

Chapter 11

Flow Cytometric Allergy Diagnosis: Basophil Activation Techniques

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Abstract

The basis of flow cytometric allergy diagnosis is quantification of changes in expression of basophilic surface membrane markers (Ebo et al., Clin Exp Allergy 34: 332–339, 2004). Upon encountering specific allergens recognized by surface receptor FcεRI-bound IgE, basophils not only secrete and generate quantifiable bioactive mediators but also up-regulate the expression of different markers (e.g., CD63, CD203c) which can be detected by multicolor flow cytometry using specific monoclonal antibodies (Ebo et al., Cytometry B Clin Cytom 74: 201–210, 2008). Here, we describe two flow cytometry-based protocols which allow detection of surface marker activation (Method 1) and changes in intragranular histamine (Method 2), both reflecting different facets of basophil activation.

Key words Basophil activation tests, CD203c, CD63, HistaFlow

1 Introduction

Basophil activation assays have predominantly focused on histamine and leukotriene release assays. However, time-consuming procedures were needed to isolate cells, to incubate with activators, and to quantify (sometimes labile) mediators. Two decades ago, the discovery of the markers CD63 (a.k.a. gp53) and subsequently CD203c (a.k.a. ENPP-3) facilitated the development of a novel technique to analyze and quantify allergen-specific in vitro activation of basophils: the so-called basophil activation test (BAT) [2]. Briefly, the BAT relies on a flow cytometric identification and quantification of various specific activation markers on the surface membrane or activated proteins inside the cells. These changes can be detected and quantified on a single-cell level using specific monoclonal antibodies coupled to fluorochromes. Practically, basophils are identified by specific markers such as CCR3/CD3 [3], CD123/HLA-DR [4], or IgE/CD203c [5]. Of these markers, only CD203c is lineage specific. Subsequently, after activation with

an allergen, the upregulation or the appearance of specific surface activation markers, such as CD63 and/or CD203c, can be measured. Activation of basophils can also be quantified by means of intracellular molecules, such as phosphorylation of MAPK (p38MAPK) [6, 7] or STAT5 (pSTAT5) [8]. At present, BAT has proven to be a rapid and reliable diagnostic method that allows the diagnosis of allergy to various allergens like food (including pollen-related food allergies), natural rubber latex, hymenoptera venom, and certain drugs. In addition, the technique has proven to be useful in non-IgE-mediated reactions, such as in drug hypersensitivity as well as in the detection of IgG anti-FcεRI or anti-IgE autoantibodies in chronic autoimmune urticaria.

Finally, it was recently shown that intracellular histamine and its release by in vitro-activated basophils can successfully be analyzed by multicolor flow cytometry (HistaFlow) [9] applying an enzyme-affinity fluorochrome method, based on the affinity of the histaminase diamine oxidase for its substrate [10]. We developed a flow cytometric technique (HistaFlow) which enables the measurement of histamine release by individual basophils in combination with cell membrane markers. Conjugating diamine oxidase to fluorochromes together with phenotyping activated basophils, histamine content, and release can be quantified in whole blood with standardized flow cytometry techniques.

2 Materials

All solutions must be prepared with sterile ultrapure water. We use a Millipore Milli Q System and water purification is performed with the use of a reversed osmosis system followed by ultra-purification to attain 18 MΩ at room temperature.

2.1 Blood Sample

10 mL peripheral heparinized blood is obtained and immediately brought to the laboratory. Incubation and analysis must be completed within 8 h (*see* **Note 1**).

2.2 Reagents

1. Interleukin-3 (IL-3)

IL-3 is sometimes used to preactivate (“prime”) basophils. It must be noted that IL-3 can upregulate CD203c.

- (a) Stock solution: 10 μg IL-3 in 10 mL water, divide per 100 μL in sterile tubes. Store at −20 °C.
- (b) Storage solution: Dilute 100 μL stock solution in 5 mL BAT buffer and divide per 500 μL. Store at −20 °C.
- (c) Working solution: Dilute storage solution with an equal volume of buffer; prepare daily before use. The final concentration is 10 ng/mL.

2. BAT buffer

- (a) A BAT buffer is prepared under STERILE conditions just before use.
- (b) Dilute 1.9 mL Hanks' balanced salt solution 10× concentrated (HBSS with Ca^{2+} and Mg^{2+}) with 17 mL H_2O .
- (c) Add 0.6 mL 7.5 % NaHCO_3 .
- (d) Add 0.4 mL 1 M Hepes.
- (e) Adjust pH to 7.4.

3. Lysis solution

Lysis solution (BD Biosciences) is diluted 1:10 with water and stored at room temperature.

4. Phosphate-buffered saline (PBS 1×)

- (a) PBS (10× concentrated without Ca^{2+} or Mg^{2+}) is diluted with water to obtain an isotonic PBS (1×) buffer. Adjust pH to 7.4.

5. PBS-EDTA

Dissolve 10 mmol/L Na_2EDTA in PBS (1×). Adjust pH to 7.4.

6. PBS- NaN_3

Dissolve 0.5 g/L NaN_3 in PBS (1×). Adjust pH to 7.4. Store at room temperature.

7. PBS-0.1 % BSA

Mix 50 mL PBS with 0.66 mL BSA (albumin bovine fraction V 7.5 %); pH should be 7.4.

8. Anti-human IgE Alexa Fluor®488 (aIgE)

Since anti-IgE (Sigma-Aldrich, Clone GE-1) is currently not available conjugated with an Alexa Fluor®488 Dye, this antibody is labeled in house using the procedure by the manufacturer Life Technologies described in Subheading 3 below.

9. CD63-PE or CD63-FITC

Anti-CD63-PE or anti-CD63-FITC (BD Biosciences) must be stored at 2–8 °C in the dark. This antibody is ready to use (*see Note 2*).

10. Anti-CD123-PE/anti-HLAdr-PERCP/anti-CD63-FITC

All antibodies available commercially ready to use (BD BioSciences) (*see Note 2*).

11. Mouse anti-human IgE (mHu-antiIgE)

- (a) This antibody (BD Biosciences, clone G7-18) is used as a positive control to stimulate basophils. Stock solution: 0.5 mg/mL in BAT buffer.
- (b) Working solution: Between 1 and 10 $\mu\text{g}/\text{mL}$ in BAT buffer.

12. CD203c-APC
Anti human CD203c (clone NP4D6) labeled with APC (BD Biosciences or BioLegend), ready to use (*see* **Note 2**).
13. Allergens
Allergens can be protein extracts, recombinant proteins, or drugs. They must be water soluble and used in concentrations which are not toxic for basophils (*see* **Note 3**).
14. DAO-PE or DAO-V500 (BD Biosciences)
 - (a) Diamine oxidase or histaminase (Sigma-Aldrich) has been conjugated with phycoerythrin or Horizon V500 at the laboratories of BD Biosciences.
 - (b) Prepare a working solution 1:1 (DAO-PE) or 1:20 (DAO-V500) in PBS/EDTA. Every new batch has to be titrated and measured with a flow cytometer in order to find the optimal staining concentration for intracellular histamine.
15. Lysis/fixing solution (BD Biosciences)
 - (a) BD Phosflow Lyse/Fix buffer (5× concentrate: BD Biosciences) is used to lyse and fix whole blood for use with intracellular staining techniques. Store at room temperature.
 - (b) Dilute 1:5 just before use in H₂O.
16. PBS-TX100
Working solution is 0.1 % Triton X-100 in PBS: Add 0.1 mL pure Triton X-100 (Sigma-Aldrich) to 100 mL PBS (1×). Store at room temperature.
17. Calibration particles
Rainbow Calibration Particles six peaks or eight peaks (BD Biosciences). Store undiluted at 4 °C in the dark. Mix well before use. Dilute 3–5 drops in 1 mL buffer. Use immediately.

3 Methods

3.1 Flow Cytometric Basophil Activation Technique: Membrane Markers (Method 1)

All procedures are carried out at room temperature, in a laminar air-flow cabinet when necessary, unless otherwise specified (*see* **Note 7**).

3.1.1 Alexa Fluor®405 Labeling of Mouse Monoclonal Anti-human IgE

1. Labeling procedure: Mouse monoclonal anti-human IgE (clone GE-1) is diluted in NaHCO₃ (0.1 mol/L in H₂O, pH 8.3) at a concentration of 5 mg/mL.
2. Alexa Fluor 488 carboxylic acid, succinimidyl ester, and triethylammonium salt (Life Technologies, Molecular Probes) can be stored at –20 °C for a maximum of 3 months. When dissolved in DMSO, the solution becomes unstable. A working solution is made fresh by dissolving 1 mg in 0.1 mL DMSO.

3. Mix 2 mL anti-IgE in NaHCO_3 slowly with 0.1 mL Alexa Fluor[®] 488 working solution for 1 h at room temperature in the dark. Mix regularly.
4. Dialyze overnight with PBS at 4 °C.
5. Add NaN_3 until a final concentration of 0.05 % is reached. Store at 2–8 °C.
6. Working solution: 1:50 in PBS- NaN_3 . Store at 2–8 °C. The working solution has to be titrated every time a new batch is made (*see also Note 2*).

3.1.2 Preparation of Positive and Negative Controls (Exclusion of Non-responders)

1. Incubate 100 μL blood with 100 μL negative (BAT buffer) or positive control (anti-IgE) during 20 min at 37 °C.
2. Using the procedure described below, stain with aIgE-AF488 and CD203c-APC (*see Note 4*) or CD123/HLA-Dr and CD203c-APC (*see Note 4*).
3. When basophils do not react one can decide to stimulate with two concentrations of the allergen to exclude non-reactivity to anti-IgE (*see Note 5*).

3.1.3 Allergen Procedure

1. All blood samples, buffers, and allergens are prewarmed without mixing for 15 min at 37 °C in warm water bath (WWB) (*see Note 6*).
2. Prepare incubation tubes and add 100 μL allergen solution to the allergen tubes. Dilutions depend on the allergen used (*see Note 3*). Use BAT buffer as a negative control or for FMO evaluation (*see Note 2*).
3. Add anti-IgE as positive control to a positive control tube. Add 100 μL BAT buffer to a negative control tube.
4. Add to all tubes 100 μL prewarmed whole blood.
5. Incubate during 20 min at 37 °C in WWB.
6. Stop reaction by adding 1 mL PBS-EDTA.
7. Spin at $200 \times g$ for 10 min at 4 °C without setting on the brake of the centrifuge. Remove supernatant.
8. Add 20 μL aIgE-AF488 and 10 μL anti-CD63-PE (or in case at 10 μL CD123/HLADr/CD63). Add 5 μL aCD203c-APC.
9. Incubate on ice for 20 min in the dark.
10. Add 2 mL lysis solution and incubate at room temperature for 20 min.
11. Spin for 10 min at $250 \times g$ at room temperature. Remove supernatant.
12. Add 2 mL PBS and spin again for 10 min $250 \times g$ at room temperature. Remove supernatant.
13. Add 300 μL PBS- NaN_3 . The tubes are now ready to measure with a flow cytometer.

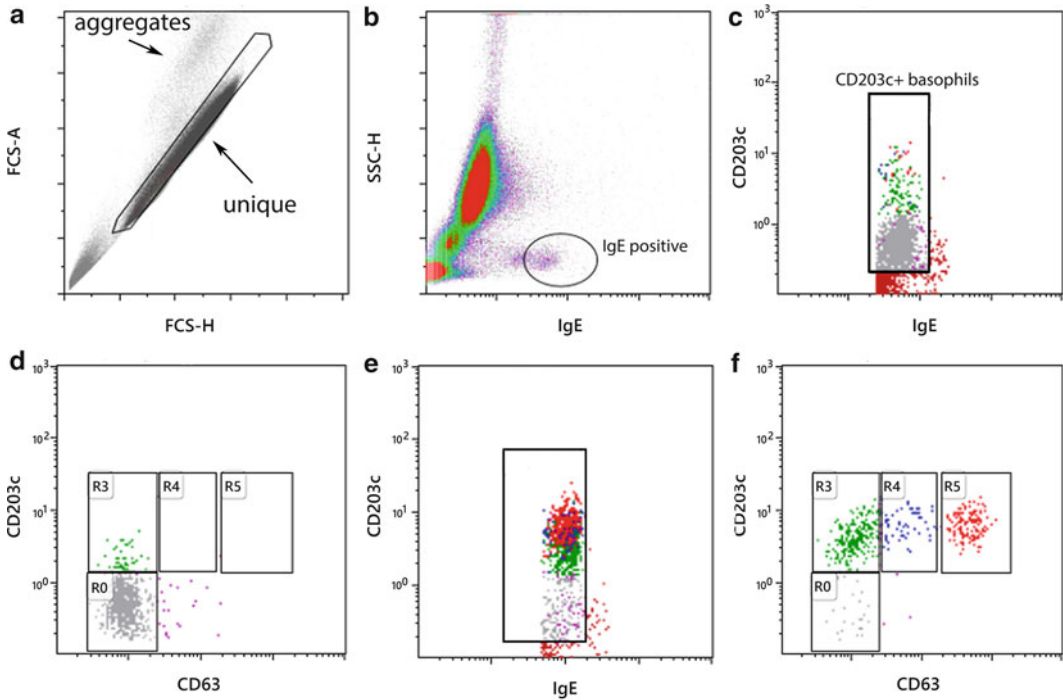


Fig. 1 Basophil selection and activation. Panel **a**: Aggregates are avoided by selecting unique cells based on FCS (forward scatter) area and FCS height plot. Plot **b**: IgE high positive cells are gated out. Panel **c**: Gated cells are IgE⁺ and CD203⁺ (without stimulation). Panel **d**: Activated basophils in control setting: R0 are CD203c⁺CD63⁻, R3 are CD203c^{hi}CD63⁻, R4 are CD203c^{hi}CD63^{low}, R5 are CD203c^{hi}CD63^{hi}. Panels **e** and **f** are equal to **c** and **d** but after activation with anti-IgE as a positive control

14. Count at least 500 basophils which are defined as aIgE⁺ and CD203c⁺.
15. Store data in an FCS-formatted file, which can be analyzed with flow cytometric software.
16. Define CD203c^{hi}CD63⁻ basophils and CD203c^{hi}CD63^{hi} as activate basophil populations (*see* Fig. 1).
17. Expression as %CD203c^{hi}CD63⁻ or %CD203c^{hi}CD63^{hi} basophils by subtracting % positive control or allergen with % negative control.

3.2 HistaFlow: Basophil Activation Technique with Intracellular Staining of Histamine (Method 2)

All procedures are carried out at room temperature, in a laminar airflow cabinet when necessary, or unless otherwise specified. The procedure is identical as in Method 1 (except volumes) until step 9.

3.2.1 Preparation of Positive and Negative Controls (Exclusion of Non-responders)

1. Incubate 200 μL of blood with 200 μL negative (BAT buffer) or positive control (anti-IgE) for 20 min at 37 °C.
2. Using the procedure described below, stain with aIgE-AF488 and CD203c-APC (*see Note 4*)
3. When basophils do not respond one can decide to stimulate with two concentrations of the allergen to exclude non-reactivity to anti-IgE (*see Note 5*).

3.2.2 Allergen Procedure

1. All blood samples, buffers, and allergens are prewarmed without mixing for 15 min at 37 °C in warm water bath (WWB) (*see Note 6*).
2. Prepare incubation tubes and add 200 μL allergen solution to the allergen tubes. Dilutions depend on the allergen used (*see Note 3*). Use BAT buffer as a negative control or for FMO evaluation (*see Note 2*).
3. Add anti-IgE as positive control to a positive control tube. Add 100 μL BAT buffer to a negative control tube.
4. Add to all tubes 100 μL prewarmed whole blood.
5. Incubate during 20 min at 37 °C in WWB.
6. Stop reaction on ice and add 1 mL PBS-EDTA.
7. Spin at 500 $\times g$ for 10 min at 4 °C without setting on the brake of the centrifuge. Remove supernatant.
8. Add 20 μL anti-IgE-AF405, 10 μL anti-CD63-FITC, and 5 μL anti-CD203c-APC.
9. Incubate at 4 °C for 20 min in the dark.
10. Add 2 mL Phosflow Lyse/Fix work solution and incubate at 37 °C in WWB for 20 min.
11. Spin for 10 min at 500 $\times g$ at room temperature. Remove supernatant.
12. Add 2 mL 0.1 % PBS-TX100 and spin again for 10 min 500 $\times g$ at room temperature. Remove supernatant.
13. Add 100 μL 0.1 % PBS-TX100 and 10–40 μL (depending on titration) DAO-V500.
14. Stain for 45 min at 37 °C in WWB. Mix several times during stain procedure.
15. Add 2 mL PBS and spin at 500 $\times g$ for 10 min at room temperature. Remove supernatant.
16. Add 300 μL PBS- NaN_3 .
17. The tubes are now ready to be measured with a flow cytometer.
18. Count at least 1,000 basophils, defined as aIgE⁺ and CD203c⁺.
19. Store data in an FCS-formatted file, which can be analyzed with flow cytometric software.

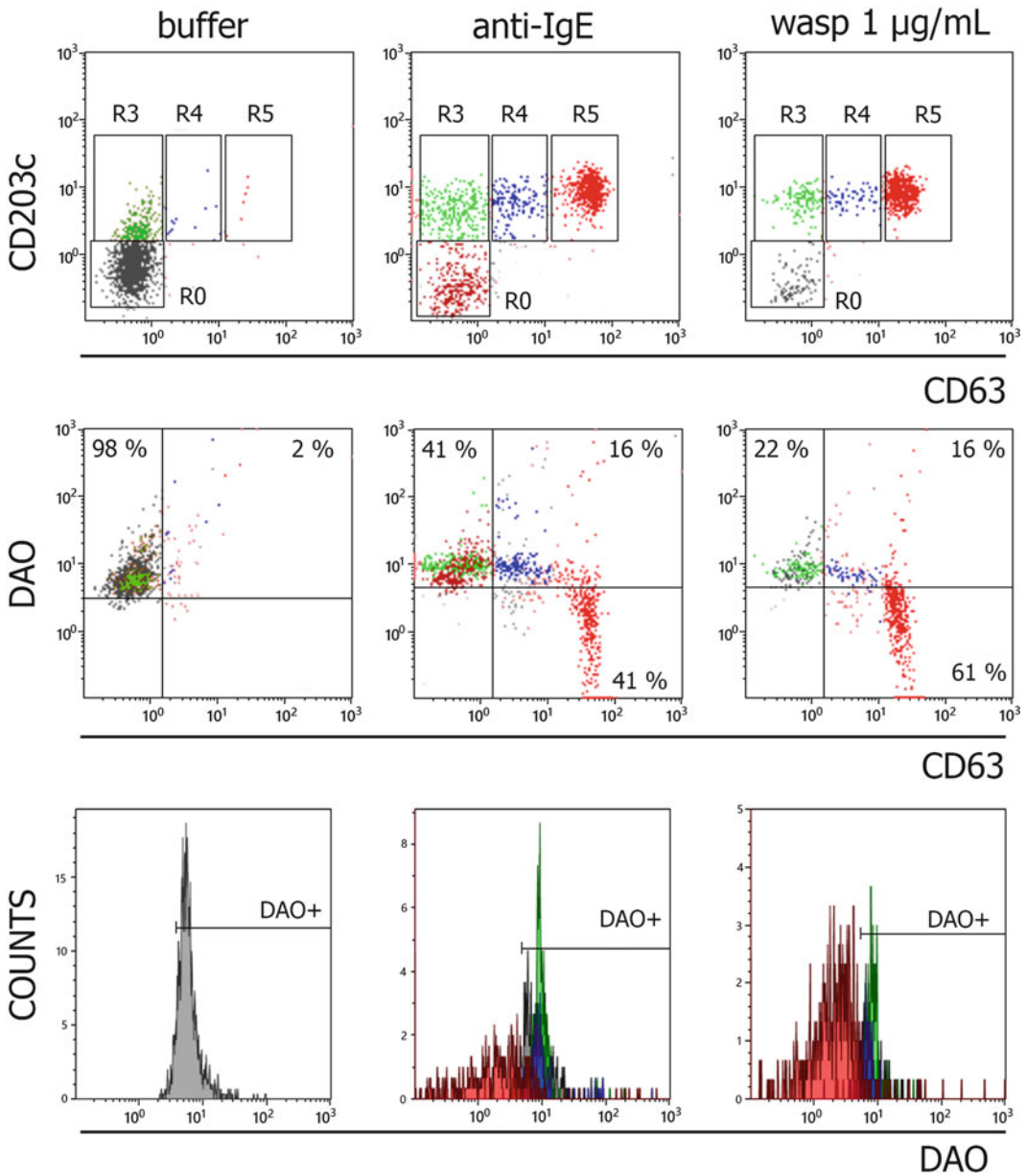


Fig. 2 HistaFlow analysis: Basophils are selected as described in Fig. 1. See also legend Fig. 1 for R0–R5 definition. The *left* column displays results of buffer (negative control), the *second* column shows results of positive control, and the *right* column displays results of activation with wasp antigen in a wasp allergic patient. DAO results (histamine content) show histamine release in 61 % of the basophils after activation with wasp antigen

20. Define $CD203c^{hi}CD63^{-}$ basophils and $CD203c^{hi}CD63^{hi+}$ as activated basophil populations. Define histamine content using the compensation setting described below. This setting must be unique for every sample. Figure 2 shows an example of HistaFlow analysis of basophils stimulated with wasp venom in a wasp allergic patient.

Table 1
Compensation method

Tube	1	2	3	4
Type	CD63–DAO–	CD63–DAO+	CD63++DAO+	CD63++DAO–
Explanation	Negative cells	DAO+ cells without activation	DAO+ and <i>intracellular</i> CD63+ staining <i>WITHOUT</i> stimulation	DAO and CD63 staining <i>WITH</i> stimulation
Incubation/activation procedure (<i>see</i> Method 2)				
Whole blood	200 µL	200 µL	200 µL	200 µL
BAT buffer	200 µL	200 µL	200 µL	–
mHu-antiIgE (pos. control)	–	–	–	200 µL
Incubation (<i>see</i> Method 2)				
Membrane-staining procedure				
CD63	None	None	None	none
IgE/CD203c	20 µL/10 µL	20 µL/10 µL	20 µL/10 µL	20 µL/10 µL
Lyse/fix (<i>see</i> Method 2)				
Wash with PBS_0.1 % TritonX				
Intracellular staining procedure (<i>see</i> Method 2)				
PBS-TX100	100 µL	100 µL	100 µL	100 µL
CD63	–	–	10 µL	10 µL
DAO	–	10 µL	10 µL	10 µL
Population by correct compensation (<i>see</i> Fig. 4)	<i>Grey cell in gate C</i> (no staining for DAO and CD63)	<i>Green cells in gate D</i> (DAO+ staining alone)	<i>Blue cells in gate E</i> (all basophils are positive for DAO and CD63; note that there is no CD203c upregulation)	<i>Red cells in gate F</i> (histamine-releasing cells)

3.3 Compensation Procedure

A functional assay like the HistaFlow technique needs an optimal flow cytometric compensation setting which is not always necessary when measuring populations, as long as the populations can be differentiated. In the case of histamine release, we must be aware that not all basophils release the same amount of histamine; hence different populations can (and must) be observed.

One could also explain this by comparing it with taking a picture with a digital camera. You need a correct exposure to see all the details. To do so, one needs a correct exposure time or shutter speed (=PMT setting in flow cytometry) and a correct aperture (=compensation setting in flow cytometry).

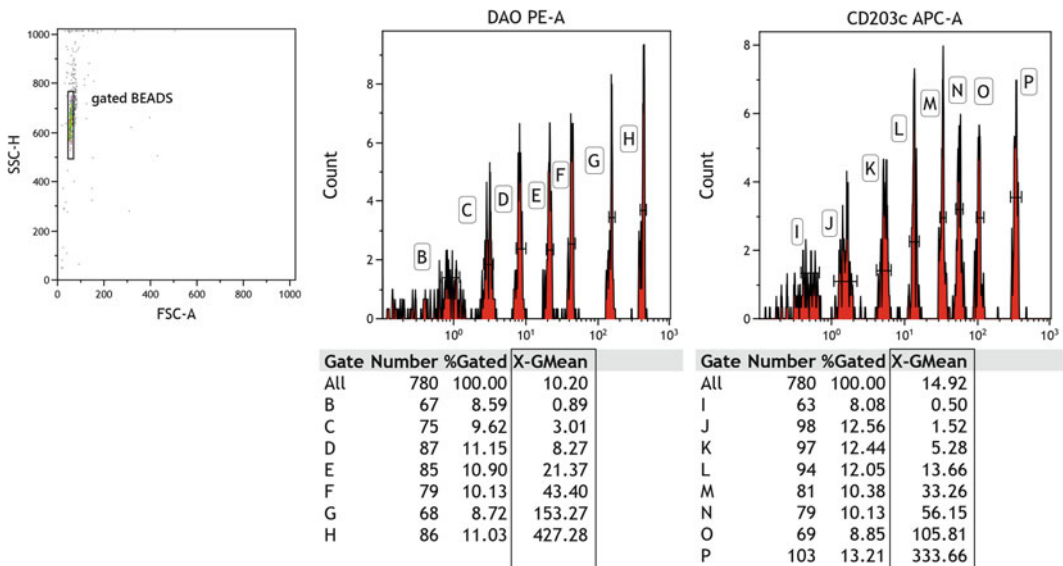


Fig. 3 Selection of standardized beads (*left*) and calculation of geometric mean of eight different intensities (*middle: PE, right: APC*). Results are shown in column X_Gmean

This is in fact what we do by combining the data of the four tubes in the compensation setting of the HistaFlow technique (*see* Table 1).

The first tube is without staining for DAO/CD63. These are the histamine-negative basophils. The second tube is DAO staining alone. These are the DAO (or histamine)-positive basophils.

The third tube is special: DAO as well as CD63 staining AFTER fixation and WITHOUT stimulation: this will give us the optimal staining for all populations.

The fourth tube is DAO and CD63 after activation (just like a positive control): This tube gives us the possibility to optimize the compensation setting (under an optimal PMT setting). By fine-tuning one will easily observe the different population releasing basophil populations.

After staining each tube separately, we can mix them to find out the optimal setting or one can measure every tube individually and, using software, add all the data in one data file. Afterwards we measure all tubes of the same patient with this setting.

Ensure that you repeat this for every donor due to potential differences in histamine content.

3.4 Calibration Procedure

1. Measure working solution of diluted beads with the identical flow cytometric settings as for the HistaFlow analysis (*see* Fig. 3).

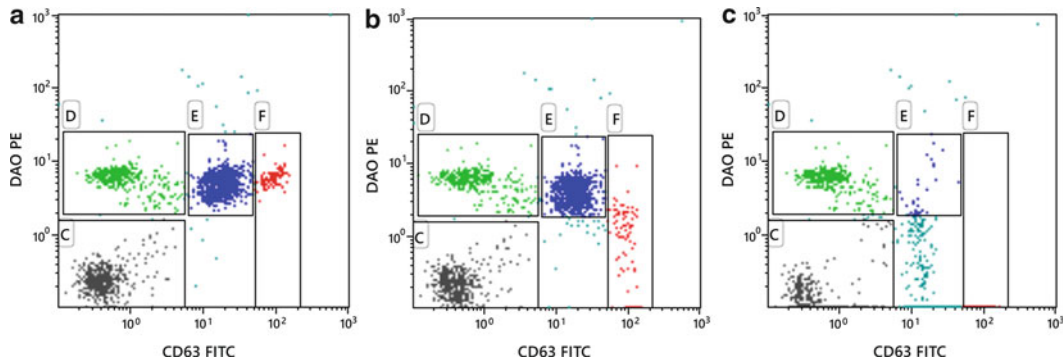


Fig. 4 Correct compensation setting. Basophils were selected as CD203⁺antiIgE⁺. Panel **a** shows undercompensation, Panel **b** is correctly compensated, and Panel **c** is overcompensated

2. Make a calibration graph (lin-log) with the use of the channel number and the median fluorescence intensity (MFI) as described by the manufacturer (*see* Fig. 4).
3. Use this calibration curve to calculate the MFI of the histamine-containing cell population.

4 Notes

1. Although different BAT techniques are described using EDTA or even citrate-treated blood, the best results are obtained with heparinized blood as basophil activation is very calcium dependent and using chelators or calcium-binding products can have deleterious effect on test results. Anti-histamines, which target the histamine receptors, have no influence on basophil activation tests in contrast to corticosteroids and immune-suppressive drugs.
2. All antibodies can be used labeled with different fluorochromes. Be aware that correct titration is necessary. This can be done by using different dilutions of the antibody and, using the correct flow cytometric settings, looking for the optimal resolution in comparison with an FMO (fluorescence minus one) measurement as a control.
3. Allergens can be protein extracts, recombinant proteins, or drugs. They must be water soluble and used in concentrations which are not toxic for basophils. To test toxicity, incubate basophils with different concentrations of the allergen or mouse anti human-IgE (positive control) for 20 min at 37 °C and measure viability using propidium iodide or Live/Dead viability/Cytotoxicity Kit for mammalian cells (e.g., Life Technologies). Do not use preparations for skin testing. These solutions contain preservatives or sometimes modified proteins which can inhibit

basophil activation in vitro. Be aware that food allergens are very difficult to standardize. The optimal concentration of an allergen to stimulate basophils must be determined in a dose-response curve experiment including about ten well-defined patients positive to the antigen and ten controls negative to the allergen. Because such a response can result in a bell-shaped curve, in routine practice at least two concentrations have to be used to define a positive result.

4. Basophils can be differentiated with different markers. Basophils are IL-3 receptor (CD123) positive and HLA-DR negative (monocytes and dendritic cells are HLA-DR positive and also CD123 positive). Because CD203c is the only lineage marker known until now for basophils (mast cells are also positive, but they do not circulate in the blood), we prefer to define basophils as CD203c⁺/IgE⁺. Nevertheless the CD203c density in resting basophils can be too low to differentiate cells.
5. In about 5–10 % of the patients, basophils do not respond to a positive control, be it anti-IgE, fMLP, or anti-Fc ϵ RI. In such a case one can decide to stimulate with the allergen. Nevertheless if the allergen also gives a negative response, the procedure has to be stopped because it could be a false-negative response (the so-called non-responder). Results will be inconclusive.
6. Basophil activation results in an immediate response (1 min) that lasts for about 20–30 min at maximum response. To obtain highly reproducible results, all solutions are best pre-warmed to 37 °C.
7. Wear gloves, lab coat, and safety glasses while handling all human blood products. Dispose of all pipettes, etc. into bagged waste collection bins. Wipe work surfaces with disinfectant before and after running tests.

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