A hand-operated microfluidic sample preparation-to-analysis workflow for simplifying the basophil activation test

Nicolas Castaño[a], Kaiser Chua[a], An Nguyen[a], Wyeth Marshall[a], Grady H. Hofmann[a], Jonathan Lee[a], Mindy Tsai[b,c], Sayantani B. Sindher[c,d], Kari C. Nadeau[c,e], R. Sharon Chinthrajah[c,d], Stephen J. Galli[b,c,f], and Sindy K.Y. Tang[a,c]*

[a] *Department of Mechanical Engineering, Stanford University*

[b] *Department of Pathology, Stanford University*

[c] *Sean N. Parker Center for Allergy and Asthma Research, Stanford University*

[d] *Department of Medicine, Division of Pulmonary, Allergy and Critical Care, Stanford University*

[e] *Department of Environmental Health, T. H. Chan School of Public Health, Harvard University*

[f] *Department of Microbiology and Immunology, Stanford University*

*E-mail: sindy@stanford.edu

ELECTRONIC SUPPLEMENTARY INFORMATION

Supplemental Methods

Reagents and material

For blood collection, we acquired green-top, vacutainer sodium heparin blood collection tubes from BD Biosciences (catalog #368037, San Jose, CA). We used 5 mL round-bottom, polystyrene FACS tubes from Corning Falcon (Corning, NY) for flow cytometry, and 15 mL Nunc conical tubes from Thermo Fisher Scientific (catalog# 339650, Carlsbad, CA) for collecting fixed blood from μF-prep devices. We introduced blood to μF-prep devices using BD Microtainers from BD Biosciences (catalog# 365965, San Jose, CA). We purchased RPMI-1640 medium from Gibco (Grand Island, NY). The two positive control stimulants were goat polyclonal anti-human IgE from Thermo Fisher Scientific (Carlsbad, CA) and N-Formylmethionyl-leucyl-phenylalanine (fMLP) from MilliporeSigma (St. Louis, MO). We prepared peanut extract following an in-house protocol previously described.[1]

Our barcode stain panel consisted of antibody stains for CD45, HLA-DR, CD203c, CD193, CD123, and CD63. Various combinations of fluorophores were used for some markers, but the clones were the same across all stain panels (see Table S1). Stain cocktails were also supplemented with True-Stain Monocyte Blocker purchased from BioLegend (catalog# 426103, San Diego, CA) and BD Horizon Brilliant Stain Buffer ("BD buffer") from BD Biosciences to mitigate interaction between BV stains (catalog# 566349, San Jose, CA).

Our wash buffer consisted of 5% BSA and 2 mM EDTA in PBS (without calcium or magnesium). We stored anti-IgE, fMLP, and allergen extracts at -80˚C and the rest of the reagents at 4˚C. To lyse RBCs and fix the samples for storage, we used an RBC Lysis/Fixation Solution ("lyse/fix buffer") purchased from BioLegend (catalog# 420302, San Diego, CA).

Blood samples

All blood samples were collected into heparinized tubes from the Stanford Blood Center (Palo Alto, CA) or through the Sean N. Parker Center for Allergy & Asthma Research (SNP) with informed consent under Stanford University's International Review Boards IRB #52850. We stored them at 4 °C for 4 to 6 hours before bringing them to room temperature prior to the start of experiments.

Additional details on the fabrication of the μF-prep device

The cost of the resins used to print the μF-prep device was $16/device. We achieved high device reproducibility, due to relatively large channel diameters (1.25 mm) and part orientation on the printer build platform to facilitate effective resin drainage from the channels. While we used stereolithographic 3D printers to prototype the μF-prep devices in this study, we anticipate that injection molding will be needed for mass production.

Preparation of stimulation-stain cocktails for μF-prep

For the stain portion of the cocktail, we added 1 μL per stain for labelling CD193, CD123, CD63, CD203c, HLA-DR, and CD45, 3 μL of monocyte blocker, and 25 μL of BD buffer. The volume of the stain portion in the stimulation-stain cocktails was 34 μL. The stimulation portion (anti-IgE, peanut extract, fMLP, or RPMI) comprised the remaining 16 μL and was added at concentrations that would yield the desired final concentration of the stimulation-stain cocktail before the addition of blood (e.g., 5 μL of 20,000 ng/mL anti-IgE diluted with 11 μL of RPMI added to 34 μL of stain for a final concentration of 2,000 μg/mL anti-IgE in the stimulation-stain cocktail). The concentrations of stimulants in the stimulation-stain cocktails, prior to the addition of blood, were 2, 20, 200, 2,000, or 20,000 ng/mL of peanut extract, 20, 100, 200, 500, 1,000, or 2,000 ng/mL of anti-IgE, or 2 μM fMLP. On the same day as the experiment, we pre-loaded eight syringes with eight stimulation-stain cocktails each with a total volume of 50 μL. The pre-loaded devices were stored at room temperature for less than 30 min before use.

Conventional BAT

We conducted BATs with heparinized blood samples that were kept for 4 to 6 hours at 4˚C. Prior to beginning the BAT, the blood was brought to room temperature, like the blood used for μF-prep. 50 μL of blood was mixed with 50 μL of a dose of a stimulant in RPMI. We stimulated blood with peanut extract at 1, 10, 100, 1,000, or 10,000 ng/mL, anti-IgE at 1,000 ng/mL, fMLP at 1 μM, or just RPMI (i.e., negative control).

We stimulated blood *ex vivo* for 15 minutes. Samples were incubated at 37˚C in a water bath. Following the incubation period, basophils were quenched by adding 900 μL of ice-cold 2.5 mM EDTA in PBS, and the tubes were

centrifuged at 500 G for 5 minutes at 4˚C. We performed all centrifugations with these settings unless specified otherwise. The supernatant was aspirated, the pellet was vortexed, and the antibody surface markers stains were added. We used stains from the same panel as the µF-prep experiments that were run in parallel with the conventional BAT controls. The stain cocktail included antibody labels for CD193, CD123, CD63, CD203c, HLA-DR, and CD45. The conventional BAT samples were not pooled, thus we only used one combination of CD193 and CD123 stains (e.g., anti-CD193 on BV421 and anti-CD123 on Alexa Fluor 700 or APC). Antibody stain cocktails contained 1 µL of each stain, 3 µL of monocyte blocker, and 25 µL of BD buffer (34 µL total per tube). We stained blood samples for 20 minutes on ice, then added 3 mL of wash buffer, centrifuged the tubes, and aspirated the supernatant. We vortexed the pellet thoroughly prior to lysis and fixation to ensure all cells were resuspended and adequately exposed to lyse/fix buffer. We added 900 µL of room temperature 1x lyse/fix buffer, immediately mixing the mixture with a brief pulse on the vortex mixer. The tubes were left in the dark at room temperature for 30 minutes before topping them off ~4 mL with wash buffer. We centrifuged the tubes, aspirated the supernatant, vortexed, and repeated this wash process after adding ~3 mL of wash buffer to the tubes. For flow cytometry, we left ~250 µL after the final aspiration step. In our hands, the total time to prepare these tubes for flow cytometry was ~3 hours.

Flow cytometry

All data were acquired with a Cytek Aurora flow cytometer (Cytek Biosciences, Fremont, CA), a spectral flow cytometer which measures the entire emission spectrum of fluorophores rather than specific wavelengths like traditional flow cytometers.[2] Prior to each session with the Aurora, we ran SpectroFlo QC beads from Cytek Biosciences (catalog# B7-10001, Fremont, CA). The QC routine adjusted gains of each channel as needed to ensure day-to-day detector consistency. We used UltraComp eBeads from Thermo Fisher Scientific (catalog# 01-2222-42, Carlsbad, CA) as reference controls to provide the emission spectra of each fluorophore so that the Aurora can unmix raw data into distinct fluorescent intensities for each marker.

Manual gating analysis and data annotation

We analyzed all flow cytometry data using FlowJo™ v10.8 Software (TreeStar, Ashland, OR). All workspaces shared common biex transformation parameters (negative decade and width of data compressed around zero) to ensure consistency in how data were represented and gated. The following parameters for each marker were used: negative =

1, width = -200 for CD63; negative = 1, width = -1000 for CD193, CD123, and CD203c; negative = 1, width = -500 for HLA-DR; and negative = 0, width = -200 for CD45. We gated basophils from singlets as $CD45^{+}/SSC^{low}CD203c^{high}/HLA\text{-}DR^{-}/CD123^{+}CD193^{+}$ (Fig. S3),[3–5] and verified through backgating so that no stained basophils were excluded from parent gates. We used the upper bound of CD63 expression in the negative control to establish the placement of the $CD63^{+}$ gate. Each specific experiment and condition assessed had its own negative control to reference. Manual gating provided the barcode index (BCI) required for training and testing the basophil classifier, and it provided $CD63^{+/-}$ labels to compare with the labels generated by the $CD63^{+/-}$ gating routine.

We used FlowKit to read Flow Cytometry Standard (FCS) files and loaded all data into Pandas DataFrames. To be consistent with how data was represented in FlowJo, we applied the same biex transforms used FlowJo workspaces to the FCS data frame using FlowKit's WSPBiexTransform class. We determined the membership of each event to a specific barcoded basophil population (i.e., 1 if the event is in the gate, 0 if not) by analyzing manually gated FlowJo workspaces with FlowKit's Workspace class.[6] Then, we encoded the membership of each event with a BCI: 0 for non-basophils and 1 to 8 for each barcode group. Similarly, we labelled basophils as $CD63^{+/-}$ (1 if the basophil is $CD63^{+}$, 0 if it is $CD63^{-}$). We also annotated all events with a donor ID, stain panel ID, and various meta data features (i.e., BAT type, incubation time, days stored, blood volume). The barcode parser used these identifiers to reference look-up tables and assign a stimulation condition to each BCI.

Automated gating pipeline

The automated gating pipeline was written in Python and consisted of an XGBoost barcoded basophil classifier, a data parser to assign conditions linked the BCIs of basophil populations, an algorithm to detect high CD63 baseline, and a $CD63^{+/-}$ gating routine (Fig. 2A, Fig. S7). First, the basophil classifier, an XGBoost model, unpooled the sample by labelling events as non-basophils or basophils from one of the eight barcoded populations. We used all flow cytometer parameters (FSC-A, FSC-H, SSC, autofluorescence, CD45, HLA-DR, CD203c, CD63, CD123, and CD193) and categorical features (incubation time, stain panel ID, and days stored) as input features to train and test the basophil classifier. After assigning BCIs the barcode parser used predefined BCI look-up tables, defined for each sample, to assign a test condition (i.e., RPMI, a dose of peanut extract or anti-IgE, or fMLP) to each BCI. Next, the RPMI negative control of each sample set was programmatically evaluated for high baseline activation using two criteria: 1)

the z-score of the CD63 standard deviation exceeded a threshold of 2.5,[7] or 2) the CD63 intensity distribution contained multiple peaks, with a second peak higher than the first. In cases of high baseline activation, all conditions associated with that RPMI control were manually gated and were omitted in all plots and analysis except in Fig. 3B. Next, parsed basophil data without instances of high baseline passed to the $CD63^{+/-}$ gating routine which used the negative control within a set of µF-prep test conditions to establish a cut-off for $CD63^{+/-}$ basophils. This cut-off value was determined as the 97.5 percentile value of the negative control[8] after outliers were removed, i.e., retaining basophils with CD63 fluorescent intensity < 75^{th} percentile value + 1.5(interquartile range). The cut-off was applied to all test conditions within a µF-prep experiment, including the negative control (with all basophils). The CD63 gating routine also converted data of individual basophils to population-level statistics data frames (i.e., calculate $\%CD63^{+}$). After verifying that our automated gating pipeline was classifying basophils and labelling $CD63^{+/-}$ basophils accurately, we relied on the pipeline to generate results for µF-prep experiments in subsequent analyses.

Across all experiments, we collected a total of ~136 million events, of which ~540,000 were basophils. To reduce computational costs, we applied a conservative $CD45^{+}/SSC^{low}/HLA\text{-}DR^{-}$ thresholding routine to reduce the data frame size to ~76 million cells. We trained and evaluated our models on all the data we collected with µF-prep, including experiments where we varied the blood volume and the storage time. We also supplemented our training and evaluation data with experiments where we incubated blood samples with anti-IgE or peanut extract for 5 to 30 minutes. From the manual gating analysis, each event was labelled with a BCI (1 to 8 and 0 for non-basophils) and a binary $CD63^{+/-}$ label (1: $CD63^{+}$, 0: $CD63^{-}$). We trained the basophils classifier with 70% of all data (~53 million non-basophils, ~378,000 basophils). We tuned the model hyperparameters of the basophil classifier using a Bayesian optimization method from BayesOpt which maximized the F1 score of model predictions.[9] For the basophil classifier we also included CD193-1, CD193-2, CD123-1, CD123-2, CD123-3, and CD123-4 for the two CD193 stains and the four CD123 stains.

Marker	Clone	Fluorophore	Stock concentration [µg/mL]	Catalog number	Stain panel ID	Supplier
CD193	5E8	BV421	50	310714	A, B, C, D, E	BioLegend (San Diego, CA)
		BV605	100	310716	A, D	
		BV711	200	310731	B, C, E	
CD123	6H6	Alexa Fluor 700	50	306040	C, D, E	
		APC	100	306012	A, B, E	
		BV605	100	306026	C	
		BV785	100	306032	A, B, D	
		PE	50	306006	A, B, C, D, E	
		PE/Cy5	50	306008	A, B, C, D, E	
CD203c	NP4D6	BV510	200	324622	A, C, D	
		BV605	100	324620	B, E	
CD63	H5C6	FITC	50	561924	A, B, C, D, E	BD Biosciences (San Jose, CA)
CD45	HI30	Alexa Fluor 700	500	304024	A	BioLegend (San Diego, CA)
		BV510	100	304036	B, E	
		APC	100	304037	C, D	
HLA-DR	L243	PE/Cy7	200	307616	A, B, C, D, E	

Table S1.

Various stain panels used for µF-prep barcoding and their IDs. The antibody stains against a given marker always used the same clone even though the fluorophore differed across stain panels. Index values N=1-2 and N=1-4 were assigned to CD193 and CD123 stains, respectively, based on the index of the row in the look-up tables where barcodes and conditions were defined for each experiment. The complexity index of these stain panels ranged from 2.18 to 2.54, well within the acceptable range of complexity for a mixture of cells with 10 stains.[10]

	conv-prep + manual gating	µF-prep + automated gating
Sample preparation		
Maximum delay between blood draw and sample preparation (i.e., basophil activation + staining)	<24 hours	< 24 hours
Total sample prep time per patient sample (for 8 stimulation conditions per patient)	~3 hours (range: 2.75 to 3.75 hours) (including 30 min incubation, 20 min staining on ice, and 30 min lyse/fix)	<47 min (range: 46 to 48 min) (including 15 min incubation + 30 min lyse/fix)
Total active user engagement time for sample prep per patient sample (for 8 stimulation conditions)	~1.5 hours (range: 1.25 to 2.25 hours)	<2 min (range: 1 to 3 min)
Number of manual sample prep steps for the user (pipetting, centrifugation, aspiration, vortex-mixing, etc.)	~90	4
Lab equipment required for sample prep	pipette, centrifuge, water bath, ice bucket	none (uses custom, portable device)
Flow cytometry analysis		
Maximum delay between blood draw and flow cytometry analysis	<24 hours	up to 7 days (stored at 4°C)
Flow cytometry preparation time	N/A (included in sample prep time above)	15 min to wash and transfer to a FACS tube
Flow cytometry run time	~32 min (range: 30 to 42 min), 8 tubes	6 min (range: 5 to 7 min), single tube
Flow cytometry data analysis	~1 hour (range: 0.75 to 1.5 hours), manual gating and compilation of results	<1 second (range: 0.5 to 1.5 seconds), automated analysis pipeline with no manual input
Total assay time (sample prep + analysis) to extract basophil activation level (e.g., %CD63+ dose response curves)	**4.5-5 hours**	**1.38 hours**

Table S2.

Comparison of procedural differences for microfluidic BAT sample preparation (µF-prep) plus the automated gating pipeline and conventional BAT (conv-prep) plus a manual gating pipeline. The sample preparation comparison assumes eight stimulation conditions and focuses on the steps involved in sample preparation, specifically up to the stage, but before, the user would send the sample to a centralized lab for flow cytometry analysis. The flow cytometry comparison focuses on additional steps taken by a centralized lab to conduct run flow cytometry and conduct the data analysis. The approximate flow cytometry run times were extracted from meta data of FCS files.

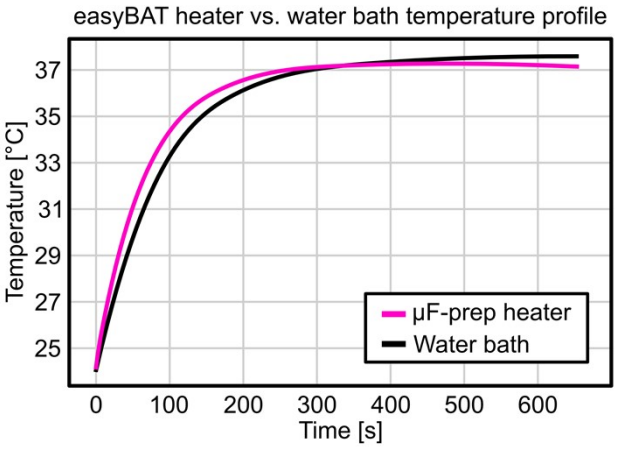

Figure S1.

Temperature profile of the µF-prep heater compared with that of the water bath used in conv-prep.

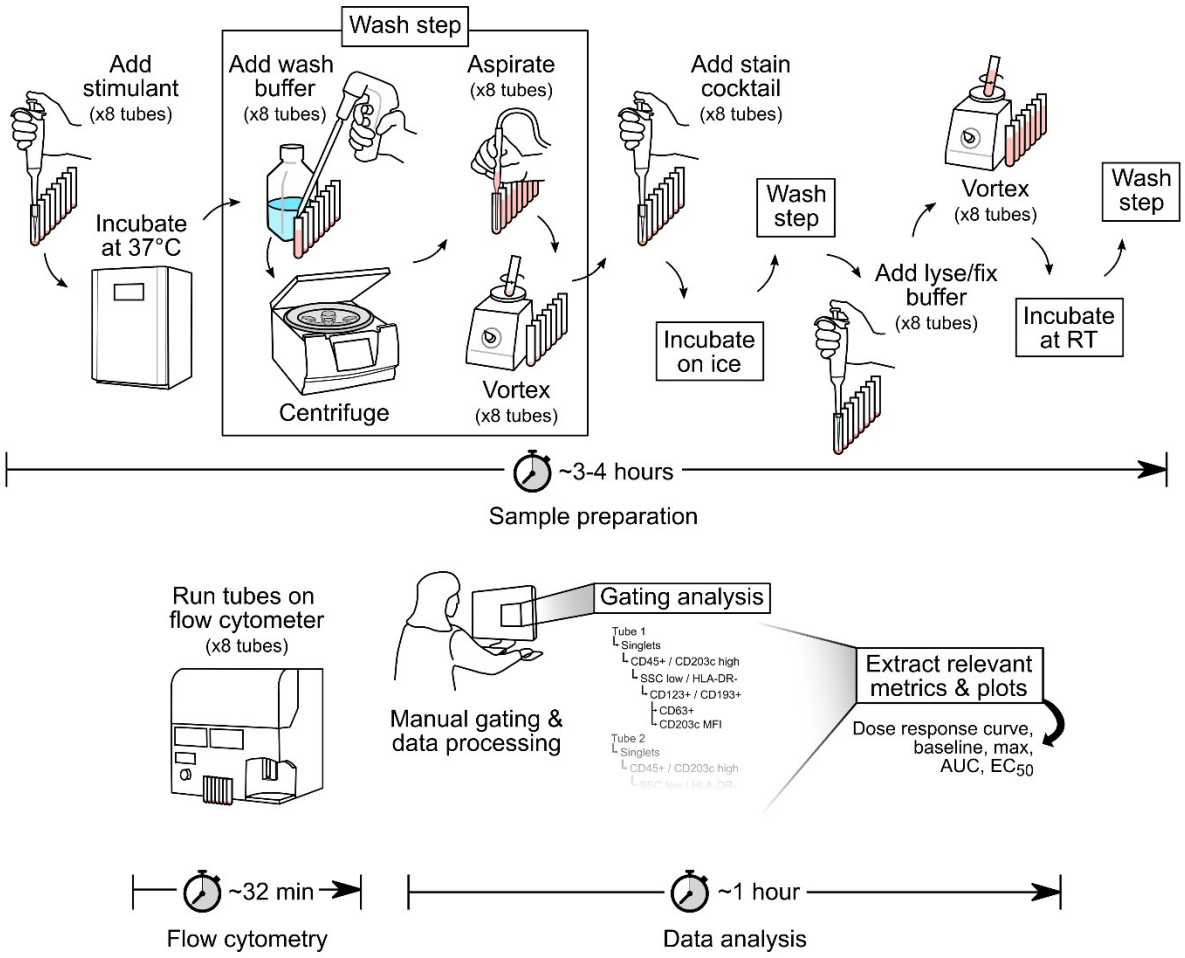

Figure S2.

Schematic representation of the conventional BAT workflow. Essential laboratory equipment includes pipettes, an incubator, a centrifuge, and a vortex mixer. The efficiency and accuracy of conventional BAT processes are heavily dependent on the skill and precision of the laboratory personnel.

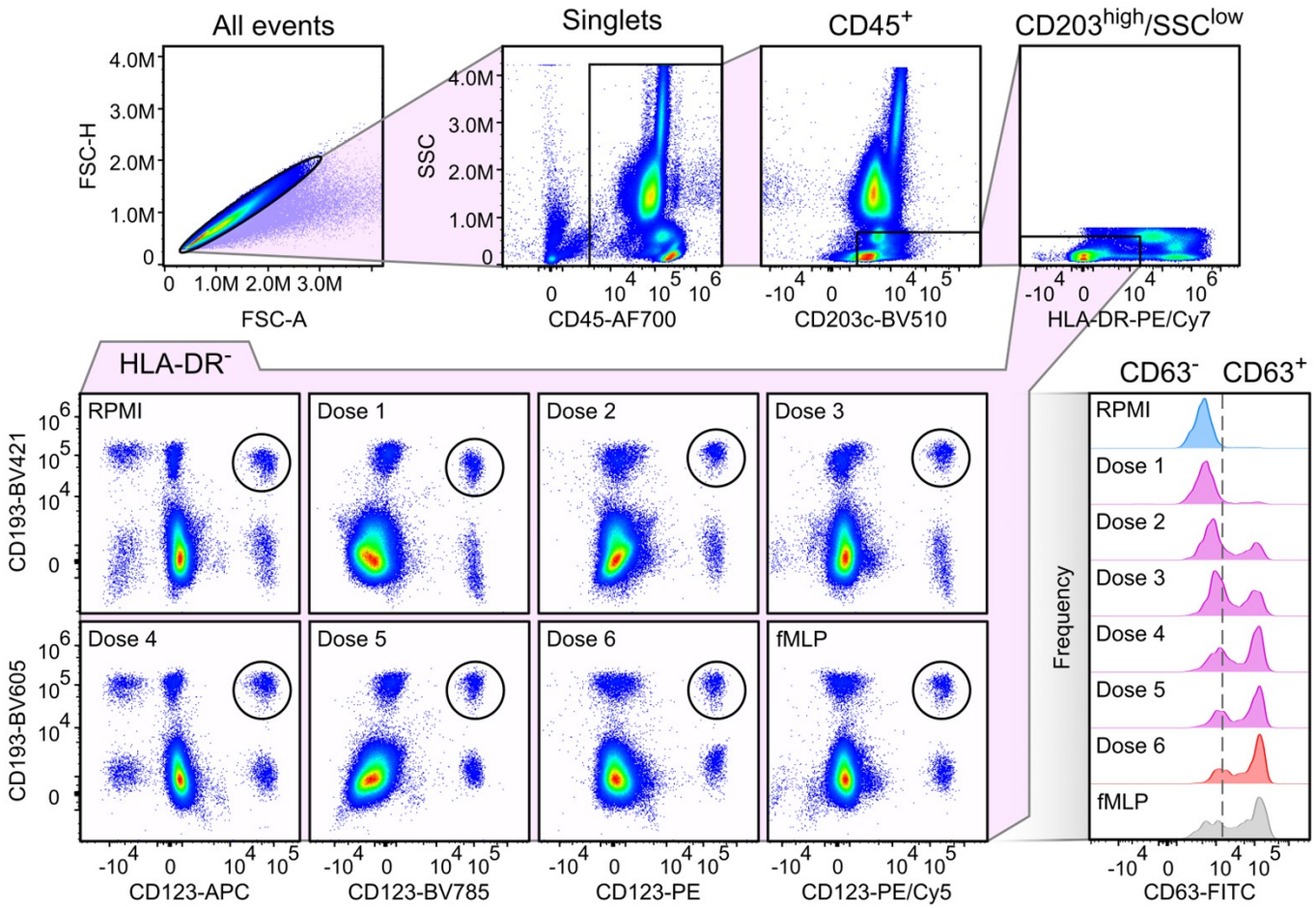

Figure S3.

Representative example of the manual gating pipeline of barcoded basophils (stain panel A, Table S1) pooled into one tube for flow cytometry. All basophil barcoded populations share singlet, $CD45^{+}$, $CD203c^{high}/SSC^{low}$, and $HLA\text{-}DR^{-}$ gates. Each basophil barcode population is easily distinguishable from other barcodes on CD123 and CD193.

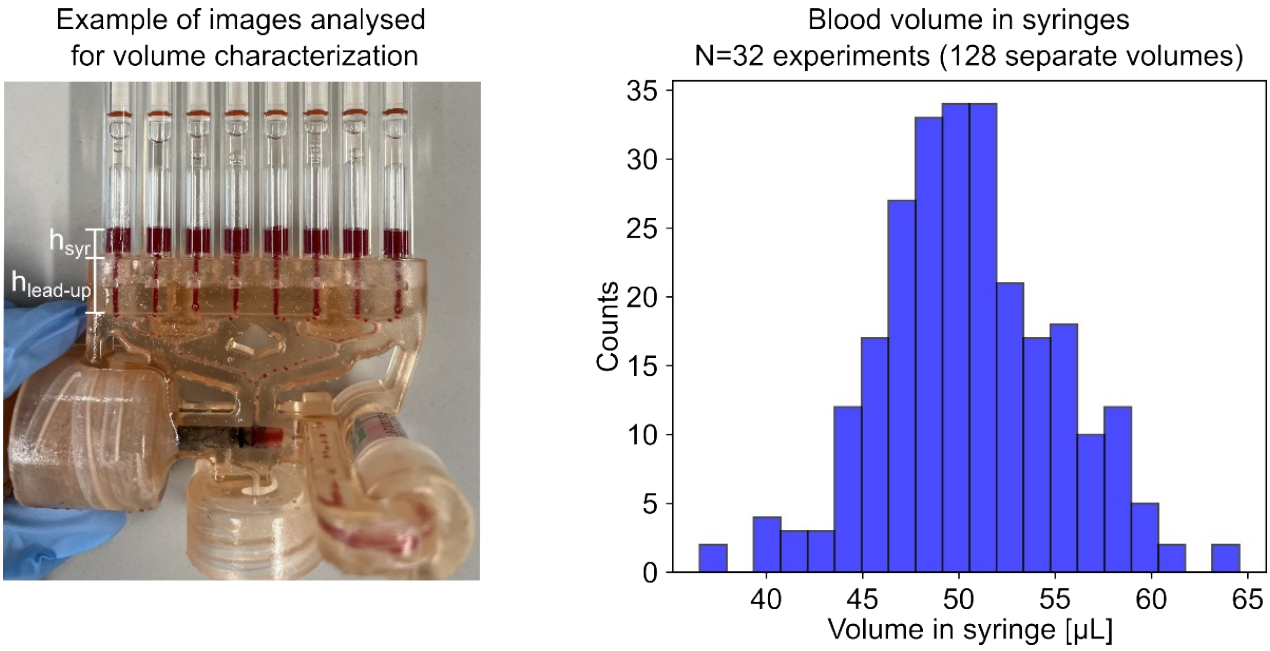

Figure S4.

We approximated the dead volume of blood subtracting the total volume across each syringe from the input blood volume of 450 µL. We determined the blood volume in each syringe by measuring the heights h_{syr} and $h_{lead-up}$ of the continuous blood column in two section of known inner diameter (3.175 mm syringe inner diameter and 1.25 mm lead-up channel inner diameter) using ImageJ. We measured h_{syr} and $h_{lead-up}$ for each syringe, and the syringe outer diameter as a reference measurement, twice and used the average to determine the volume in each syringe. We performed this approximation for 32 separate experiments across 5 devices. The average and standard deviation of blood volume in each syringe was 50.6 ± 2.8 µL

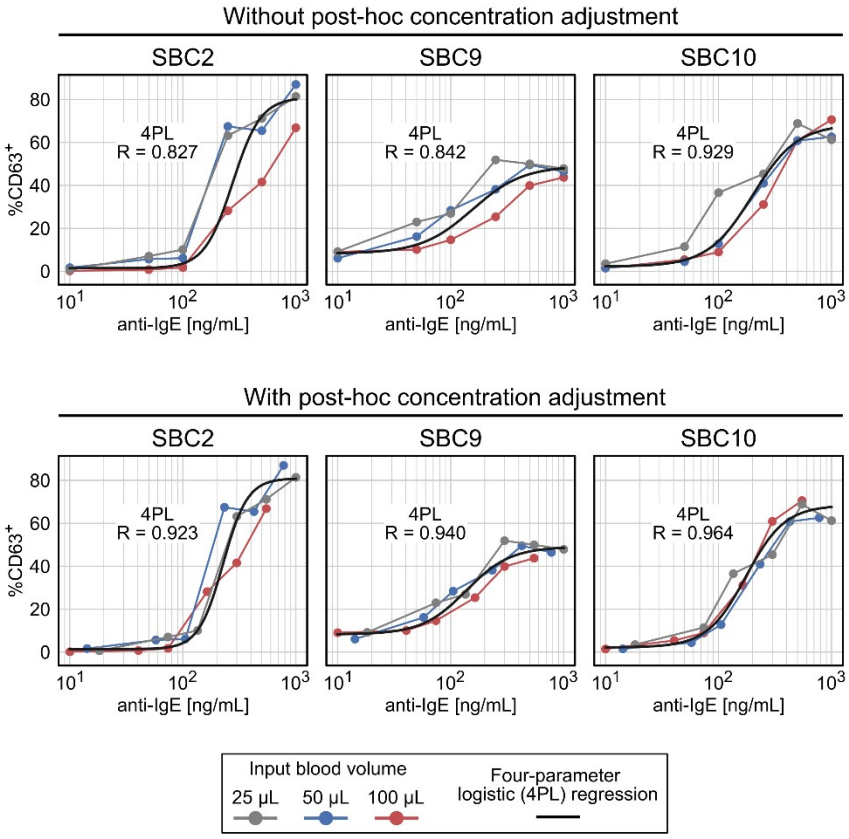

Figure S5.

We examined the influence of different input blood volumes, other than 50 µL as was used in other experiments, on the anti-IgE dose-response curves. By using blood volumes of 25 µL and 100 µL, the effective concentration of target anti-IgE was adjusted to 1.33 times and 0.67 times the intended doses, respectively. Following a post-hoc correction of anti-IgE concentrations, the dose-response data were better fitted by four-parameter logistic (4PL) regressions, as indicated by increased R-values compared with those from the original, non-adjusted dose responses.

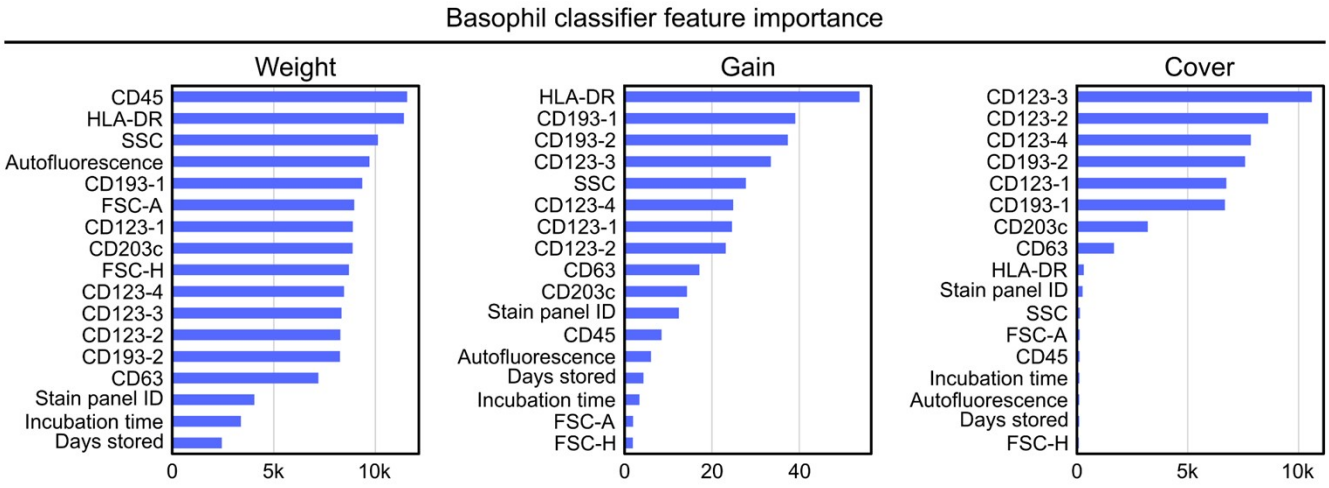

Figure S6.

The average importance of features for the basophil classifier. Weight describes the number of times a feature is used to split the data across all trees (i.e., count of how often a feature is used in the model). Gain describes the contribution of each feature to the model by considering the improvement in accuracy brought by a feature to the splits it is used in (i.e., how much a feature contributes to making more accurate predictions). Cover describes the relative quantity of observations related to a feature (i.e., how many times on average a feature is used in a split across all trees, weighted by the number of training instances a node is responsible for).

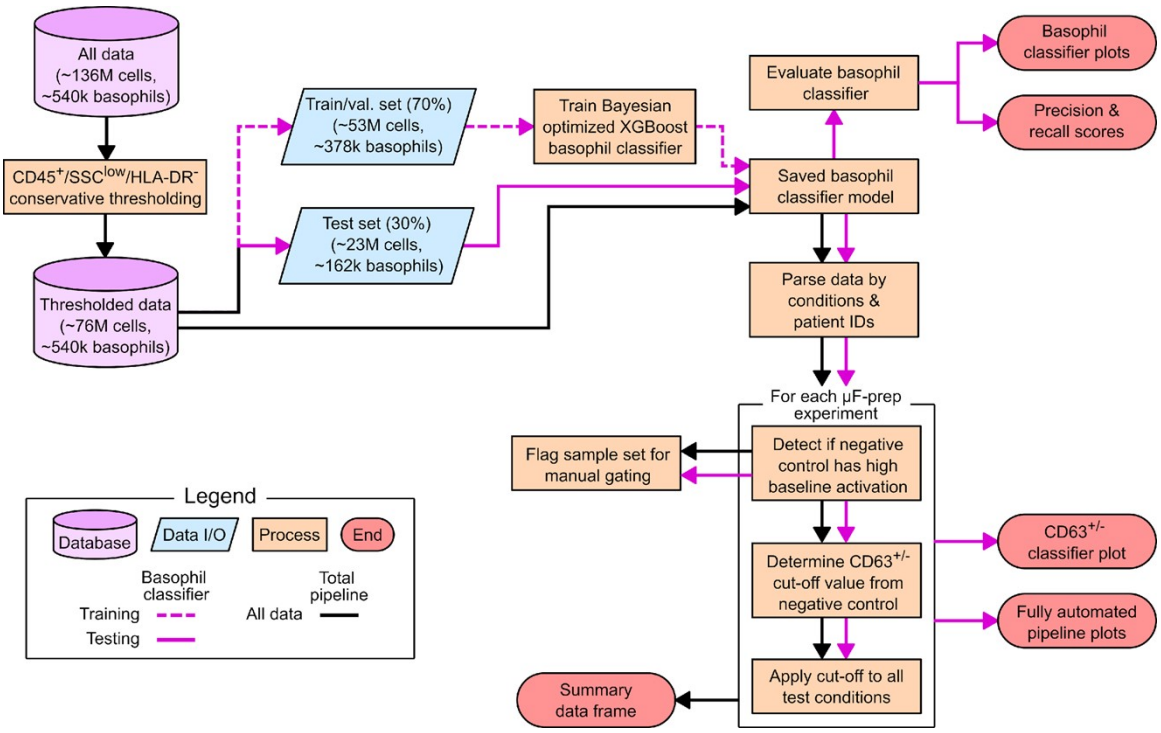

Figure S7.

Flowchart outlining the automated analysis pipeline for basophil classification and CD63$^{+/-}$ gating. The dataset consisted of approximately 136 million cells including 540 thousand basophils. After a conservative thresholding routine on CD45^{+}/SSC^{low}/HLA-DR^{-}, 76 million cells were used for the basophil classifier with a 30/70 test/train split. The performance of the Bayesian-optimized XGBoost model plus the CD63$^{+/-}$ gating routine was evaluated with the test set (solid magenta arrow) (see Fig. 2D, E, and F). The fully automated pipeline was applied to all data (solid black arrows) to generate a population-level summary data frame (i.e., each row contains statistics of separate barcoded basophil populations). From this data frame, other BAT metrics were derived, e.g., area under the dose response curve (AUC).

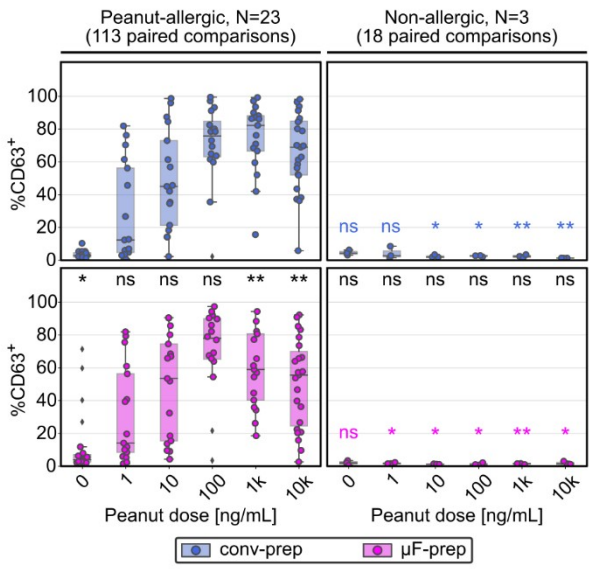

Figure S8.

Same plot as Figure 3B in the main text but separating µF-prep and conv-prep into different graphs. Tukey-style box and whisker plots show %CD63$^+$ activation measurements of peanut-allergic (PA) and non-allergic (NA) subjects. Wilcoxon signed-rank tests were used for paired comparisons between µF-prep and conv-prep (black font). Mann-Whitney U-tests were used to compare between PA and NA for both µF-prep and conv-prep (magenta and blue font, respectively, annotated on the non-allergic plot). The Benjamini and Hochberg method was used to adjust *P* values (i.e., *Q* values) by correcting for false discovery rates during multiple hypothesis testing. *$Q < 0.05$, **$Q < 0.01$, and ns is not significant ($Q > 0.05$).

Movie S1. Movie showing the F-prep device being used.

References

1. Castaño, N. *et al.* Combining avidin with CD63 improves basophil activation test accuracy in classifying peanut allergy. *Allergy* (2023). doi:10.1111/all.15930

2. Nolan, J. P. & Condello, D. Spectral flow cytometry. *Curr. Protoc. Cytom.* **Chapter 1,** Unit1.27 (2013).

3. Santos, A. F. *et al.* Distinct parameters of the basophil activation test reflect the severity and threshold of allergic reactions to peanut. *J. Allergy Clin. Immunol.* **135,** 179–186 (2015).

4. Santos, A. F. *et al.* Biomarkers of severity and threshold of allergic reactions during oral peanut challenges. *J. Allergy Clin. Immunol.* **146,** 344–355 (2020).

5. Santos, A. F., Bécares, N., Stephens, A., Turcanu, V. & Lack, G. The expression of CD123 can decrease with basophil activation: implications for the gating strategy of the basophil activation test. *Clin. Transl. Allergy* **6,** 11 (2016).

6. White, S. *et al.* Flowkit: A python toolkit for integrated manual and automated cytometry analysis workflows. *Front. Immunol.* **12,** 768541 (2021).

7. Yang, J., Rahardja, S. & Fränti, P. Outlier detection: How to threshold outlier scores? in *Proceedings of the International Conference on Artificial Intelligence, Information Processing and Cloud Computing' ' - AIIPCC '19* 1–6 (ACM Press, 2019). doi:10.1145/3371425.3371427

8. Bezinge, L. *et al.* Basophil activation test: How many basophils are enough? *Allergy* (2024). doi:10.1111/all.16391

9. Martinez-Cantin, R. BayesOpt: A Bayesian Optimization Library for Nonlinear Optimization, Experimental Design and Bandits. *arXiv* (2014). doi:10.48550/arxiv.1405.7430

10. Cytek Cloud. at <https://cloud.cytekbio.com/login?source=standlone>