ONLINE DATA SUPPLEMENT

A flow cytometric method for isolating cystic fibrosis airway macrophages from expectorated sputum

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SUPPLEMENTAL METHODS

Leukocyte labeling for FACS

Following incubation with FcR blocking reagent, cells were pelleted and resuspended in FACS buffer at $1x10^7$/ml, and up to $1x10^7$ cells in 1ml of buffer were stained for cell sorting. Smaller numbers of cells were used for isotype control staining, and to calibrate compensation settings. Cells were incubated for 30 min on ice with antibody cocktails: Calcein AM (eBioscience, San Diego, CA/ ThermoFisher, Hudson, NH), anti-CD15 (Miltenyi Biotec, Clone VIMC6), anti-CD14 (BD Pharmingen, San Diego, CA, Clone MϕP9, or eBiosciences/ThermoFisher, Clone 61D3), Annexin-V (eBioscience), and propidium iodide (eBioscience). Cells were then washed in FACS buffer, and resuspended in RPMI lacking phenol red. To minimize cell clumping during sorting, the final cell suspension was filtered through a 70μM cell strainer, and DNase (Sigma Aldrich, St. Louis, MO) was added at a final concentration of 1mg/ml.

Density gradient separation

Solubilized and filtered sputum specimens were centrifuged over Ficoll-paque (GE Healthcare from Fisher Scientific) at 800xg, room temperature, 15 minutes, no brake, using a swinging bucket rotor centrifuge. The cell layer that accumulated between the Ficoll-paque and media layer (defined as "mononuclear cells" when this method is used to fractionate peripheral blood cells) was recovered.

Magnetic cell isolation and purification

Solubilized and filtered sputum specimens were incubated MACS CD15 Microbeads (Miltenyi Biotec) as described in the manufacturer's instructions using an LS column (Miltenyi Biotec).

Statistical Analyses

Data are expressed as mean +/- standard error of the mean (SEM) unless otherwise specified.

SUPPLEMENTARY FIGURE LEGENDS

Figure E1: Protocol to solubilize sputum and reduce debris. Steps in blue boxes represent steps from published protocols for processing sputum.

Figure E2: **Histology of sputum leukocytes following different methods for isolating macrophages**. Sputum specimens and cells were prepared as noted (see Figure 1 for sample number references), and then ~30,000 leukocytes were applied to a microscope slide via cytospin and stained with Romanowsky stain (Diff-Quick). All images are the same as for specimens in Figure 1, but enlarged to show detail. Each method has been tried on at least 3 different sputum specimens. Images shown represent cells isolated by different methods starting with the same individual sputum specimen.

Figure E3: **Elimination of aggregates and debris in CF sputum using different cell isolation protocols**. Cell preparations were acquired on the FACS Aria II. Total events were plotted for forward scatter-height (FSC-H) vs. forward scatter-width (FSC-W), which separates events based on size; see Figure E4 for further description of this method. Left lower quadrant contains subcellular debris. Left upper quadrant contains larger aggregates. Right upper quadrant contains cell aggregates or "doublets" (most prominent in samples 3 and 4.) Right lower quadrant contains single cells.

Figure E4: Unstained and isotype control staining of sputum to demonstrate determination of gates. Sputum from subject 39 was processed as described and 50,000 events of specimens that were unstained, stained with isotype control antibodies (isotype antibody mixture also contains calcein blue), or stained with specific antibody panel were acquired on the flow cytometer. Numbers of events, and percent of parent population (in parentheses) are indicated on each plot.

Figure E5. Flow cytometric parameters forward scatter height (FSC-H) and forward scatter width (FSC-W) can be used to differentiate debris, single cells, doublets and aggregates. **A.** Gating strategy to identify debris and cells. Numbers indicate percent of total events in each gate. **B.** Replotting of populations identified in panel "A" for calcein content, which indicates cell viability. Variability in calcein positivity, measured as mean fluorescence index (MFI), reflects populations of varying size (large cells will have higher calcein MFI) or the presence of cellular doublets or aggregates. **C**. Replotting of populations identified in panel "A" for forward

scatter-area (FSC-A) vs. side scatter-area (SSC-A). D. Table depicting variability in percentages of viable and single cells in sputum specimens from different subjects.

Figure E6: Examples of variability of sputum specimens between different subjects: flow cytometric evaluation of abundance of debris, aggregates, and single cells. Top panels display total events for sputum specimens from 3 different subjects prepared by the protocol described in this manuscript. Bottom panels depict calcein positivity of gated populations from the upper panels. Note that subject 3 usually provides specimens with copious salivary contamination, and thus a high abundance of squamous cells that fall into "aggregate" gate.

Figure E7: Examples of variability in sputum specimens acquired from the same subject on different days. Specimens were obtained from subject 18 on different days over several months, and flow cytometry was used to assess single cells, debris, and doublets. Panels **A-E** depict FSC-H vs FSC-W for total events from each specimen. Inset panels show calcein positivity for the gated cells populations: grey peaks = debris, red peaks = single cells, blue peaks = doublets.

Figure E8: Gating strategy for identifying apoptotic and necrotic cells in sputum specimens using propidium iodide (PI) and annexin. Top panel: To confirm annexin and PI staining, THP-1 cells (a monocytic cell line) were treated with heat (65^{o}C for 60 min, then allowed to sit for 1 hr), Staurosporine (5μM for 3 hr), or irradiation (XRT: 150 mJ/cm^2 and allowed to sit for 2 hour), and then stained with the same panel used to stain CF sputum cells. Bottom panel: the same gating strategy as in the upper panel, applied to a sputum specimen that was analyzed either unstained or stained with the fluorescent panel.

Figure E9: Calcein+Annexin+ PI- apoptotic cells bind both anti-CD15 and anti-CD14 antibodies. **A.** Total events from antibody stained solubilized sputum were plotted for Calcein positivity. Calcein positive events were plotted for anti-CD15 and anti-CD14 antibody binding (**B**), and annexin-V and propidium iodide binding (**C**). Populations identified in panels A, B, and C were replotted in overlay plots to determine binding of anti-CD15 (**D**) and anti- CD14 (**D, E**) antibody binding

Figure E10: **Flow cytometry reveals that anti-CD15 and anti-CD14 specific antibodies bind to dead cells, debris, and aggregates. A.** Total events from antibody stained solubilized sputum were plotted for FSC-A vs

SSC-A, and a gate was drawn around the area associated with leukocytes. **B.** Leukocyte gate events were plotted for anti-CD14 and anti-CD15 antibody binding. **C.** Leukocyte gate events were also plotted for viable (calcein+) cells. **D.** Viable cells from panel "C" were plotted for identification of single cells and doublets. **E.** Single cells from panel "D" were then plotted for CD14 and CD15 expression. Gates "a" and "b" indicate macrophages and neutrophils, respectively. **F.** Doublets and single cells from panel "D" are plotted for CD15 and CD14 binding as in panel "E". **G.** Isotype control for this same specimen demonstrating correct placement of gates in panels "E". **H.** Populations "a" (CD14+/CD15-, macrophages) and "b" (CD14-/CD15+, neutrophils) from panel "E", and populations "c" (CD14+/CD15+, double positive events, likely apoptotic cells and/or aggregates) and "d" ($CD14^{lo}$/CD15-, likely subcellular debris) from panel "B" were plotted for calcein expression. **I-L.** Images are photographs of sorted cells after cytospins and staining with Diff-Quik. **I.** Cells in population "b" in panel "E" (CD14-/CD15+ cells) have morphology consistent with neutrophils. **J.** Cells in population "a" in panel "E" (CD14+/CD15- cells) have morphology consistent with macrophages. **K.** "doublets" in panel "D"; black arrowhead denotes a macrophage, asterisks denote neutrophils.

Figure E11: Most sputum neutrophils that are not retained by the magnetic column following incubation with anti-CD15 antibody-conjugated beads do express CD15. Solubilized sputum was treated to remove CD15+ cells using magnetic bead isolation as described in methods. Specimens were stained for flow cytometry, and gating for analysis of CD15+ and CD14+ cells was performed as described. **A.** Isotype control, total cells**. B.** Total solubilized sputum, prior to passage over the magnetic column. **C.** Cells in the sample that passed though the column ("flow through").

Figure E12. Longitudinal evaluation of CF sputum leukocytes reveals changes in relative and absolute abundance of sputum macrophages that occur temporally with changes in symptoms. Subject 18 sent sputum specimens (on ice) by overnight mail on the indicated dates. Sputum was processed as described for flow cytometry. Time period **A** indicates onset of a pulmonary exacerbation, defined by the clinic staff. Time period **B** indicates administration of antibiotics for the exacerbation. Time period **C** indicates that the subject called clinic with pulmonary exacerbation symptoms, but the subject opted not so start antibiotics. Time period **D** indicates that the subject called to report that she was feeling better; "it might have been a cold".

Table E1: Variability in abundance of viable, single cells in CF sputum specimens from a single subject, as determine by flow cytometry

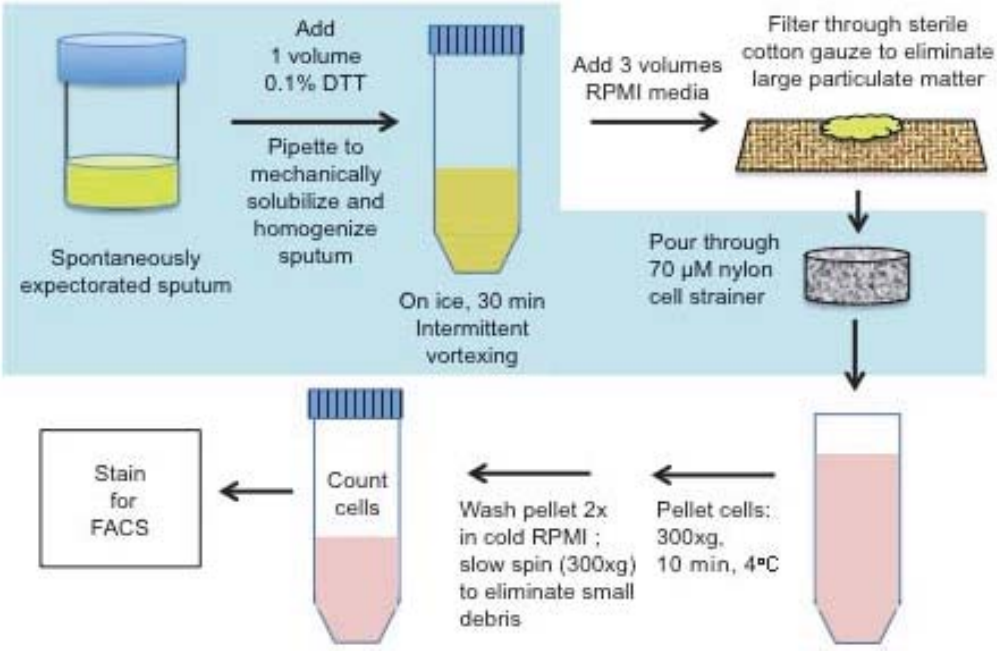
	Total cells		Single cells	Calcein$^+$ cells	Single, viable cells	
	% calcein$^+$	% single cells	% calcein$^+$	% single cells	CD14+/CD15- cells	CD14-/CD15+ cells
Mean	18.9	17.6	62.3	84.8	3.3	70.7
Median	20.3	17.4	64.2	87.0	2.4	68.8
SEM	2.2	2.6	5.2	3.1	1.5	7.2
Range	11-25	9-25	40-83	75-93	0.7-7.8	55-90

n = 6 specimens collected over 3 months for % calcein+ and single cells, and n = 4 for abundance of CD14+/CD15- cells and CD14-/CD15+ cells

Table E2: Proportion of total CF sputum sample events that are sorted as CD14+CD15- macrophages and CD14-CD15+ neutrophils

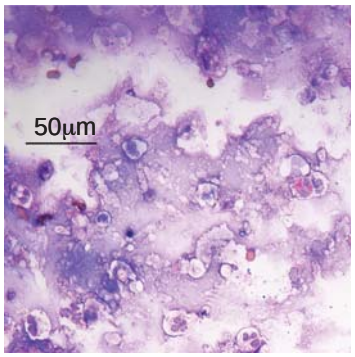
	Number of CD14+CD15- cells sorted per total sputum events sorted	Number of CD14-CD15+ cells sorted per total sputum events sorted
Mean	0.0086	0.21
Median	0.0079	0.15
SEM	0.0014	0.051
Range	0.0013 – 0.034	0.043 - 0.46
Number of specimens	26	10
Number of subjects	15 (2 specimens from subject 3, 7 specimens from subject 18)	7 (2 specimens from subject 3, 3 specimens from subject 18)

Supplemental figure E1

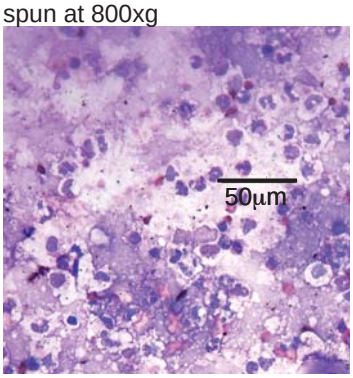


Supplemental figure E2

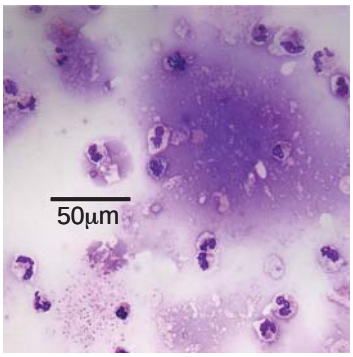
Sample 1: Sputum treated with 0.1% DTT



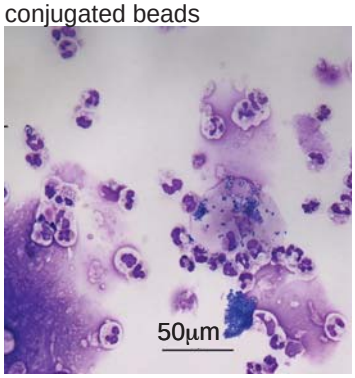
Sample 2: Sputum + 0.1% DTT, strained and filtered, spun at 800xg



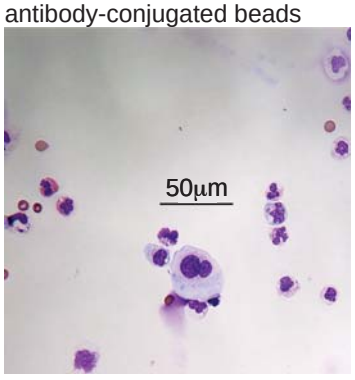
Sample 3: Sputum prepared per new protocol for FACS.



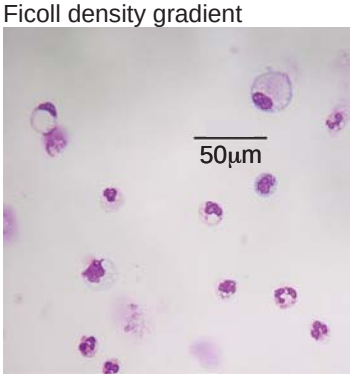
Sample 4: Cells bound to anti-CD15 antibody-conjugated beads



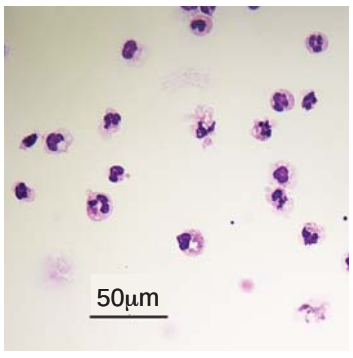
Sample 5: Flow through of column with anti-CD15 antibody-conjugated beads



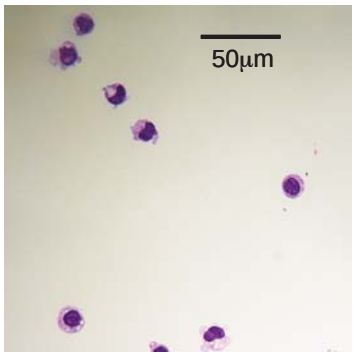
Sample 6: Cells at the interface of layers following Ficoll density gradient



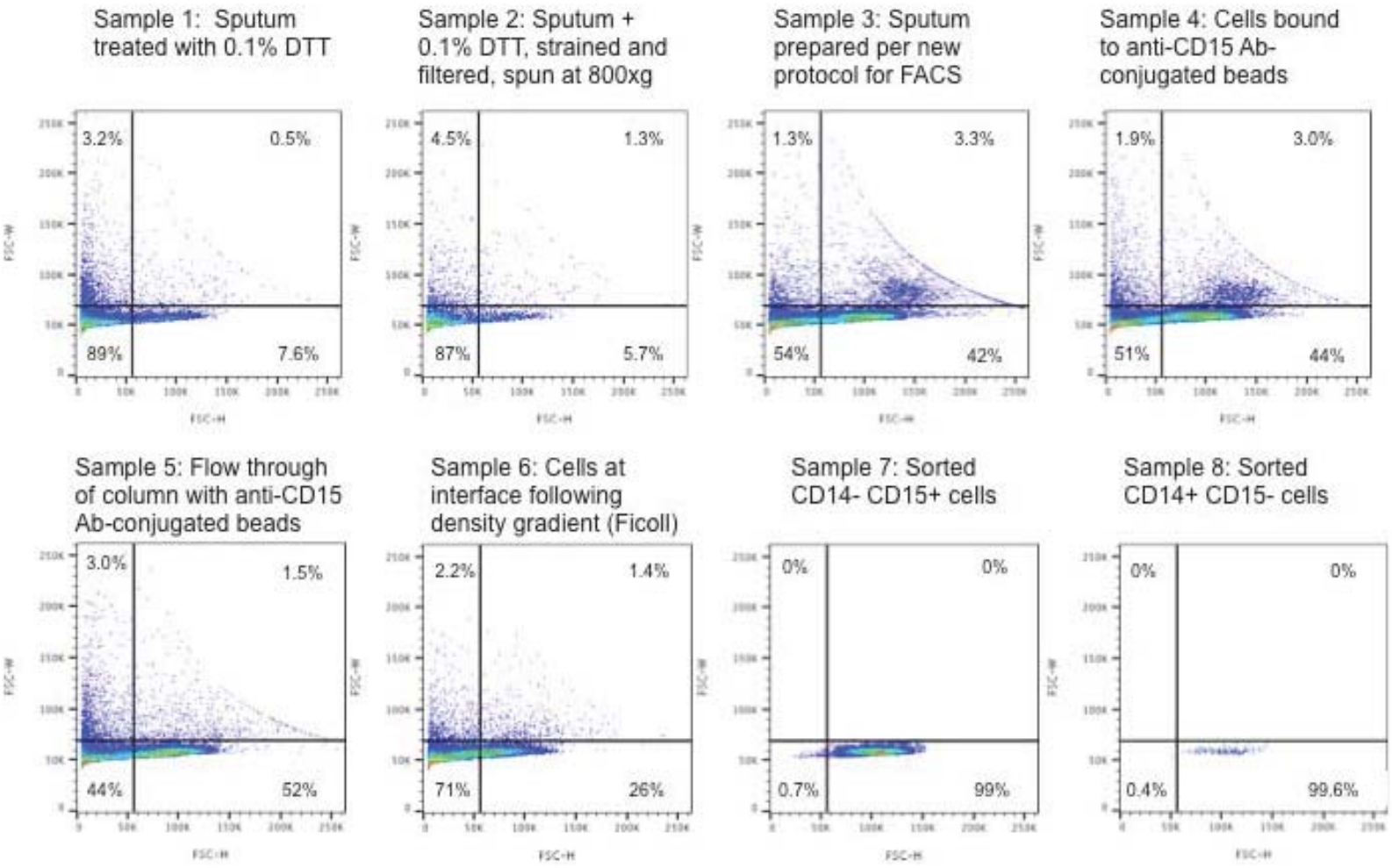
Sample 7:
Sorted CD14-/CD15+ cells



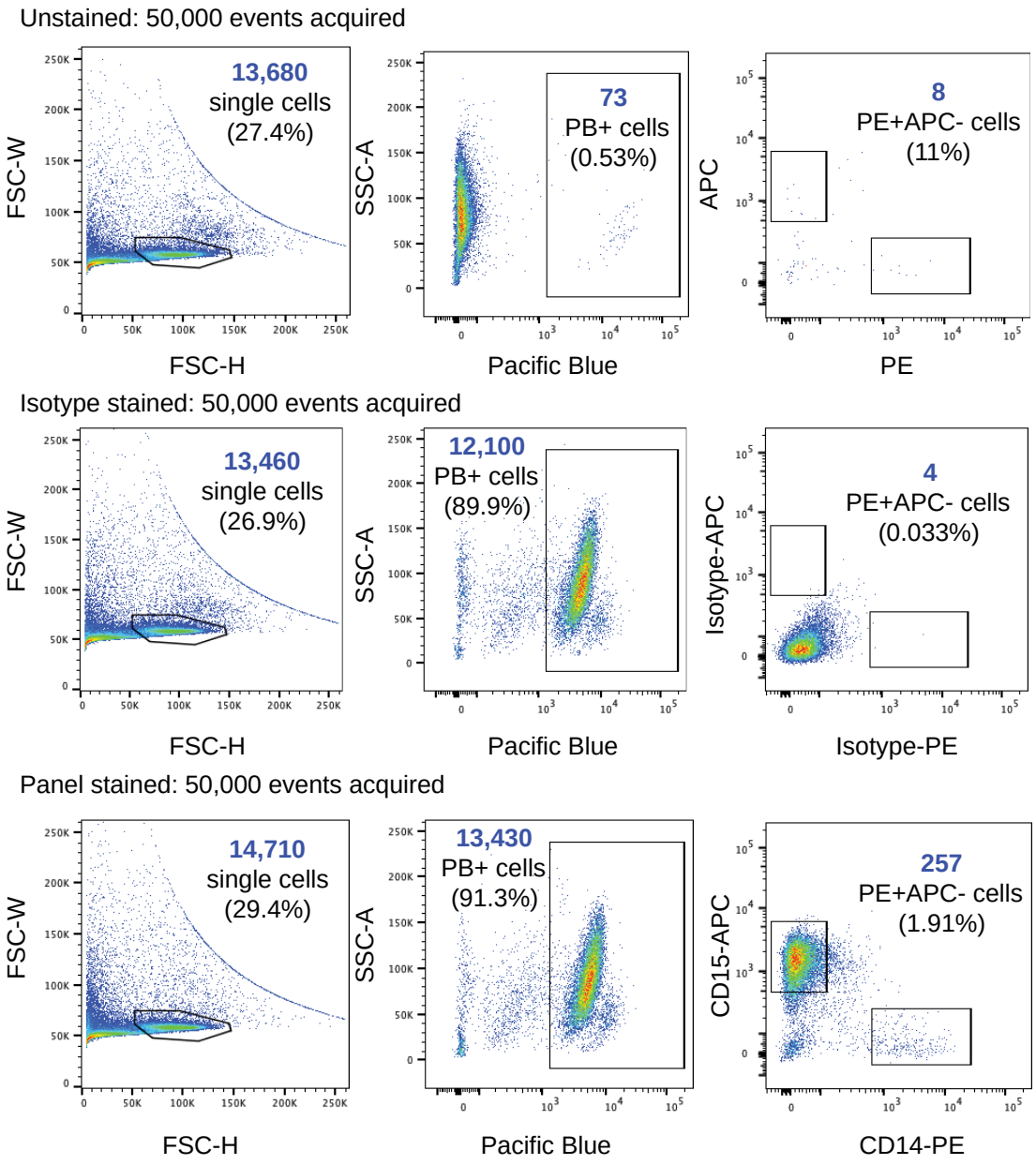
Sample 8:
Sorted CD14+/CD15- cells



Supplemental figure E3



Supplemental figure E4



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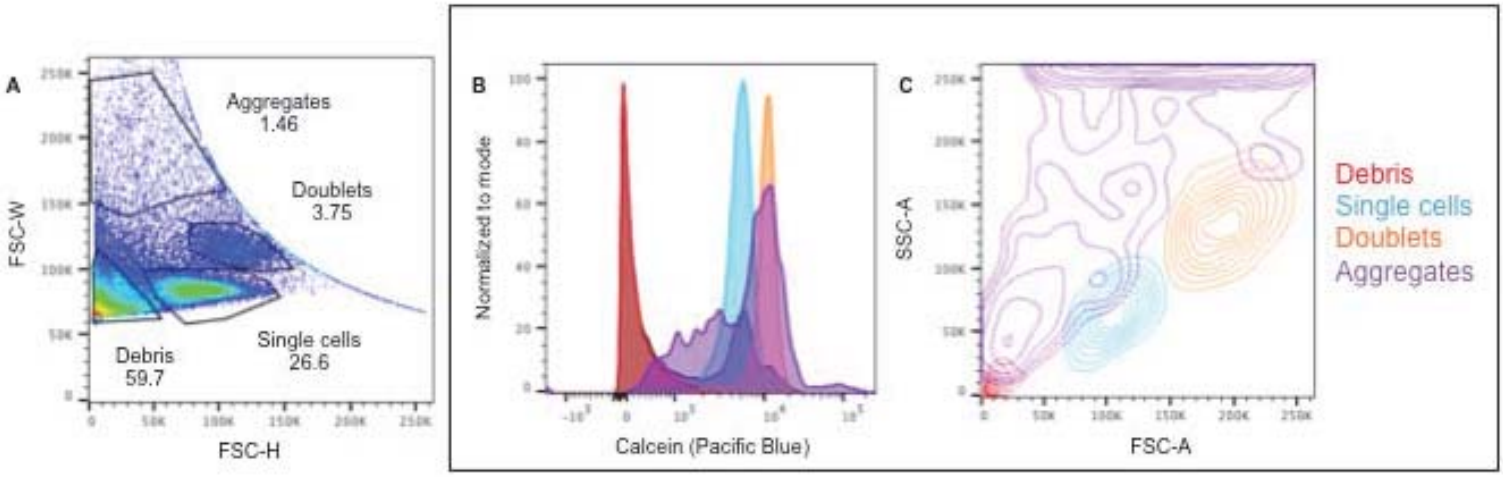

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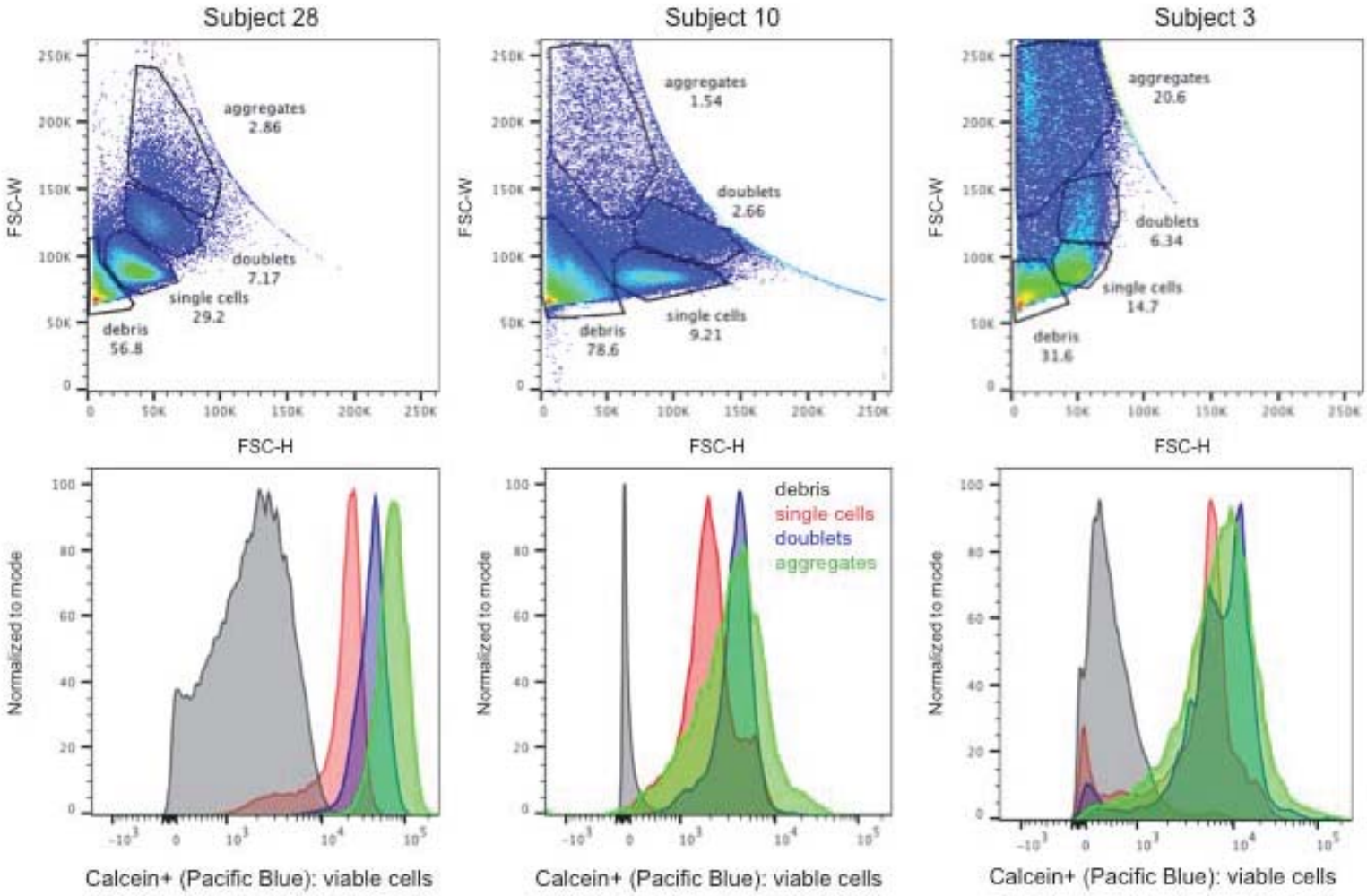
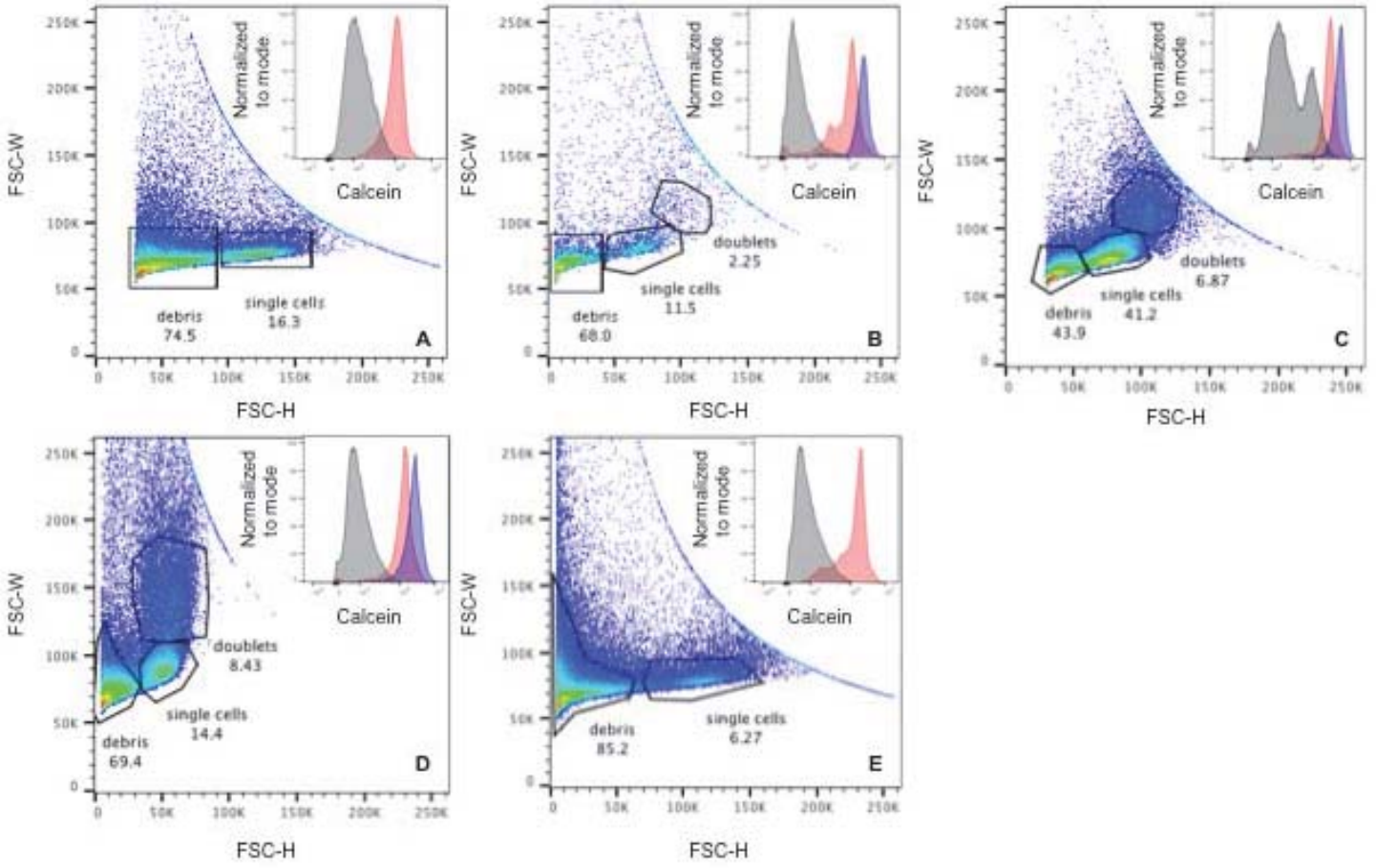
	Total cells		Single cells	Calcein$^+$ cells
	% calcein$^+$	% single cells	% calcein$^+$	% single cells
Mean	32.9	20.2	81.0	51.0
Median	24.3	17.3	85.1	52.40
SEM	6.2	3.9	3.7	6.4
Range	11-78	20-51	56-94	18-83

n = 11 sputum specimens from 5 different subjects; repeat specimens from subjects 3 & 18 were obtained on multiple days over several months. SEM = standard error of the mean.

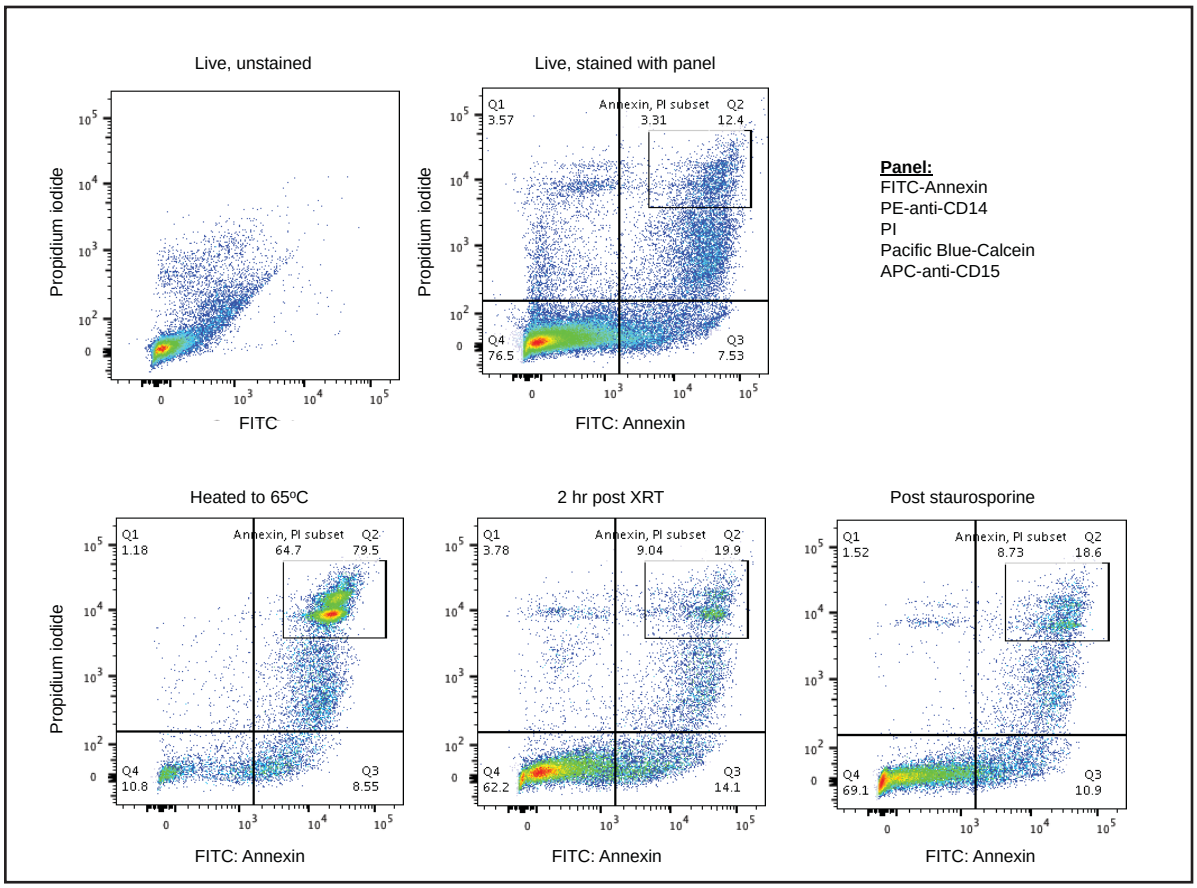
Supplemental figure E6

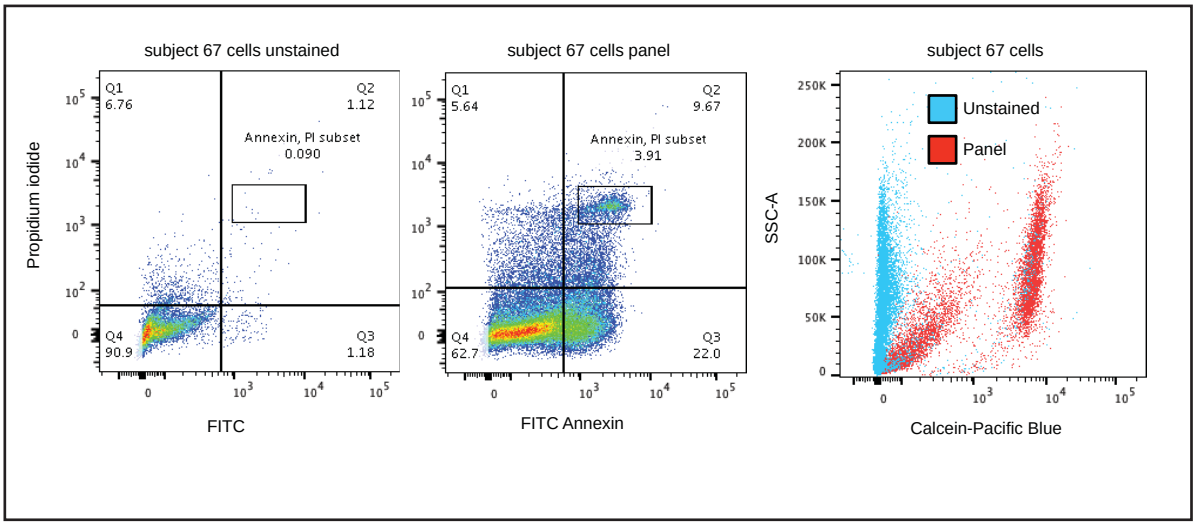


Supplemental figure E7

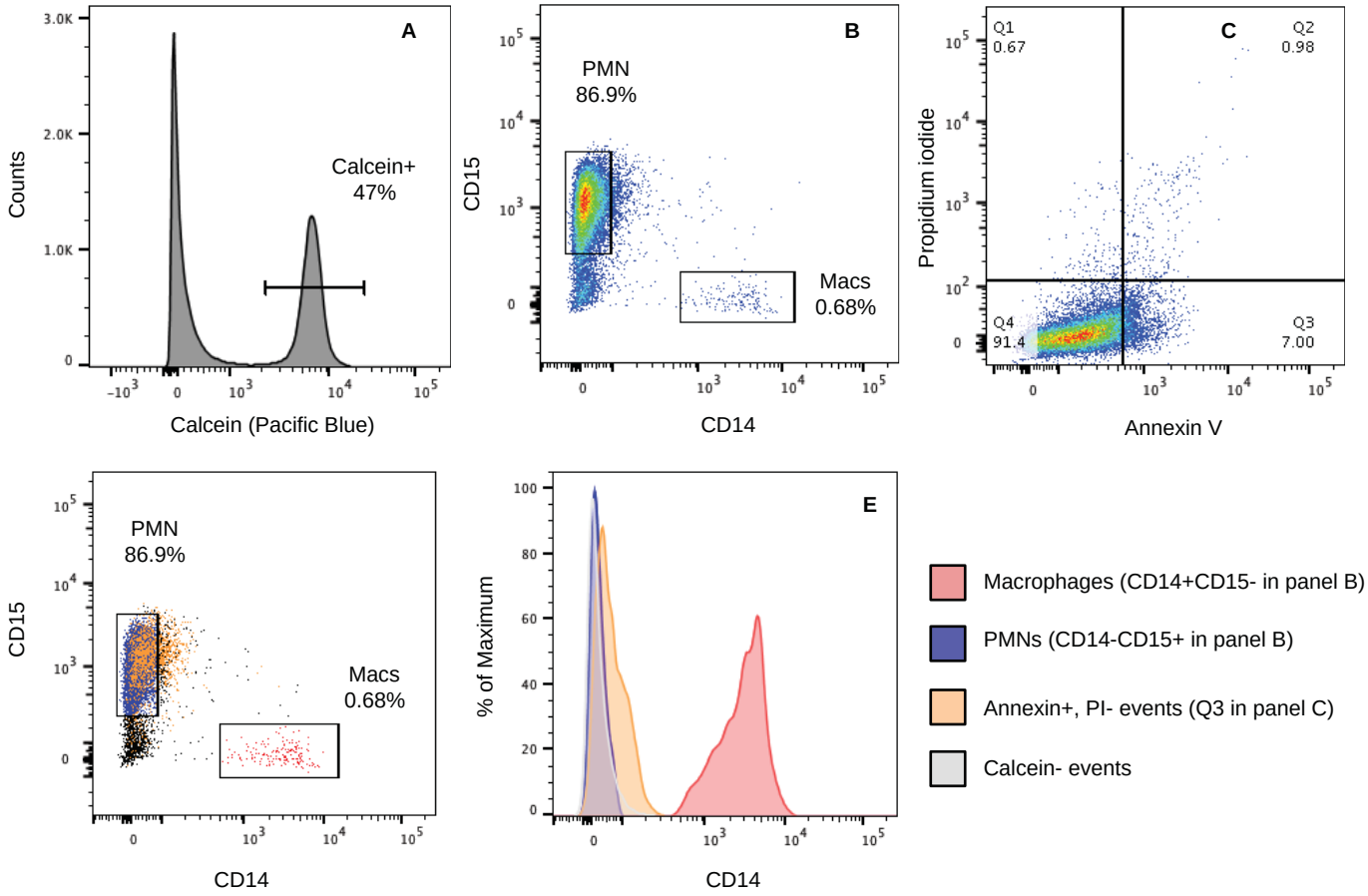


Supplemental figure E8





Supplemental figure E9



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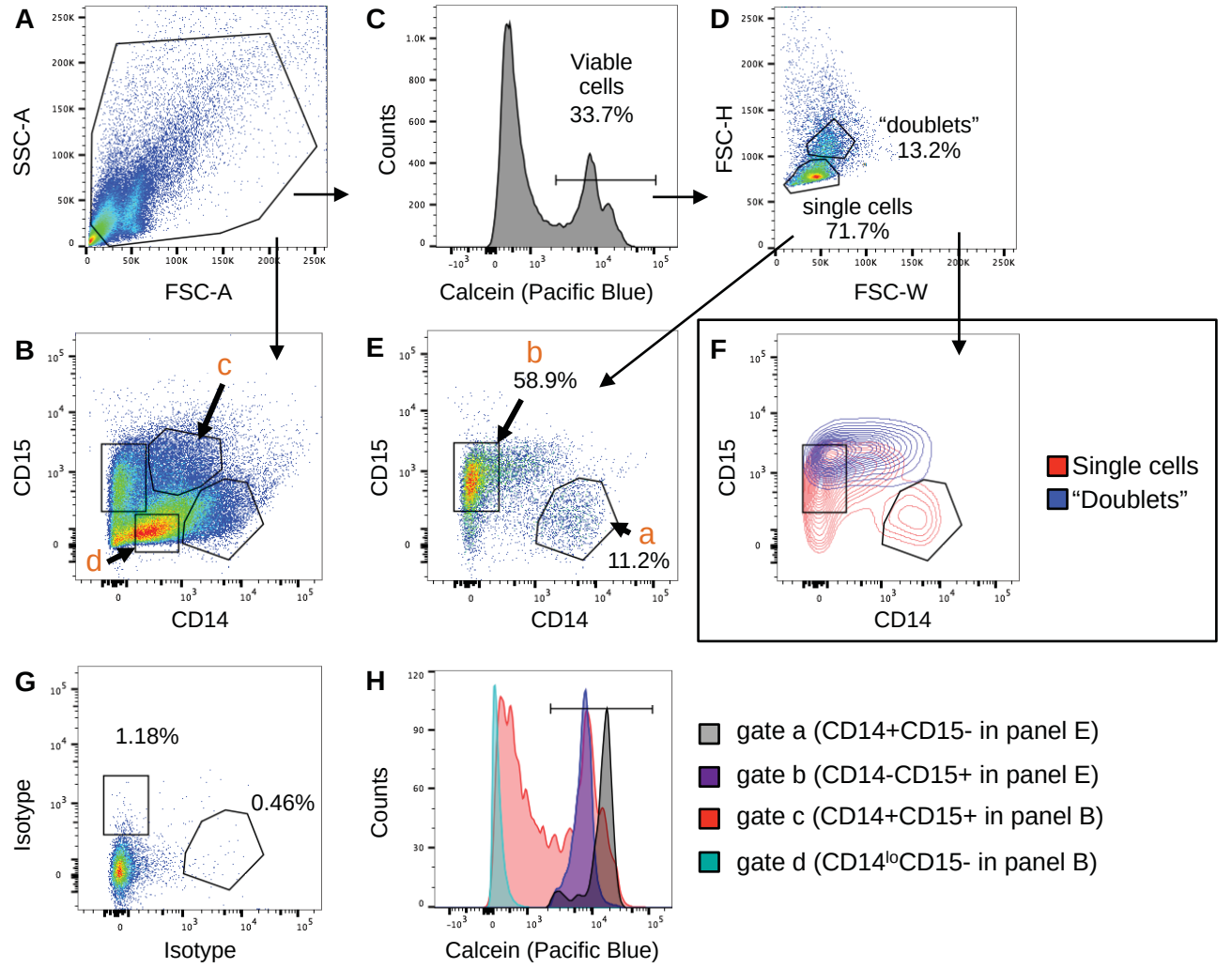
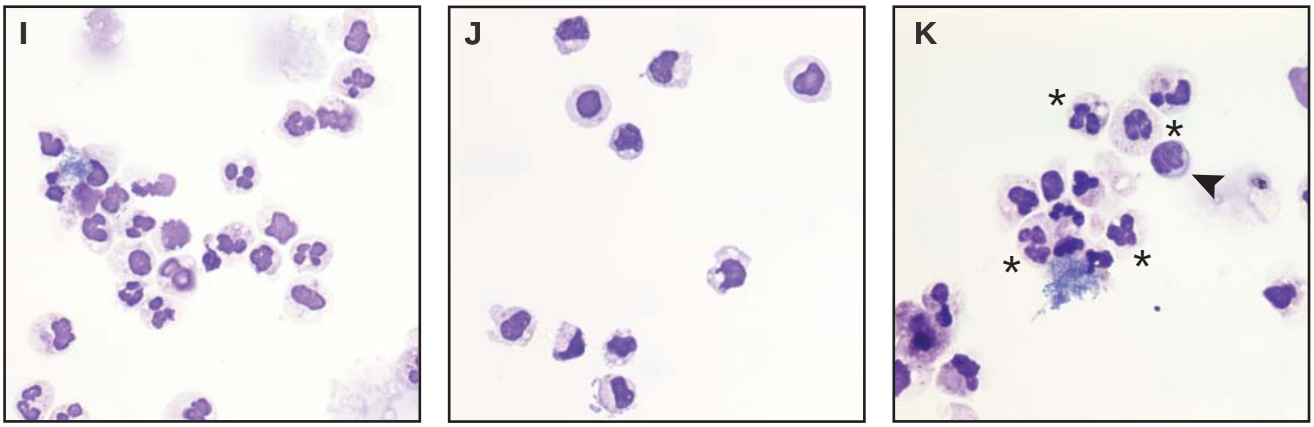
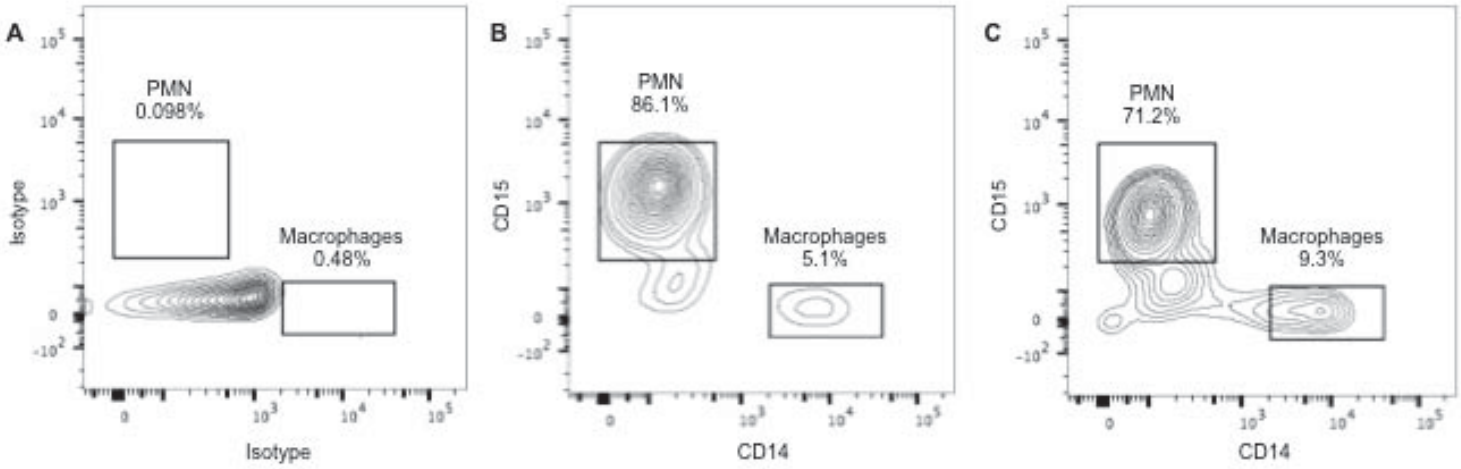




Supplemental figure E11



Supplemental figure E12