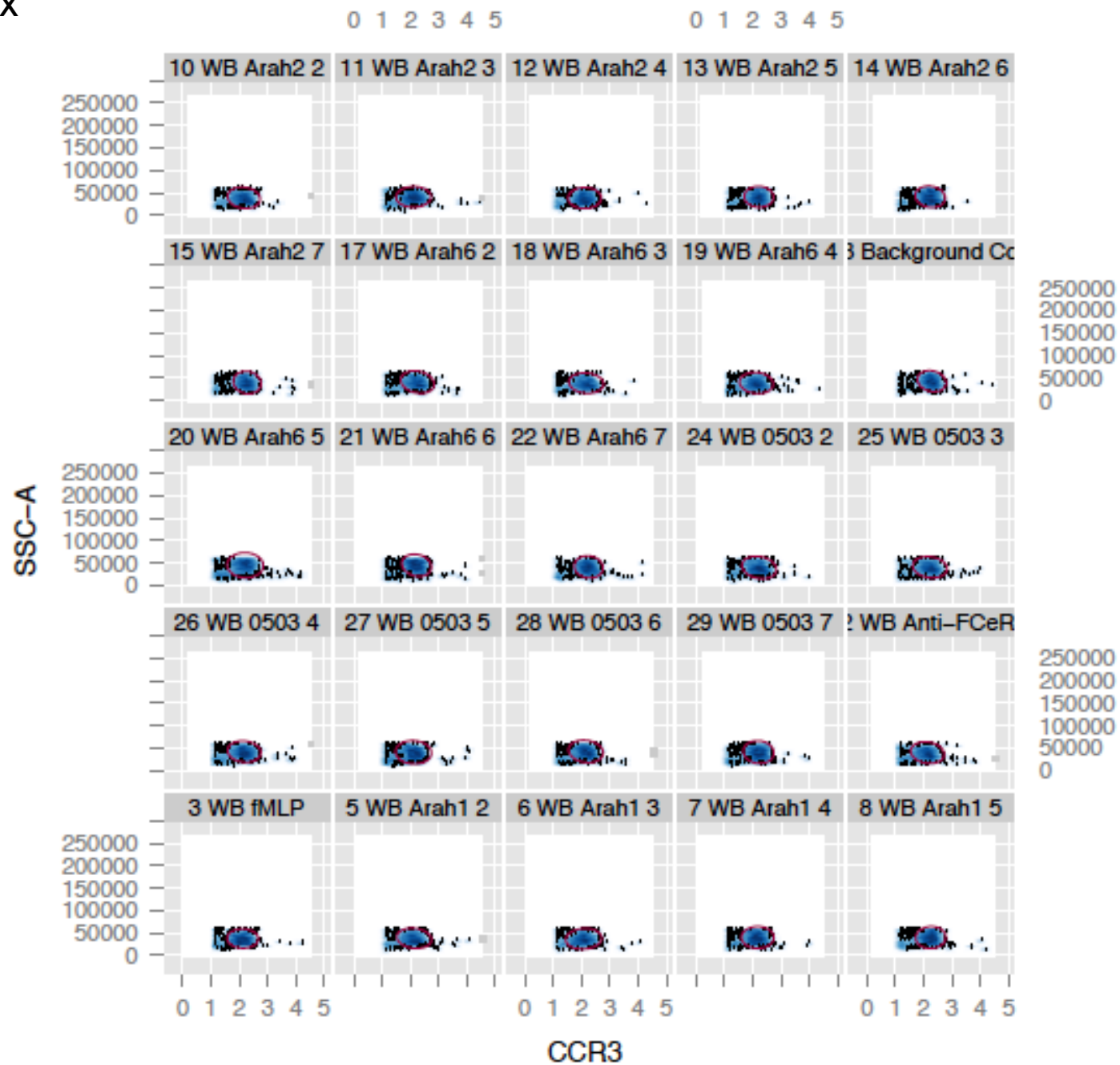
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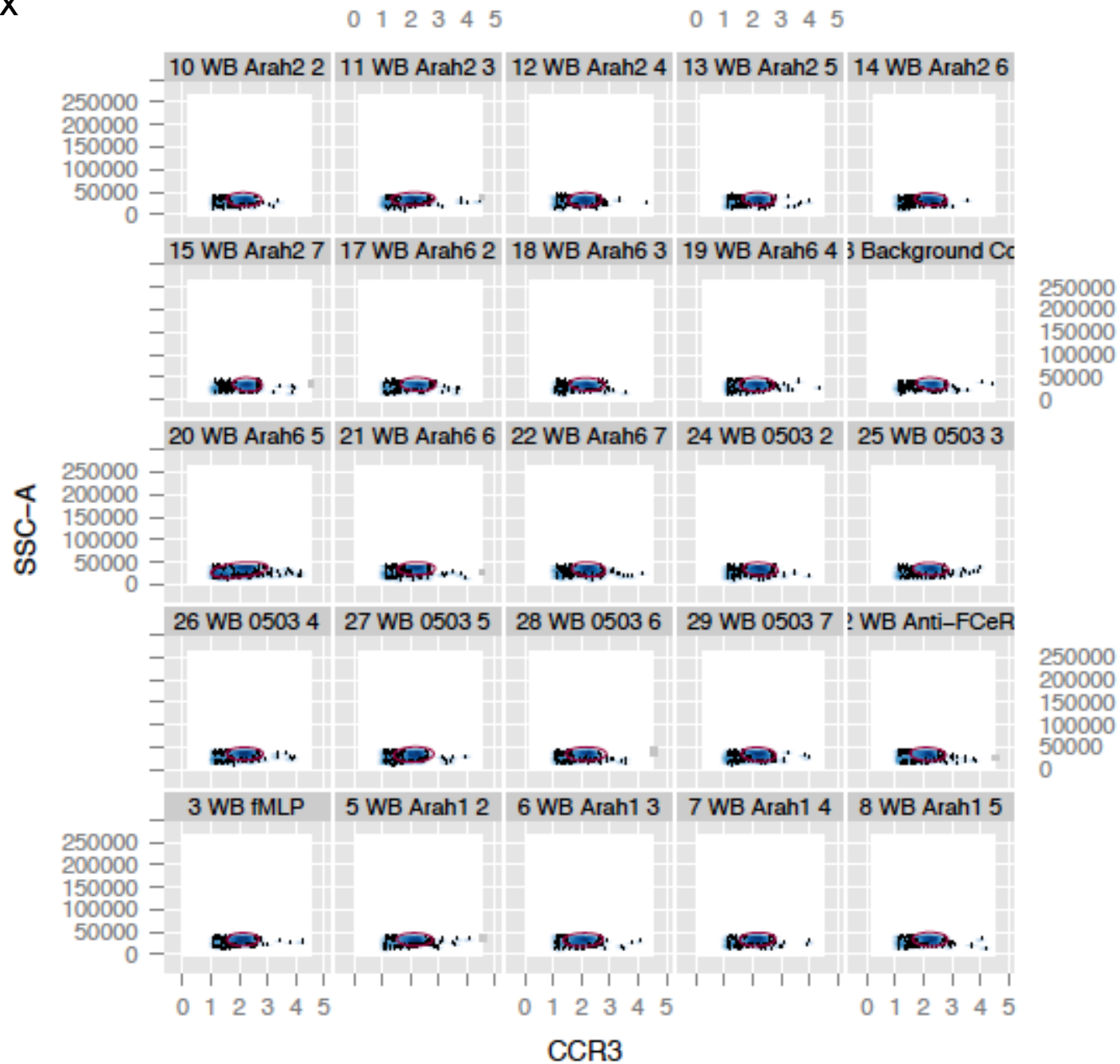
Appendix



Basophil km2, n=2

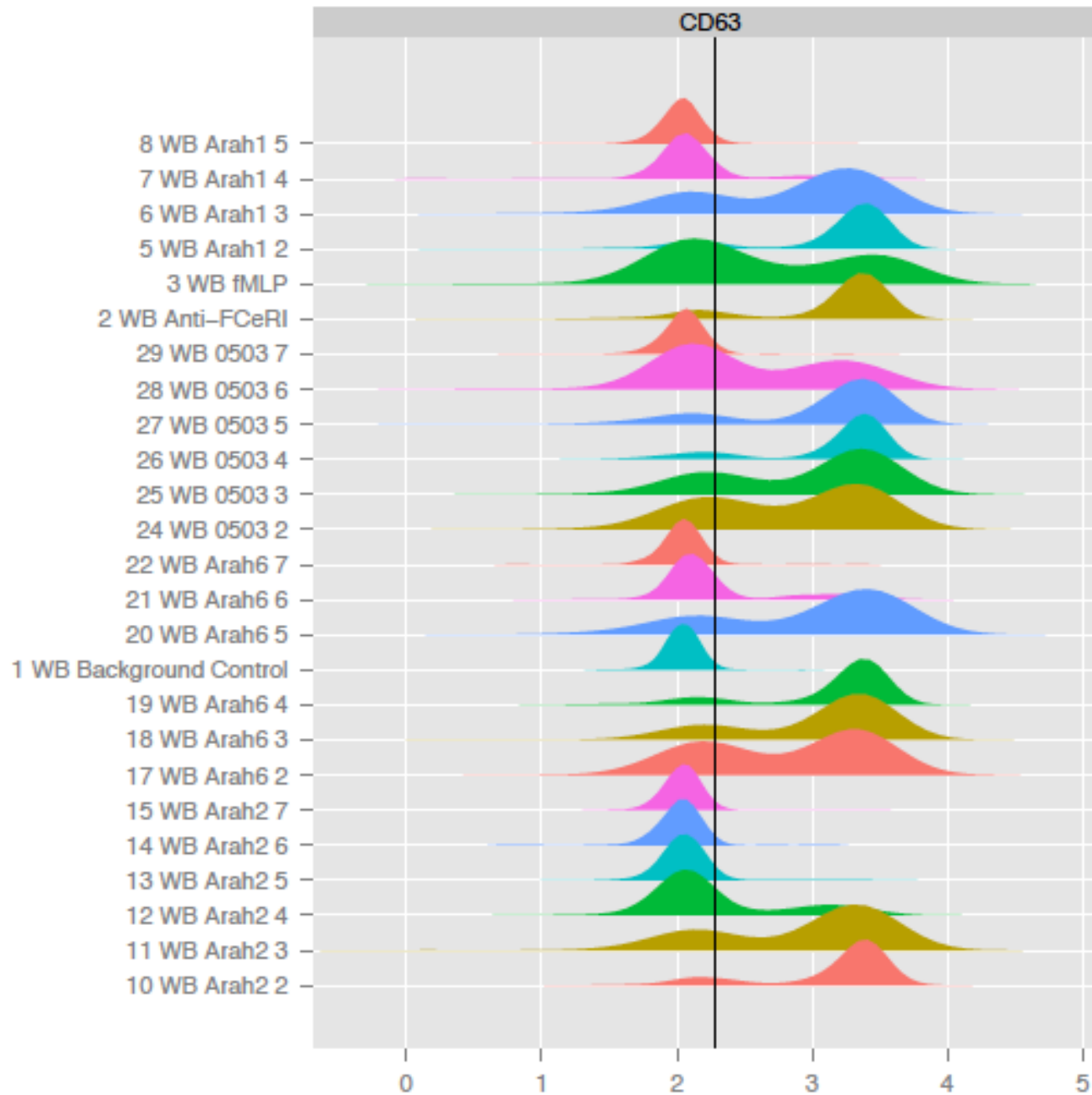


Basophil km3, n=2



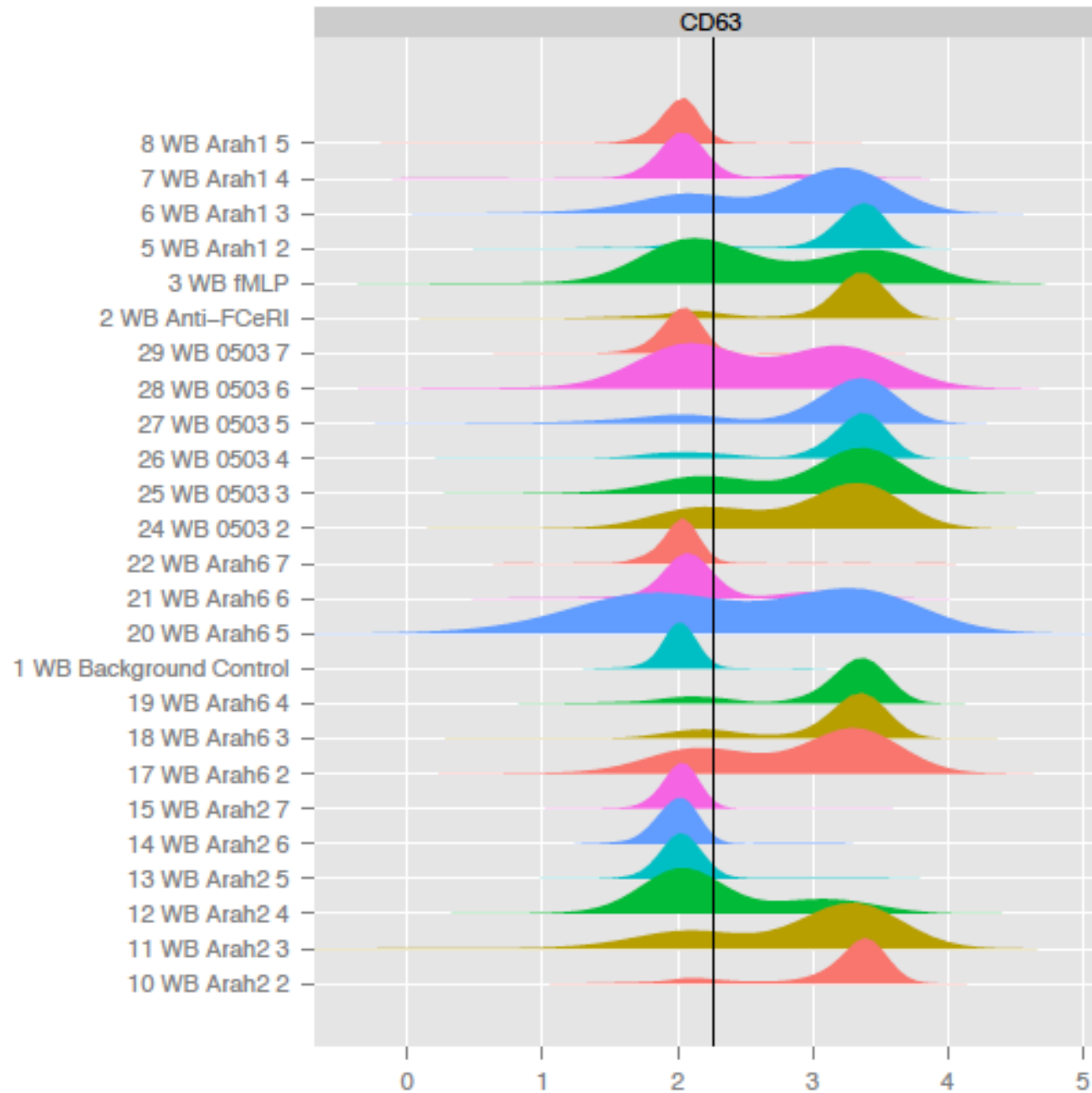
Appendix

Norm2Filter km2, CD63 Gate on Medium



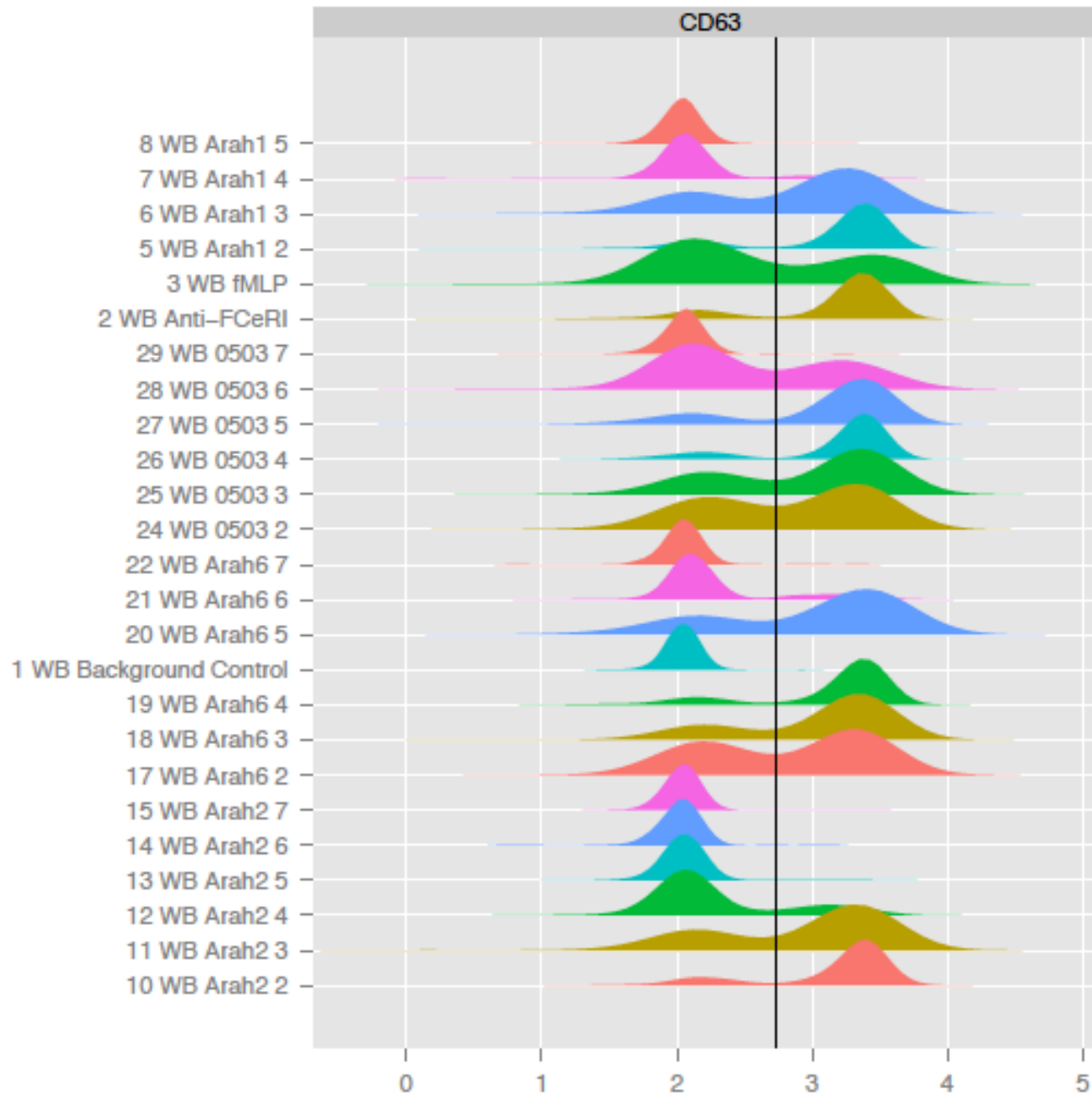
Appendix

Norm2Filter km3, CD63 Gate on Medium



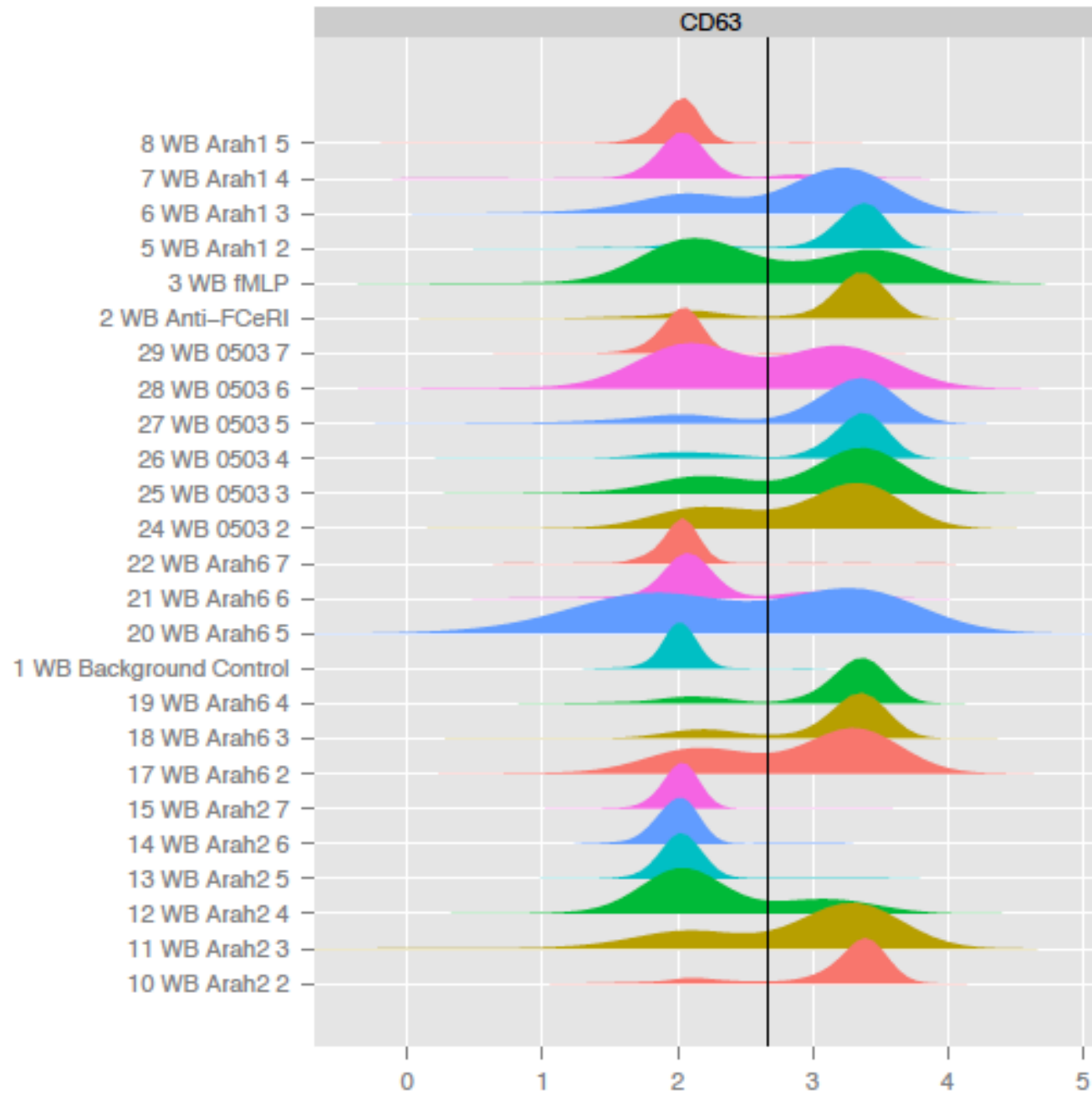
Appendix

Norm2Filter km2, CD63 Gate on Anti-IgE



Appendix

Norm2Filter km3, CD63 Gate on Anti-IgE



1. Experiment Overview

1.1. Purpose

The purpose of this experiment is to quantify and characterize activated peripheral basophils in peanut allergic subjects undergoing peanut oral immunotherapy after allergen stimulation.

1.2. Keywords: basophils, peanut allergy, basophil activation tests

1.3. Experiment Variables:

Time on peanut oral immunotherapy (PNOIT)

1.4. Organization

1.4.1.Name: Massachusetts General Hospital, Center for Immunological and Inflammatory Diseases

1.4.2. Address: 149 13th Street, Room 8.318, Charlestown, Massachusetts, 02129, United States.

1.5. Primary Contact

1.5.1.Name: Sarita Patil, MD

1.5.2. Email: sarita.patil@mgh.harvard.edu

1.6. Date

Peanut Oral Immunotherapy (PNOIT) Trial at Massachusetts General Hospital started on August 26, 2011 and is currently ongoing.

Flow cytometry analysis performed at visits: pre-PNOIT, weeks (approximately): during PNOIT: 0, 4, 8, 12, 16, 36, 52-58, at the end of PNOIT, after 4 additional weeks of peanut avoidance, and then every 6 months during the follow-period.

1.7. Conclusions

Suppression of allergen-stimulated basophil activation occurs during active PNOIT and increases slightly post-avoidance.

1.8. Quality Control Measures

Basophil counts, basophil CCR3 and SSC MFI were recorded. Visualization of

basophil gating was performed on all autogated samples.

1.9. Other Relevant Experiment Information

2. Flow Sample/Specimen Details

2.1. Sample/Specimen Material Description

2.1.1.1. Biological Sample Description: 2 mL of peripheral blood collected in EDTA-anticoagulated tubes.

2.1.1.2. Biological Sample Source Description: Homo sapiens. Subjects were recruited from a single-center trial of peanut oral immunotherapy (NCT01324401).

2.1.1.3. Biological Sample Source Organism Description: 2 mL of peripheral blood collected in EDTA-anticoagulated tubes.

- *Taxonomy:* Homo sapiens

- Age: 7-21 years of age.

- *Gender:* Male and female

- *Phenotype:* Diagnosis of peanut allergy based on:

- o Clinical history of reaction to peanut within one hour of ingestion and either a positive skin prick test (>8 mm wheal) or elevated peanut-specific IgE (CAP FEIA >10 kU/L).

- *Genotype:* all-inclusive

- *Treatment:* Subjects underwent PNOIT in a single-center, randomized trial (3:1, active:placebo) using commercially available peanut flour (Golden). Patients recruited into the active arm underwent a one-day modified rush protocol for a maximum dose of 12mg, followed by a build-up period with escalating peanut doses every 2 weeks which included blood draws at every month (solid arrows), starting at 2 weeks. The build-up period was followed by a maintenance period of 3 months at 4000mg followed by an oral food challenge to assess tolerance.

- *Other Relevant Biological Sample Source Organism Information:* Patient material was de-identified. Specimens were labeled using subject number, visit date, and visit name.

2.1.2.Environmental Samples

N/A

2.1.3. Other Samples

N/A

2.2. Sample Characteristics

Peripheral blood from subjects undergoing PNOIT.

Expected/analyzed types of cells: Identification of SSC^{lo}, CCR3^{hi} basophils, identification of activated basophils as CD63^{hi} basophils.

2.3. Sample Treatment Description

Using the FlowCAST® assay (BÜHLMANN Laboratories, Schönenbuch, Switzerland) and following the manufacturer's protocol, 0.05 mL of peripheral blood was incubated with peanut allergens: Arah1, Arah2, Arah6, whole peanut extracts (0503 or Golden peanut flour) for 15 minutes at 37°C along with fluorescent anti-CCR3 and -CD63 antibodies. Control conditions included a medium-only negative control, a positive control involving the crosslinking of the high-affinity Fc epsilon receptor (anti-FcERI), and a positive control independent of FcERI signaling, involving stimulation with *N*-Formylmethionyl-leucyl-phenylalanine (fMLP).

2.4. Fluorescence Reagent Description

Each sample has been stained according to the following table:

Optical Detector	FL1	FL2	FL3	FL4	FL5	FL6
Reporter	FITC	PE				
Antibody	CD63	CCR3	-	-	--	-

3. Instrument Details

3.1. Instrument Manufacturer

BD Biosciences <http://www.bdbiosciences.com/home/>

3.2. Instrument Model

BD LSRIITM Flow Cytometer SORP (Special Order Research Product)

Serial number H47100174. Manufacture date August 2008.

<http://www.bd.com/resource.aspx?IDX=17868>

3.3. Instrument Configuration and Settings

3.3.1.Flow Cell and Fluidics

From August 2008 to present: The LSRII and Fortessa has a fixed-alignment cuvette flow cell.

3.3.2.Light Sources

From August 2008 to April 2012: The instrument has not been altered; three-laser base configuration with ACDU.

- 488nm Coherent Sapphire solid state; 50mW
- 640nm Coherent Cube solid state; 40mW
- 405nm Cube solid state; 50mW

From April 2012 to present: The LSRII was altered with the addition of

- 561nm Sapphire HP; 100mW.

No alterations of the Fortessa.

3.3.3.Excitation Optics Configuration:

LSRII: From August 2008 to April 2012: The instrument has not been altered.

LSRII: From April 2012 to present: See figures below.

Fortessa:See figure below.

3.3.4.Optical Filters

From August 2008 to April 2012: The instrument has not been altered.

From April 2012 to present: See figures below.

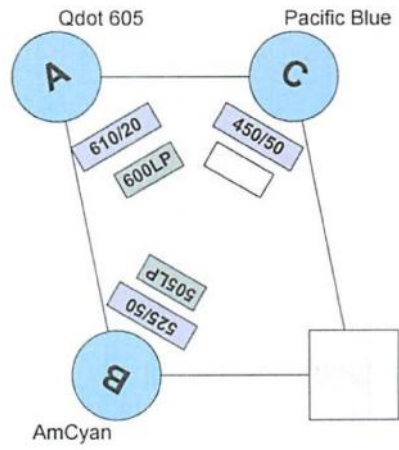
For both the LSRII and Fortessa (Table 1)

3.3.5.Optical Detectors

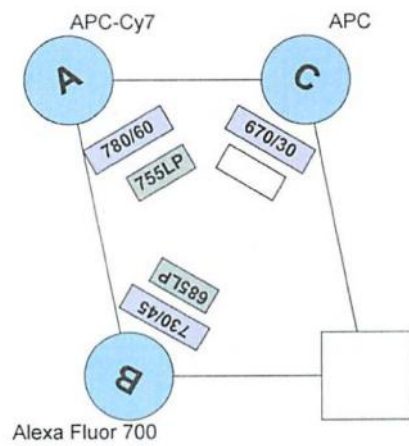
 Detector voltages varied by experiment.

3.3.6.Optical Paths: Same for the LSRII and Fortessa

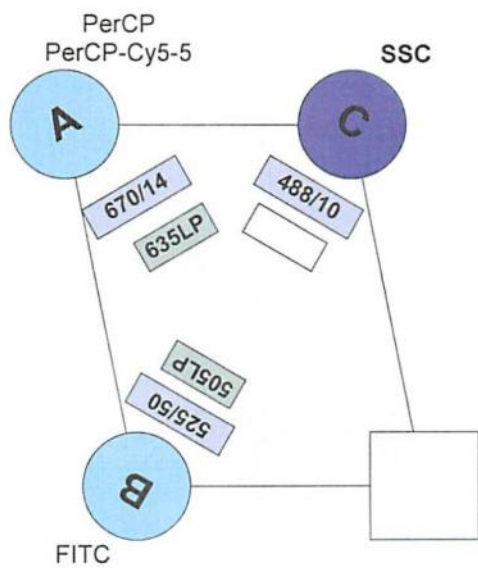
Violet Laser
(405nm)



Red Laser
(633nm)



Blue Laser
(488nm)



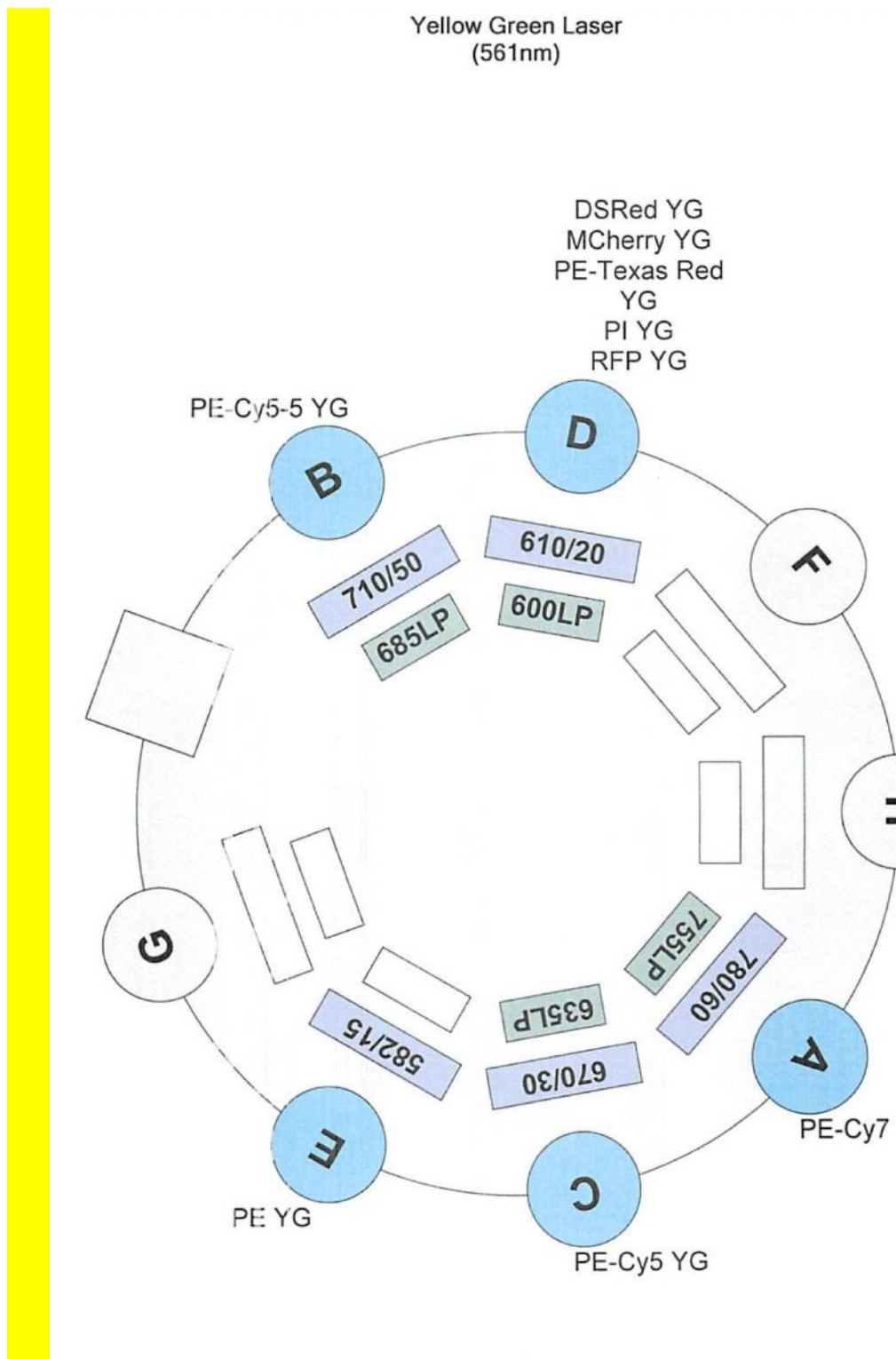


Figure 1: Optical detectors configuration.

Detector Array (Laser)	PMT	BP filter	Dichoric Filter	Intended Dye
Violet Laser (405nm)	A	610/20	600LP	Qdot 605
	B	525/50	505LP	AmCyan
	C	450/50	Blank	Pacific Blue
Red Laser (633nm)	A	780/60	755LP	APC-Cy7
	B	730/45	685LP	Alexa Fluor 700
	C	670/30	Blank	APC
Blue Laser (488nm)	A	670/14	635LP	PerCP, PerCP-Cy5-5
	B	525/50	505LP	FITC
	C	488/10	Blank	SSC
Yellow Green Laser (561nm)	A	780/60	755LP	PE-Cy7 YG
	B	710/50	685LP	PE-Cy5-5 YG
	C	670/30	635LP	PE-Cy5 YG
	D	610/20	600LP	DS Red YG, MCherry YG, PE-Texas Red YG, PI YG, RFP YG
	E	582/15	Blank	PE YG

Table 1: Optical detectors configuration details

3.4. Other Relevant Instrument Details

4. Data Analysis Details

4.1. List-mode Data Files

FCS data files can be obtained by contacting PNOIT Principal Investigator Dr. Wayne Shreffler after this work has been published.

4.2. Compensation Description

Set per experimental protocol.

4.3. Data Transformation Details

4.3.1. Purpose of Data Transformation Visualization and gating.

4.3.2. Data Transformation Description FlowJo default visualization settings have been used for gating:

- Select which parameters to display with logarithmic scaling: All fluorescence parameters

- Linear channel number corresponding to left edge of log display: 3
- Number of decades to display log-converted data: 5 for pulse area parameters, 4 for pulse height parameters
- Logicle transformation enabled: number of decades: 4.5, additional negative display size: 0, width basis: -10.

4.3.3. Other Relevant Data Transformation Details: FlowJo version 8.8.7 on a Macintosh OSX 10.6.8 has been used for transforming the data.

4.4. Gating (Data Filtering) Details

We implemented a sequential negative followed by a positive gating strategy may be effective for identification of basophils as SSC^{lo}, CCR3^{hi} cells.

4.4.1. Gate Description

The gating strategy involves the following steps:

- Logical transformation, followed by removal of boundary events
- Selecting the lowest K-means subset of the SSC distribution, where k=2 or 3
- Remove CCR3^{hi} non-basophils after filtering on the bivariate distributed CD63 and CCR3 population
- Identify basophils as a bivariate normally distributed SSC^{lo}, CCR3^{hi} population
- Identify the cut-off for CD63 to identify CD63^{hi} basophils by either:
 - o Medium-only gating: cutoff set at 97.5 percentile of CD63 expression of unstimulated basophils
 - o Anti-FcERI method: the cutoff is set at the midpoint of the bimodal populations of the CD63 expression in anti-FcERI stimulated basophils

4.4.2. Gate Statistics

We captured the following statistics: Basophil counts; basophil CCR3 MFI, SSC MFI, and percentage of CD63^{hi} basophils.

4.4.3. Gate Boundaries

In the Appendix of the attached paper.

4.4.4. Other Relevant Gate Information: None.