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Quality-Controlled Sputum Analysis by Flow Cytometry

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Abstract

Sputum, widely used to study the cellular content and other microenvironmental features to understand the health of the lung, is traditionally analyzed using cytology-based methodologies. Its utility is limited because reading the slides is time-consuming and requires highly specialized personnel. Moreover, extensive debris and the presence of too many squamous epithelial cells (SECs), or cheek cells, often renders a sample inadequate for diagnosis. In contrast, flow cytometry allows for high-throughput phenotyping of cellular populations while simultaneously excluding debris and SECs.

The protocol presented here describes an efficient method to dissociate sputum into a single cell suspension, antibody stain and fix cellular populations, and acquire samples on a flow cytometric platform. A gating strategy that describes the exclusion of debris, dead cells (including SECs) and cell doublets is presented here. Further, this work also explains how to analyze viable, single sputum cells based on a cluster of differentiation (CD)45 positive and negative populations to characterize hematopoietic and epithelial lineage subsets. A quality control measure is also provided by identifying lung-specific macrophages as evidence that a sample is derived from the lung and is not saliva. Finally, it has been demonstrated that this method can be applied to different cytometric platforms by providing sputum profiles from the same patient analyzed on three flow cytometers; Navios EX, LSR II, and Lyric. Furthermore, this protocol can be modified to include additional cellular markers of interest. A method to analyze an entire sputum sample on a flow cytometric platform is presented here that makes sputum amenable for developing high-throughput diagnostics of lung disease.

Introduction

Technical advancements in the hardware and software of flow cytometers have made it possible to identify many distinct cell populations simultaneously[1,2,3,4]. The utilization of the flow cytometer in hematopoietic cell research, for example, has led to a much better understanding of the immune system[2] and the cellular hierarchy of the

hematopoietic system[5], as well as the diagnostic distinction of a multitude of different blood cancers[6,7,8]. Although most sputum cells are of hematopoietic origin[9,10,11], flow cytometry has not been widely applied to sputum analysis for diagnostic purposes. However, several studies suggest that the evaluation of immune cell populations in sputum (the most significant subset of cells) may be of great help in diagnosing and/or monitoring diseases such as asthma and chronic obstructive pulmonary disease (COPD)[12,13,14,15]. Moreover, the existence of epithelial-specific markers that can be used in flow cytometry allows the interrogation of the following most significant subset of cells in sputum, lung epithelial cells.

In addition to the ability to analyze many distinct cell populations of different tissue origins, a flow cytometer can evaluate large numbers of cells in a relatively short period. In comparison, slide-based, cytological types of analyses often require highly specialized personnel and/or equipment. These analyses can be labor-intensive, which leads to only a proportion of the sputum sample being analyzed[16].

Three critical issues limit the widespread use of sputum in flow cytometry. The first issue relates to the collection of sputum. Sputum is collected through a huff cough that expels mucus from the lungs into the oral cavity, subsequently spitting into a collection cup. Since the mucus travels through the oral cavity, there is a high chance of SEC contamination. This contamination complicates the specimen analysis, but the problem is easily rectified on a flow cytometric platform, as shown in this study.

Not everyone can produce sputum spontaneously; therefore, several devices have been developed to assist with the sputum collection in a non-invasive manner[17]. The nebulizer is one such device and has been shown to produce reliable sputum samples[18,19,20]. Although the nebulizer is a very effective way of non-invasively collecting sputum, its use still requires a setting at a medical facility with specialized personnel[21]. In contrast, handheld devices such as the lung flute[22,23,24] and the acapella[16,25] can be used at home since they are very user-friendly. These assist devices are both safe and cost-effective.

For us, the acapella gave consistently better results than the lung flute[16], and therefore, the acapella device has been chosen for sputum collections. A 3-day collection sample was decided because the primary purpose for using sputum is to develop a lung cancer detection test[16]. It has been shown that a 3-day sample increases the likelihood of lung cancer detection compared to a 1- or 2-day sample[26,27,28]. However, other methods of sputum collection may be preferable for different purposes. If a different sputum collection method is used than the one described here, it is recommended to carefully titrate each antibody or dye used for flow cytometric analysis; very little data is available on how different sputum collection methods affect the targeted proteins for flow cytometry.

The second issue dampening the enthusiasm for using sputum for diagnostics, primarily related to flow cytometry, is cell number. The problem is the collection of sufficient viable cells for a reliable analysis. Two studies demonstrated that sputum samples collected by non-invasive methods, with the help of an assist device, contain enough viable cells that can be utilized in clinical diagnosis or research studies[16,24]. However, neither of these studies addressed the issue of cell numbers concerning flow cytometry.

For the studies that form the basis for this protocol, sputum samples were collected from participants at high risk for developing lung cancer following approved institutional

guidelines for each study site. High-risk participants were defined as between 55-75 years, having smoked 30 pack-years and having not quit smoking within the past 15 years. Patients were shown how to use the acapella device according to the manufacturer's instructions[29] and collected sputum for three consecutive days at home. The sample was kept in the refrigerator until the last collection. On the final collection day, the sample was shipped to the laboratory overnight with a frozen cold pack. The samples were processed into a single cell suspension on the day they were received. With this method of sputum collection, more than enough viable cells are obtained for a reliable flow cytometric analysis.

Lastly, and related to the previous cell number issue, is the question of how to release the sputum cells from its mucinous environment. How can the cells be kept viable and create a single cell suspension that does not clog the flow cytometer? Based on initial work by Pizzichini et al.[30] and Miller et al.[31], this protocol describes an easy and reliable method for sputum processing into a single cell suspension that is suitable for flow cytometric analysis. This method has used well-established guidelines in flow cytometry[32,33,34] to develop an efficient antibody labeling strategy to identify hematopoietic and epithelial cells in sputum and provide instrument settings, quality control measures, and analysis guidelines standardizing sputum analysis on a flow cytometric platform.

Protocol

Sputum samples were collected from participants between 55-75 years, having smoked 30 pack-years and having not quit smoking within the past 15 years. These participants were at high risk of developing lung cancer. The samples were collected following approved institutional guidelines for each study site. All steps of the sputum processing are performed in a biological safety cabinet with appropriate personal protective equipment.

1. Reagent preparation before starting sputum dissociation

1. Thaw 1% Paraformaldehyde (PFA), 25 mL per sample on ice, and keep cold until use.
   CAUTION: PFA is toxic by inhalation and skin contact. Prepare a fixative according to the manufacturer's instructions and freeze at -20 °C in 25 mL aliquots until use.
2. Approximate the weight of the sample and thaw enough 0.1% Dithiothreitol (DTT) for step 2.2 and bring it to 37 °C. (Aliquots of DDT should be stored at -20 °C before use.)
3. Bring enough 0.5% N-acetyl- L-cysteine (NAC) up to 37 °C for step 2.2. (NAC should be made fresh weekly and stored at 4 °C before use.)

2. Sputum dissociation

1. Weigh the sputum sample to determine the volumes of dissociation reagents.
   NOTE: A sample is considered to be small if the initial weight is ≤3 g, medium if >3 g but ≤8 g, large if >8 but ≤16 g, and extra-large if a sample weighs >16 g. The indications small, medium, large, and extra-large will be used throughout the protocol. The quantity of reagents required for dissociation and labeling differ according to the size of the sputum sample.
2. Transfer a small sample to a clean 50 mL conical tube, a medium sample to a clean 250 mL plastic disposable bottle, or a large and extra-large sample to a clean 500

mL plastic disposable bottle. Add 1 mL/g sample weight of 0.5% NAC and 4 mL/g sample weight of 0.1% DTT.

3. Vortex at maximum speed (for 15 s), and then rock at room temperature (at maximum speed) for 15 min.

4. Dilute the sample with four volumes of Hank's Balanced Salt Solution (HBSS) (based on the total volume of sample + reagents) to neutralize the NAC and DDT; vortex quickly at maximum speed and rock at room temperature for 5 min at maximum speed.

5. Filter the cell suspension through 100 µm nylon mesh cell strainer(s) into one or more 50 mL conical centrifuge tube(s) to create a single-cell suspension.

6. Centrifuge the cells at 800 x *g* for 10 min at 4 °C. Aspirate the supernatant, combine all pellets in one 15 mL conical tube, and then wash the pellets with HBSS using the same conditions.

7. Resuspend the cell pellet in a volume of buffer determined by the initial weight of the sputum sample.
   NOTE: A small sample is resuspended in 250 µL of HBSS. A medium sample is resuspended in 760 µL of HBSS. Large and extra-large samples are resuspended in 1460 µL of HBSS.

8. Take an aliquot of the cell suspension for a live/dead cell count using Trypan Blue.
   NOTE: For a small sample, use 5 µL. For a medium, large or extra-large sample, use 10 µL. Dilute 1:10 with HBSS.
   1. Mix 10 µL of the sputum dilution with 30 µL of 0.4% Trypan Blue to achieve a final sample dilution of 1:40. Load into the counting chambers of a hemocytometer for a cell count.
      NOTE: It may be necessary to adjust the final dilution if the cell numbers are too low or too high to achieve an accurate count. Consulting Guiot et al.[20] for correctly distinguishing sputum cells from SECs and debris is strongly recommended here. This is essential for an accurate cell count.

9. From the extra-large sample, remove 50 x 10^6 cells from the total and add to a new tube with enough added HBSS to create a total volume of 1700 µL.
   NOTE: Consider this a large sample for the remainder of the protocol. Left-oversamples can be discarded or used for other purposes.

3. Antibody and viability dye staining

1. Choice of antibody and staining dye
   NOTE: **Table 1** shows the antibodies and viability dye that are used in this protocol and the cell populations they identify.
   1. Label the tubes containing the sputum cells (see **Table 2** for labels).
   2. Use 5 mL flow cytometry tubes (compatible with the flow cytometer used) for the sample tube with the unstained cells and the tube with the isotype control. Use 15 mL conical tubes for the blood and epithelial tube samples.
      NOTE: These samples will be transferred to flow cytometry tubes following antibody staining and fixation.

2. Label the compensation tubes (**Table 3**).
   NOTE: Use 5 mL flow cytometry tubes compatible with the flow cytometer being used.

3. Add the amount of HBSS, antibody and/or dye to each of the sputum cell tubes and compensation tubes as indicated in **Table 2** and **Table 3**, respectively.

NOTE: Add buffer (HBBS), antibodies, and dye to all the tubes before adding cells or compensation beads to ensure that all tubes' staining time is consistent.

4. Add the amounts of sputum cell volume listed in **Table 2** to the assay tubes.
5. Add compensation beads to the compensation tubes as listed in **Table 3**.
6. Incubate all the tubes (assay and compensation tubes) on ice, protected from light, for 35 min. Then, fill the tubes with ice-cold HBSS and centrifuge at 4 °C for 10 min at 800 x *g*.
7. For the compensation tubes, aspirate the supernatant as close to the pellets as possible and flick the pellets to loosen.
8. Add 0.5 mL of cold HBSS to the compensation tubes, store them on the ice at 4 °C and protect them from light until needed for flow cytometry analysis.
9. Aspirate the supernatant from the unstained isotype, blood, and epithelial tubes after centrifugation (step 3.6 from the antibody staining section) and loosen the pellets by flicking the tubes.

4. Fixation with 1% Paraformaldehyde (PFA)

1. Add cold 1% PFA (which should be thawed by now) to the unstained, isotype, blood, and epithelial tubes; 2 mL to the unstained and isotype tubes, and 10 mL to the blood and epithelial tubes.
2. Incubate the tubes on ice, protected from light for 1 h. Vortex quickly at maximum speed after 30 min.
3. Fill the tubes with ice-cold HBSS. Then, centrifuge tubes at 4 °C for 10 min at 1600 x *g*.
4. Aspirate as much supernatant as possible without disturbing the cell pellet and flick the tube with the fingers to loosen the cells.
5. Add 200 µL of cold HBSS to the unstained and isotype tubes.
6. Calculate the volume of HBSS for resuspension of the blood and epithelial tube according to the total cell count.
   NOTE: Resuspension volume = 0.15 x [total cell count (step 8 of sputum dissociation) / 10^6]. Use 50 x 10^6 as the cell count for an extra-large sample.
7. Store all sample and compensation tubes, protected from light on the ice at 4 °C until flow cytometry analysis is performed.
   NOTE: This protocol has not been tested for storage for more than 24 h.

5. Data acquisition on the flow cytometer

1. Apply appropriate startup procedures for the flow cytometer being used.
   NOTE: This section of the protocol assumes that the person operating the flow cytometer is trained in the use of the instrument available to them, especially concerning daily procedures including checking the stability of the optics and fluidics systems, techniques for standardizing light scatter and fluorescence intensity, as well as calculating and applying the correct compensation matrix.
2. Use the mixture of National Institute of Standards and Technology (NIST) beads to ensure that forward scatter and side scatter voltages are set to place the NIST beads to span the entire plot without placing the beads too close to the axes.

NOTE: This step is crucial to ensure that debris smaller than 5 µm can be eliminated by gating post-acquisition analysis. Depending on the flow cytometer used, be mindful that the smallest beads are not being excluded with the threshold (when using the LSR II or the Lyric) or with the high discriminator (using the Navios EX flow cytometer). For the Navios EX, a gain of 2 was used for the forward and side scatter, a voltage of 236 for forward scatter, and 250 for side scatter. For the LSR II, a forward scatter voltage of 165 and a side scatter voltage of 190 was used.

3. Set the flow rate to medium (LSR II) or high (Navios EX).
   NOTE: The medium or high flow rate for most instruments can be used to acquire the sputum tubes. It is important to note that using too slow of a flow rate or too dilute of the sample may result in the cells settling, resulting in increased vortexing which is not desired. Therefore, adhering to the calculated resuspension volume in step 4.6 should result in cellular density that can be acquired quickly but not clog the machine.
4. Adjust the voltages for each parameter used for the scatter and fluorescent parameters to place the cell populations on the scale. Use the figures with the gating strategy as guidance on how to adjust the voltages accordingly.
   NOTE: Ensure that all the parameters needed are selected in the parameter selection window before the acquisition or that data will not be acquired.
5. Acquire data for the unstained sputum sample first, followed by the isotype-stained sample, and then the blood tube and the epithelial tube.

NOTE: If the cell suspension is too concentrated to allow the flow cytometer to run the samples without clogging, the samples may be further diluted with HBSS.

Representative Results

This protocol was developed with a clinical laboratory setting in mind. The focus during the development of the protocol was on simplicity, efficiency, and reproducibility. It was found that the most time-consuming step in the processing of sputum was counting the cells. Therefore, the protocol is set up in such a way that sputum processing and cell labeling can be performed independently from cell counting without loss of time. An accurate cell count, which is still necessary to dilute the sample appropriately for an unobstructed run, can then be obtained during the antibody labeling incubation period.

This protocol uses a sputum weight measure instead of a cell count as an indication of how much antibody/dye to use for optimal cell labeling. However, there is a great degree of variation in the weight of the sputum samples; of the 126 samples that were analyzed, the weight ranged from 0.57 g to 38.30 g. **Figure 1A** shows that the correlation between sputum weight and cell yield is not strong. Therefore, the samples were divided into four categories; the median weights of the samples were 2.1 g for small samples, 5.0 g for medium samples, 11.2 g for large samples, and 22.0 g for extra-large samples (**Figure 1B**). However, there were still samples that yielded far more cells than expected based on their weight (**Figure 1C**), the majority of the samples clustered nicely. For small samples, the median cell yield was 8.0×10^6 cells, for medium samples 13.0×10^6, for large samples 35.4×10^6, and extra-large samples 93.0×10^6.

Each antibody used in this protocol was titrated. A concentration on the plateau phase (**Figure 2A**) with the

highest staining index (**Figure 2B**) of the titration curve was chosen as the working concentration. Variations in cell numbers will not dramatically alter the staining intensity. This antibody titration and testing is essential and should be set up with care for each new antibody or dye used in this protocol[34,35]. When an antibody is titrated with care, it should stain 10 to 50-fold more cells than the number of cells on which the antibody was titrated[34,35]. An example of this principle is provided in **Table 4**. The anti-human CD45-PE antibody was titrated on 1 x 10^6 cells in a total volume of 400 µL. If this antibody to staining volume is extrapolated to the protocol (see **Table 2**, which shows the CD45-PE quantity and staining volumes for each sample size), for a small sample, 0.625 x 10^6 cells will be stained, 1.25 x 10^6 cells will be stained for a medium sample, and 2.5 x 10^6 cells for a large sample (column A in **Table 4**). A 50-fold excess in cell number for each category is calculated to be 31.25 x 10^6, 62.5 x 10^6, and 125 x 10^6 cells, respectively (column B). As shown in columns C and D, the median cell number of each size category and the largest sample in each category are well within the 50-fold range.

Despite the apparent differences in size and cell yield between the various sizes of the sputum samples, the median percent SEC contamination in each category is very similar. **Figure 3A** shows the percentage of SECs found in individual samples, stratified according to their sputum weight category. The main concern was with SECs since these cells are not representative of lung tissue but of the oral cavity. However, the average SEC contamination is less than 20% for all categories. **Figure 3B** shows the percentage of viable cells found in these samples. Excluding the SECs, the average viability was approximately 72% for the small, medium, and large sample size categories. It was slightly higher (79%) for the extra-large category.

Figure 4 shows a typical gating strategy to separate the sputum cells of interest from debris, dead cells (which also includes the contaminating SECs[36]), and cell clumps. **Figure 4A** shows the use of NIST beads to set gates to eliminate debris smaller than 5 µm or larger than 30 µm, while **Figure 4B** applies this gate on sputum cells. **Figure 4C** shows a typical sputum profile when viewed as side scatter width (SSC-W) against forward scatter width (FSC-W), the width gate. This profile is used to eliminate small debris that presents itself along the SSC-W and FSC-W axes. The elimination of dead cells is shown in **Figures 4D** and **Figure 4E**. The unstained control (**Figure 4D**) is used to determine the cut-off for FVS510 positivity; cells that stain positive for FVS510 above the negative control are considered dead and will not be used in the sputum cell analysis (**Figure 4E**). Lastly, a singlet gate is applied to remove cell doublets from the analysis, which is shown in **Figure 4F**. Thus, the cells that have made it through the selection gates shown in **Figures 4B**, **Figure 4C**, **Figure 4E**, and **Figure 4F** represent live, single sputum cells ready for subsequent analysis with the antibodies used in this protocol.

The use of the anti-CD45 antibody in this labeling protocol allows the separation of live, single sputum cells into a blood ($CD45^+$) cell compartment and a non-blood ($CD45^-$) cell compartment. The latter category includes epithelial cells and other non-blood cells. The top profile in **Figure 5A** shows the use of an unstained sputum sample to set the cut-off for CD45 positivity, rendering the gates to capture $CD45^+$ cells and $CD45^-$ cells. The bottom profile of **Figure 5A** shows both these gates applied to a sputum sample stained with the anti-CD45 antibody. Staining with additional antibodies

is then used to identify blood cell-specific populations within the $CD45^+$ gate (**Figure 5B**) and other non-blood cell populations, including epithelial populations in the $CD45^-$ gate (**Figure 5C**). In both the figures, the top profiles show how isotype-stained sputum is used to set the double negative populations, whereas the bottom profiles show the antibody-stained sputum cells. **Figure 5B** (bottom) shows six different $CD45^+$ cell populations created with the cocktail of antibodies detectable in the fluorescence (FL) 1 channel (anti-CD66b, CD3, CD19 antibodies) and the anti-CD206 detectable in the FL3 channel. Sorting experiments have revealed that lung-specific macrophages reside in the gates identified with M. The presence of cells in these gates thus indicates that the sputum sample was derived from the lung and was not saliva (Bederka et al., manuscript in preparation). **Figure 5C** (bottom) shows the gating quadrants created with the anti-pan-cytokeratin (panCK) detectable in the FL1 channel and anti-epithelial cell adhesion molecule (EpCAM) antibodies detectable in the FL3 channel among the $CD45^-$ sputum cells.

This labeling protocol has been extensively tested on the Navios EX and the LSR II. Some preliminary experiments were performed on the Lyric, but without the extensive instrument optimization that was done for the other two machines. Therefore, detailed instrument settings are provided for the Navios EX and the LSR II in the Materials sheet, but not for the Lyric. For understanding the similarities and variations between these three flow cytometric platforms that can be used to analyze sputum cells, profiles obtained from the various machines can be compared in **Figure 6** and **Figure 7**. Two large sputum samples were processed, pooled, labeled, separated into three equal portions, and subsequently acquired on each flow cytometer mentioned above. **Figure 6**, which is similar to **Figure 4**, compares the gating strategy for eliminating debris, dead cells, and cell clumps. **Figure 7**, which is identical to **Figure 5**, compares the blood and non-blood profiles from the data acquired on the various flow cytometers.

Comparing the various profiles obtained from the three different flow cytometers shows that the same basic profiles can be observed with each instrument. The differences to pay attention to are the SSC-W/FSC-W (width gate) plots (**Figure 6**, second row) and the linear scales of the scatter plots (**Figures 6** and **7**). The scatter width plot generated on the Navios EX shows an inclusion gate (red box), meaning that all cells included in the gate are further analyzed; the events along the axes in the left-bottom corner are excluded. The scatter width plots on the LSR II, and Lyric do not show a similar deposit of events along these same axes. This discrepancy is likely due to the very sensitive threshold used on the Navios EX, leading to some small debris in the previous size-exclusion gate (top row of **Figure 6**). On occasion, debris is seen along the axes in the scatter width profile generated on the LSRII, but it is in the right-bottom corner. In those cases, an exclusion gate (as indicated by the dashed red box in the middle profile in row 2 of **Figure 6**) is used to eliminate these events from further analysis.

While the log scales appear slightly different on the plots between the Navios EX and the Lyric and LSR II cytometers, the Navios EX does have the option to acquire data using more decades. Implementing more decades depends on the context of the experiment and the desired sensitivity to visualize the populations of interest.

An additional notable difference between the Navios EX and LSR II and Lyric cytometers is the appearance of the profile of the singlet gate. The Navios EX cytometer is equipped with a rectangular flow cell with a different collection angle than the LSR II. The Navios EX contains three software-controlled

collection angles to optimize the forward angle light scatter to achieve the sensitivity appropriate for particle size to analyze. The polygon gate for the Navios EX cytometer will need to be adjusted to a slightly lower angle than the singlet gate for the LSRII. The singlet gate for the Lyric can be optimized to get a profile similar to that seen with the LSR II. Using the sputum sample, the area scaling factor would need to be optimized for each laser on the Lyric to achieve a singlet gate similar to the LSR II.

All the profiles shown in **Figure 4** to **Figure 7** were analyzed with FlowJo software, version 10.6. The .fcs files can be found in the FlowRepository (https://flowrepository.org), under IDs FR-FCM-Z3LX, FR-FCM-Z3MJ, and FR-FCM-Z3MM and can be used to practice the sputum analysis as illustrated in these figures.

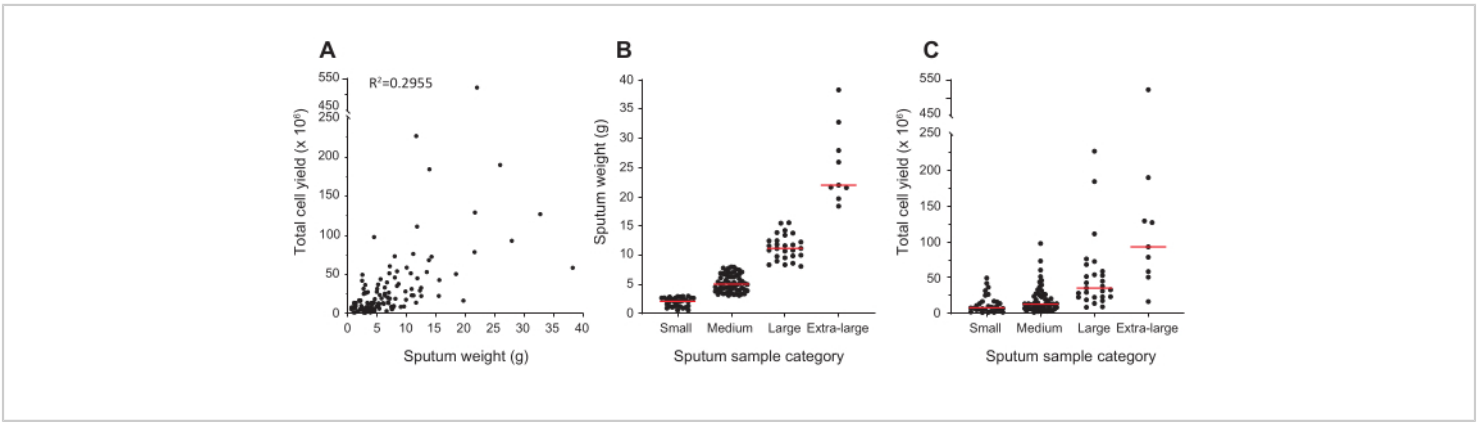

Figure 1: Sputum weights and cell yield. (**A**) Depicted is the relationship between sputum weight, determined before processing, and total cell yield, determined after processing using a hemocytometer. Each bullet represents an individual sample, 126 in total. (**B**) Sputum samples were stratified into four categories based on their weight: small for samples weighing up to 3 g, medium for samples weighing more than 3 g up to 8 g, large for samples weighing more than 8 g up to 16 g, and extra-large samples for those weighing more than 16 g. (**C**) Shown is the distribution of cell yield for the samples in each category. The red bars in (B) and (C) represent the median values in each category. Please click here to view a larger version of this figure.

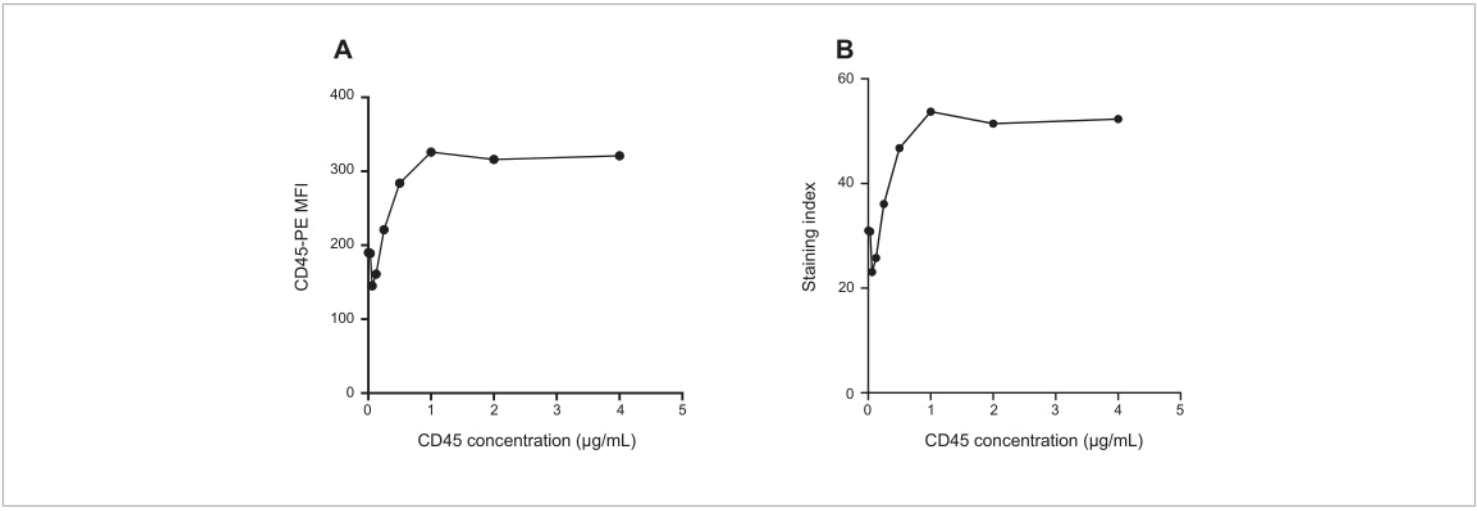

Figure 2: Antibody titration for anti-human CD45-PE. (**A**) The CD45-PE (IgG1) titration curve with the mean fluorescence intensity (MFI) of the positive population plotted versus the concentration of the CD45-PE antibody plateaus at 1 µg/mL. (**B**) Titration curve depicting the staining index versus the antibody concentration shows the highest staining index is at 1 µg/mL. Staining index was calculated as: [CD45 MFI positive population - CD45 MFI negative population] / [2 * Standard Deviation]. Based on **Figure 2A** and **Figure 2B**, 1 µg/mL was chosen as the optimal concentration for the CD45-PE antibody. Please click here to view a larger version of this figure.

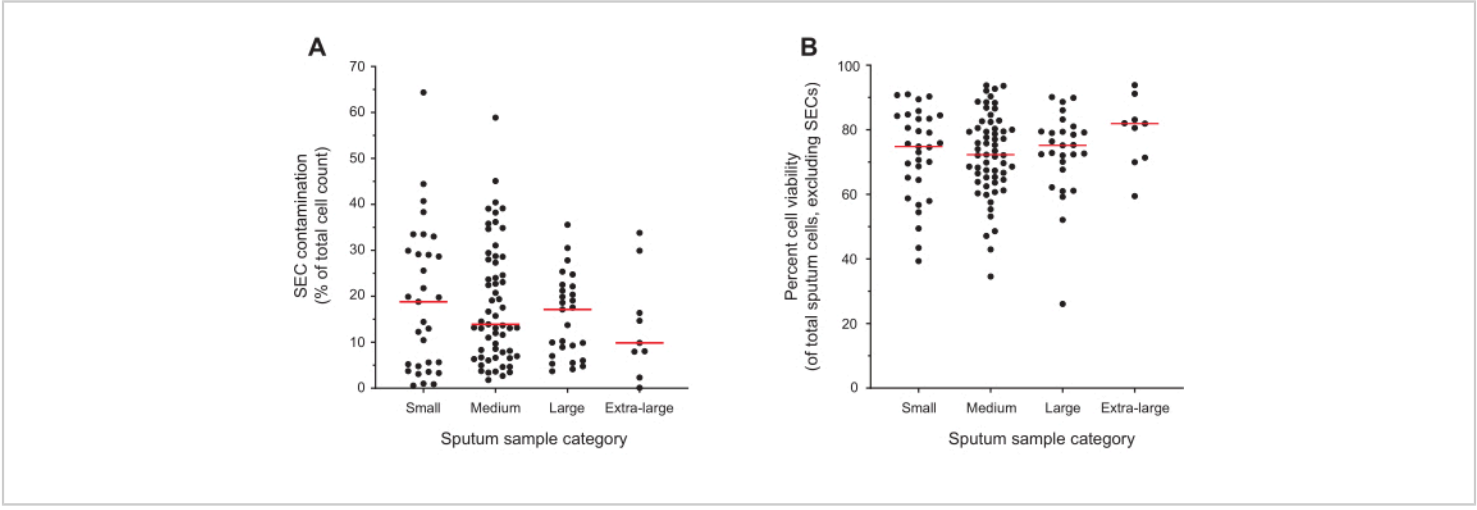

Figure 3: Proportion of SECs and dead cells in sputum samples is consistent among the four weight categories. (**A**) The proportion of SECs in sputum samples is classified according to their weight category. The percent SEC contamination was determined by hemocytometer as part of the total cell count. (**B**) Cell viability of sputum samples excluding SECS, determined by hemocytometer and the trypan blue exclusion method. For each graph, the red lines represent the median values. Please click here to view a larger version of this figure.

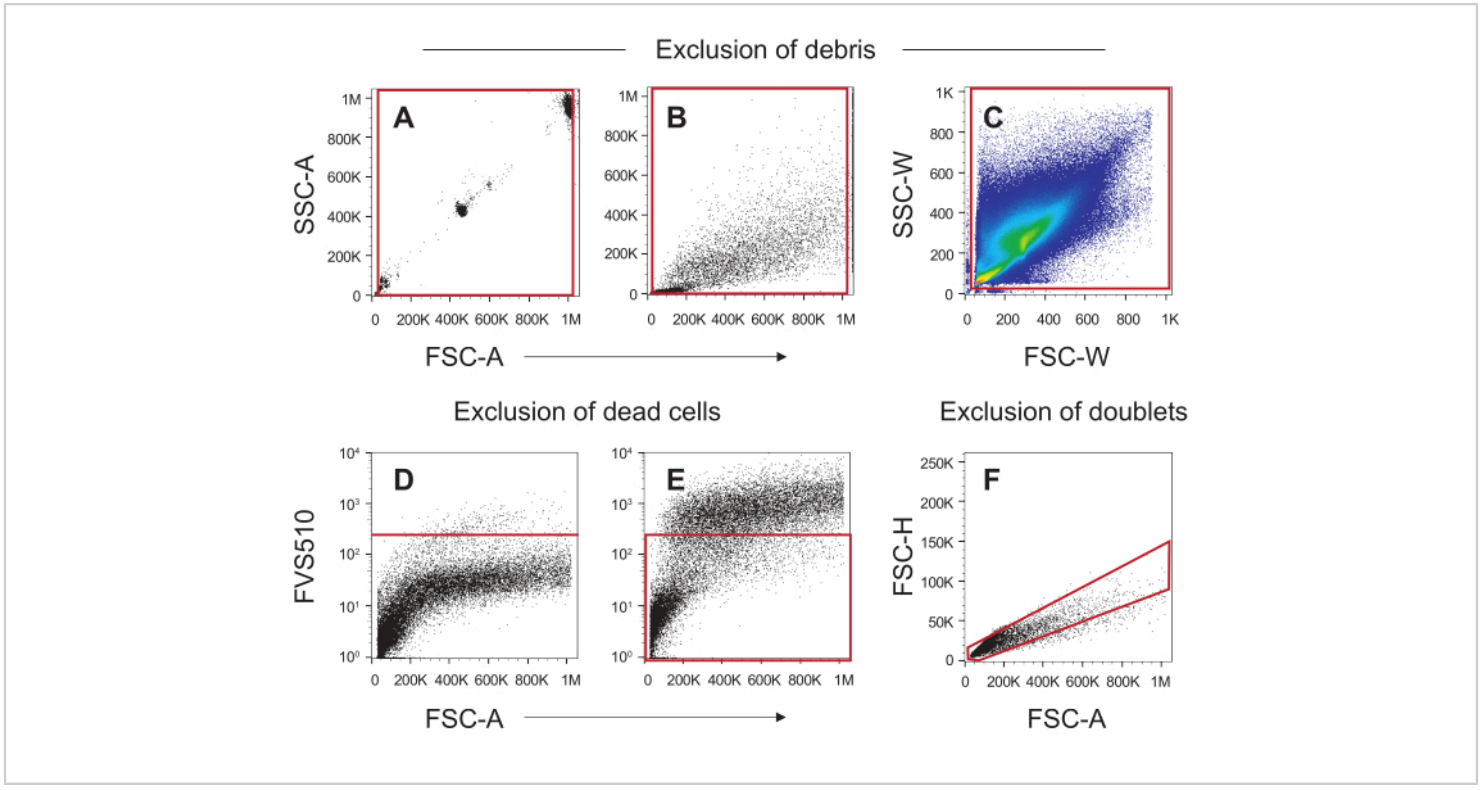

Figure 4: Gating strategy for excluding debris, dead cells, and doublets in a sputum sample. (**A**) NIST beads of sizes 5 µm, 20 µm, and 30 µm were used to set the gate indicated by the red box to exclude debris smaller than 5 µm and larger debris above 30 µm and close to the axis. (**B**) A sputum sample was acquired using the same voltages for the forward and side scatter as that of the NIST beads. The gate created in (A) was applied to exclude debris. (**C**) Small debris close to the axis was excluded in this scatter width plot. (**D**) An unstained sputum sample was used to set the cut-off (red line) for the negative, unstained population for the viability dye. **(E)** The cut-off created in D was applied to the stained sputum sample to form a gate (red box) to include live, viable cells. (**F**) The singlet gate indicated by the red polygon excludes cells that do not fall on the diagonal, eliminating doublets and/or clumps. Please click here to view a larger version of this figure.

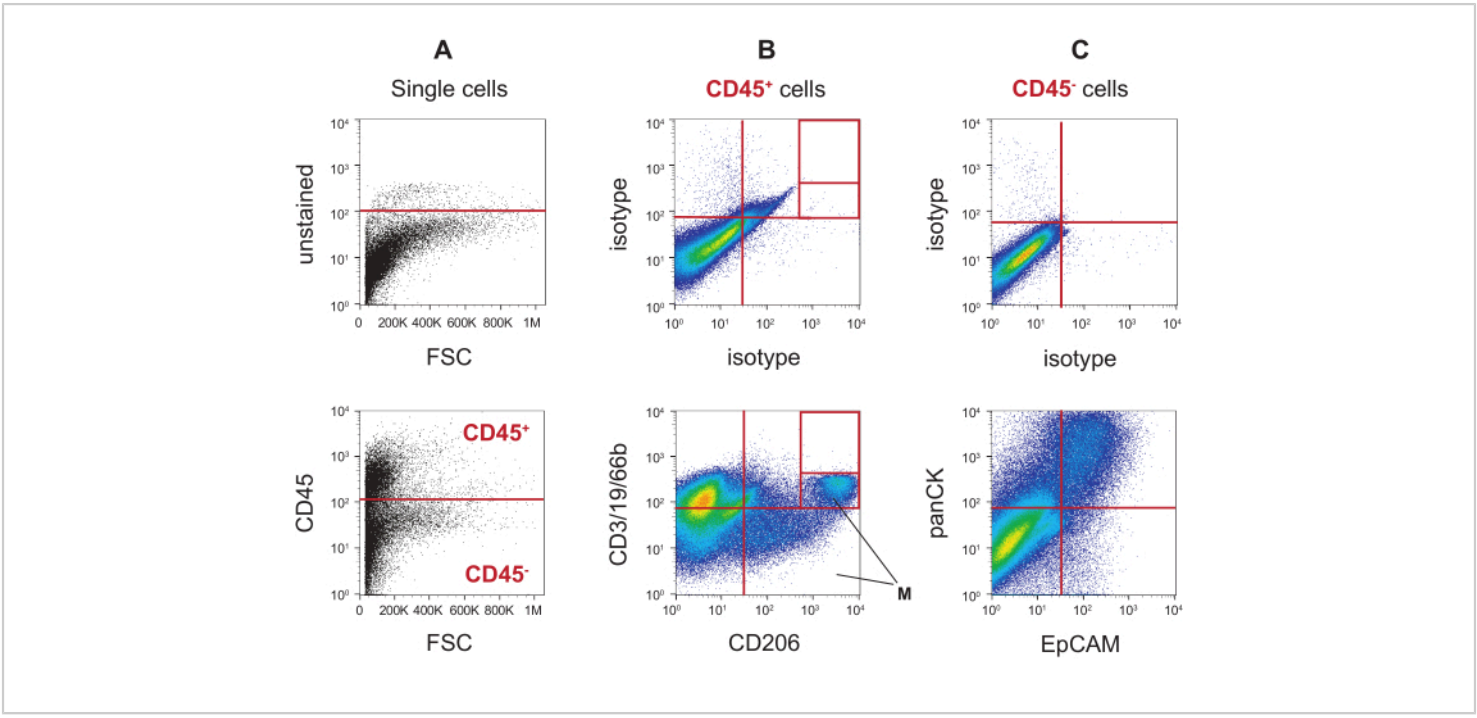

Figure 5: Gating strategy of sputum cells into blood and non-blood cell compartments. (**A**) Unstained sputum cells derived from the singlet gate are used to set the cut-off (red line) on the negative population for CD45 (top). The cut-off from the top profile is applied to the stained sputum sample (bottom) to differentiate CD45 positive ($CD45^+$) and negative populations ($CD45^-$). (**B**) $CD45^+$ sputum cells stained with isotype antibodies for FITC/AF488 are used to set the gates on the negative population (top). The same gates are applied to the $CD45^+$ sputum cells stained with blood cell markers CD3, CD19, CD66b, and CD206 (bottom). (**C**) Quadrant gates are placed on the $CD45^-$ sputum cells stained with isotype controls (top) and applied to the $CD45^-$ cells from the sputum sample stained with the epithelial markers pan-cytokeratin (panCK) and EpCAM (bottom). Please click here to view a larger version of this figure.

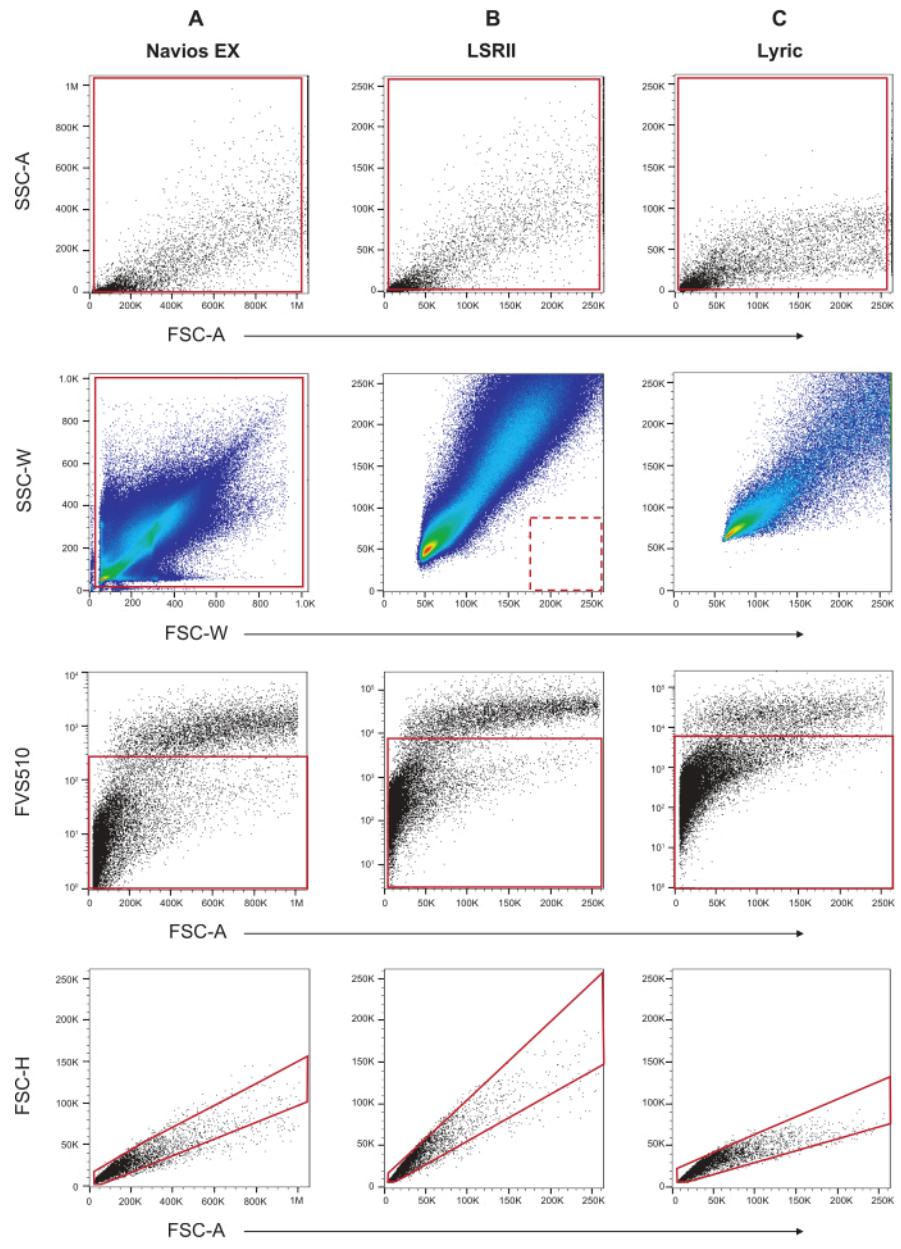

Figure 6: Gating strategy to exclude debris, clumps, and dead cells of stained sputum cells on three flow cytometric platforms. Two large sputum samples were processed, pooled, labeled, and divided into three equal portions for acquisition on the Navios EX (**A**), LSR II (**B**), and the Lyric (**C**) flow cytometers. Top row: Sputum samples were gated (red box) to exclude very small and large debris. Second row: The large red box in the Navios EX plot represents an inclusion gate that includes the cells but excludes debris. The dashed small red box seen in the LSR II plot represents an exclusion gate to eliminate debris from further analysis. Further optimization with the Lyric is needed to determine where debris exclusion gating is needed. Therefore, no gate is present in that plot. Third row: red rectangular gates include live cells for further

analysis. Bottom row: polygon gate has single cells by excluding doublets that fall outside the diagonal of the gate. Please click here to view a larger version of this figure.

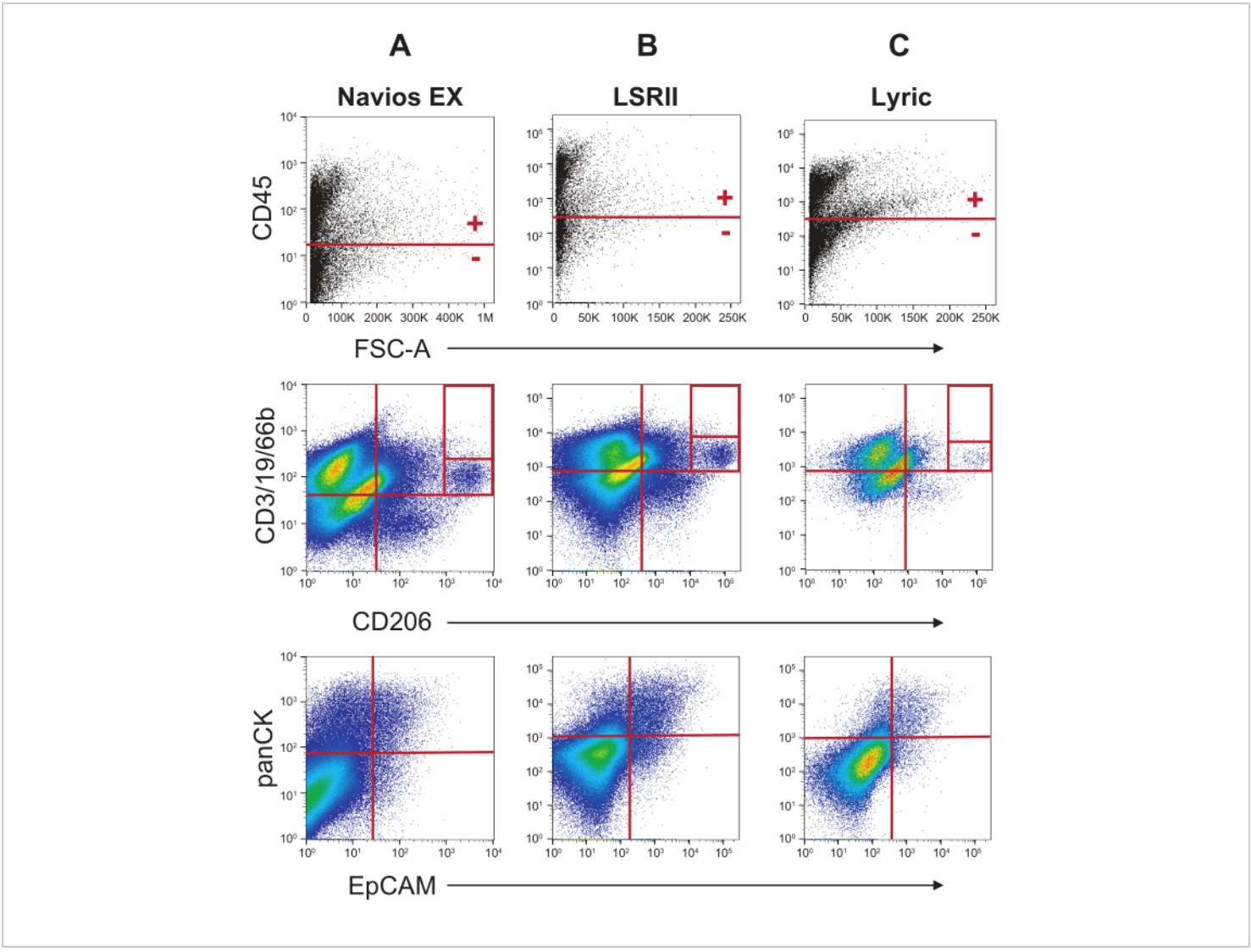

Figure 7: Gating strategy for sputum stained for blood and epithelial markers on three flow cytometric platforms. The analysis shown in **Figure 6** is continued in **Figure 7**; all viable, single cells (bottom row, **Figure 6**) were divided into $CD45^+$ and $CD45^-$ populations (first row, **Figure 7**), so that blood cell-specific markers and epithelial cell-specific markers could further delineate these populations. Second row: profile of blood cell markers CD3/CD19/CD66b and CD206 from $CD45^+$ cells. Third row: epithelial cell markers panCK and EpCAM from $CD45^-$ cells. Please click here to view a larger version of this figure.

Antibody/stain	Purpose
FVS510	Viability stain
Anti-human CD45 – PE	Pan-leukocyte marker; PE compensation
Anti-human CD66b – FITC	Granulocyte marker
Anti-human CD3 – Alexa488	T cell marker
Anti-human CD19 – Alexa488	B cell marker; FITC/Alexa488 compensation
Anti-human CD206 – PE-CF594	Lung macrophage marker; PE-CF594 compensation
Anti-human EpCAM – PE-CF594	Epithelial cell marker; PE-CF594 compensation
Anti-human pan-cytokeratin (panCK) – Alexa488	Epithelial cell marker
IgG1κ – Alexa488	CD3/CD19/panCK isotype control
IgG1κ – FITC	CD66b isotype control
IgG1κ – PE-CF594	CD206/EpCAM isotype control
Anti-human CD45 – BV510	FVS510 compensation

Table 1: Antibodies and viability stains used. List of antibodies and viability dye used in this protocol. Indicated are the cellular subsets they each identify.

Tube (Label)	Sample size*	HBSS (µL)	FVS510 (µL)	CD45 (µL)	Other antibodies (µL)				Cells § (µL)
Unstained	small	80							20
	medium	50							50
	large	50							50
					IgG1κ - Alexa488	IgG1κ- FITC	IgG1κ - PE-CF594		
Isotype control	small	60.65	0.6	10	2	6	0.75		20
	medium	30.65	0.6	10	2	6	0.75		50
	large	30.25	1	10	2	6	0.75		50
					CD206	CD3	CD19	CD66b	
Blood	small	92.875	1.5	25	1.875	5	1.25	7.5	115
	medium	65.75	3	50	3.75	10	2.5	15	350
	large	102.5	10	100	7.5	20	5	30	725
					EpCAM	PanCK			
Epithelial	small	96.5	1.5	25	10	2			115
	medium	73	3	50	20	4			350
	large	117	10	100	40	8			725
* Samples are considered small, medium or large based on weight determined in step 1 of the sputum dissociation section of the protocol.									
§ Cells should be added ***after*** all reagents have been distributed, including those in the compensation tubes (Table 3).									

Table 2: Content of tubes with sputum cells. Use this table as referenced in the protocol to add the specified volumes of antibodies and viability stain for the appropriate sample size.

Tube (Label)	Reagents added (µL)						Reagents added (drop)	
	HBSS	CD45-PE	CD206-PE-CF594	EpCAM-PE-CF594	CD19-Alexa488	CD45-BV510	CompBead (+)	CompBead * (-)
PE comp.	76	4	x	x	x	x	1	1
PE-CF594 comp.	72	x	4	4	x	x	1	1
Alexa488/ FITC comp.	60	x	x	x	20	x	1	1
BV510 comp.	60	x	x	x	x	20	1	1
* The positive (+) and negative (-) beads should be added ***after*** all reagents have been distributed and the tubes in Table 2 have been prepared.								
comp. = compensation								

Table 3: Content of compensation tubes. Use this table as referenced in the protocol to add the specified volumes of antibodies to the compensation beads.

Cell number (x 10^6)				
	A	B	C	D
Sample size	**In staining volume ***	**50-fold access ¶**	**Median sample (fold access) #**	**Largest sample (fold access) #**
Small	0.625	31.25	8.0 (12.8)	24.77 (39.6)
Medium	1.25	62.5	13.0 (10.4)	48.87 (39.1)
Large	2.5	125	35.4 (14.2)	113.5 (45.4)
* Staining volumes used in the protocol (Table 2). Cell number is extrapolated from how the titration curve was performed; 1 µg/mL of CD45-PE and 1 x 10^6 cells per 200 µL.				
¶ 50-fold access of cell numbers in Column A.				
# fold access in columns C and D are calculated by dividing the number presented in the cells of columns C or D by the number in the corresponding cell in column A.				

Table 4: Anti-human CD45-PE concentration for all samples in each sample size category.

Discussion

The cellular content of sputum includes a large variety of wide-ranging cells, often accompanied by a lot of debris[37]. In addition, sputum analysis requires a quality control that confirms the sample is collected from the lung instead of the oral cavity[38]. Therefore, it is not as simple to analyze sputum by flow cytometry as it is for blood, for example, which releases a much cleaner and homogeneous cell suspension. This protocol has addressed all these issues: providing instrument settings using specific size beads to ensure that both the smallest and largest cell populations can be detected, a gating strategy to eliminate debris, cell clumps, contaminating SECs and other dead cells, and lastly, a quality control measure for ensuring a sputum sample is from the lung instead of being mostly saliva.

There are critical steps in the protocol in working with sputum that is worth pointing out. First, cell yield can be drastically impacted by the number of nylon cell strainers used in step 2.5 of the sputum dissociation part of the protocol. Multiple strainers may be required to avoid losing too many cells due to clogging of the strainer. When the flow through the strainers has slowed down noticeably, a new strainer should be used. Second, the cell pellet of sputum samples can be very loose, especially if there is high contamination of SECs. Therefore, it is essential not to aspirate too close to the pellet when removing the supernatant after centrifuging the samples. Aspirating too close to the pellet may result in cell loss, if not loss of the entire pellet. Third, this protocol requires a fixation step. This preserves the cells and their staining profile and serves as a safety measure to protect the flow cytometer operator. Running samples on certain flow cytometers may present increased biological hazards due to

the potential for aerosol production[39]. The PFA fixation helps to protect the operator from potential pathogens in the sputum sample. Fourth, and perhaps the most challenging aspect of preparing sputum samples for flow cytometric analysis, is cell counting. Cell counting is complicated because of the large variety of cell types present in the sputum. Counting machines are often limited by the cell size range they can capture and are therefore less reliable than a hemocytometer. However, cell counting of sputum samples by hemocytometer, which is excellently described by Guiot et al.[20], is tedious and requires practice to become proficient. An accurate cell number is vital in determining the sample's final resuspension volume to allow for a reasonable flow rate and prevent clogs in the flow cytometer. The presence of the very large SECs in the sputum and the many smaller cell clumps that are not broken up by the dissociation buffer, nor caught by the filters, increase the likelihood of clogging the flow cytometer. Therefore, it is recommended to spend some time determining the best cell concentration/flow rate for data acquisition using the available flow cytometer. Additionally, ensure that the flow cytometer has the appropriate flow cell and nozzle size (or probe) capable of measuring the bigger cell populations present in the sputum.

Even when proficiency in cell counting has been achieved, it is still a time-consuming process. Therefore, the reagents' determination for cell labeling in this protocol is based on the sample size (as judged by weight) rather than cell number. This allows for more efficient use of time since the manual cell count can be completed during the time needed for the antibody and viability dye staining. However, there are three notable exceptions to this. If a sample is >16 g (the so-called extra-large samples), the manual cell count should occur before staining so that expensive reagents can be conserved by only staining 25 x 10^6 cells each for the blood and epithelial tubes. This cell number gives very reliable profiles in the settings described in this protocol. Another potential exception is the case of a very small sample. The estimation is that a minimum of 1-2 x 10^6 cells is needed for the flow cytometry analysis as presented in this protocol to generate reliable profiles (data not shown). Therefore, if a sample contains fewer than 1-2 x 10^6 cells, it may not be worth continuing with the procedure since the final result will likely be unacceptable.

Specific labeling reagents that work well on peripheral blood cells or cell lines may work very differently on blood cells contained in sputum (unpublished observations). Therefore, it is recommended to titrate each antibody or other labeling reagents according to well-established protocols[34,35] before they are used in flow cytometry experiments. Most antibody titrations should give comparable results when staining up to 10 to 50-fold; however, it is advised to do additional titrations with cell numbers higher than that range[34]. When a reagent concentration has been determined, it is essential to keep the incubation time of that reagent the same as in the actual experiment. For that reason, the protocol stresses adding all the buffer and reagents to the tubes before the cells or compensation beads are added. This order of adding reagents and cells/beads together allows for higher consistency in labeling times. If, for some reason, the experimental incubation time cannot be kept similar to that used for antibody/dye titration, the latter should be repeated with an incubation time that is feasible during experiments.

Sputum samples have been widely used as a diagnostic specimen to study the pathophysiology of various illnesses affecting the lung. Tuberculosis[40,41], COPD[13,42], asthma[43], cystic fibrosis[44,45], lung cancer[46,47,48], and rheumatoid arthritis[49] are among the many illnesses where sputum has

been studied due to the advantage of it being a non-invasively obtained specimen. More recently, during the coronavirus pandemic, sputum has been used to detect prolonged viral shedding in patients hospitalized with coronavirus disease 2019 (COVID-19)[50].

Various technologies, including reverse transcription-polymerase chain reaction (RT-PCR), protein analysis, microscopy, and flow cytometry, have been used to identify disease-specific markers in sputum. Using a flow cytometric platform to analyze sputum samples is not widely used, but its use increases. With recent advancements in the technology of flow cytometers[3,51,52], as well as advancements in the chemistries of fluorophores, generation of new antibody clones, and development of various dyes to identify dead cell populations[51,53,54], the possibility of designing antibody panels aimed at diagnosing human diseases has dramatically increased. Moreover, the recent push for automating the analysis of flow cytometric data[55,56] will eliminate the potential bias in manually reading and interpreting flow data. These new developments in flow cytometry will significantly facilitate the exploration of sputum as a clinical diagnostic.

Disclosures

All the authors are past or current employees of bioAffinity Technologies.

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