# Going with The Flow: Using Milo to Streamline Your Flow Cytometry Experiments

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## Introduction

Researchers often turn to flow cytometry and fluorescence-activated cell sorting (FACS) for single-cell intracellular protein analysis, but concerns regarding target accessibility, antibody quality and the potential for off-target antibody binding limit the production of reproducible data. All flow cytometry and FACS protocols designed for intracellular protein analysis require that cells be fixed and permeabilized before probing for your protein of interest. Depending on your target, optimization of detergent stringency and class of fixing agent may also be required. Although this technique is necessary to preserve protein localization, it can negatively affect the detection specificity of other intracellular targets or the ability to simultaneously pick up cell surface proteins for analysis.


Transcription factors are a group of proteins that are regularly found in the nucleus, bound to DNA or other proteins, making them particularly difficult to access and analyze by flow or FACS. As such, combinations of polymerase chain reaction (PCR) and Western blotting methods are often used to assess their expression. Although PCR may provide reliable interpretations regarding the transcriptional regulation of a protein, no conclusions can be made on its final expression. Further, transcription factors are also uniquely expressed in different cell types with varying cell-to-cell expression. Transcription factor expression can vary widely, even in a sample containing the same cell-type—a level of heterogeneity that neither PCR nor traditional Western blotting can assess. There is, therefore, a need for a more analytical proteomic approach that can evaluate expression parameters on a single-cell level. In this application note, we focus on profiling the protein expression heterogeneity of PU.1, a critical transcription factor in hematopoiesis and macrophage function and susceptibility to conditions like leukemia and sepsis[1,2]. Using multiple cell types, we demonstrate Milo's ability to quantitate protein expression and the heterogeneity of intracellular proteins using one simple and streamlined workflow. We then compare the data to the performance of standard approaches using flow cytometry.

## How Milo Simplifies Flow Experiments

Milo™ performs single-cell protein analysis without the need to fix and permeabilize cells for the targeting of intracellular proteins, saving you time and hassle while improving detection efficiency. Cells are chemically lysed before analysis, making intracellular compartments more accessible. You can use the large catalog of Western blot antibodies to detect protein targets, which provides greater assay flexibility and enables the detection of proteins that don't have high-quality, flow-validated antibodies. This means those hard-to-get-to intracellular proteins like transcription factors are now just as easy to measure as cytosolic or surface proteins! Milo also incorporates a size-based separation step before antibody probing, giving you molecular weight and antibody specificity information for additional confidence in the accuracy of target detection. This can be particularly important for phosphorylated targets where antibodies

tend to be a little messy and can have non-specific binding that you wouldn't otherwise see in a flow assay. Milo's fast and simple workflow processes approximately 1,000 single cells per run and measures protein expression for up to 12 targets per cell. Finally, Scout™ Software quantitatively automates data analysis. The overall workflow is flexible and can be tailored to your protein target and samples of interest.

## Materials and Methods

### CELLS, ANTIBODIES AND REAGENTS

The high PU.1-expressing macrophage cell line, RAW264.7, and the PU.1-negative embryonic kidney, HEK293T, cell lines were purchased from ATCC (Manassas, VA) and maintained in DMEM supplemented with 10% FBS (ThermoFisher Scientific, Waltham, MA). The low PU.1-expressing early hematopoietic cell line HPC-7[3] was cultured in IMDM supplemented with 5% FBS and 50 ng/mL mSCF at a cell density of 300 x $10^3$ cell/mL. All cells were kept at 37 ˚C and 5% $CO_2$. Primary granulocyte-macrophage progenitors (GMPs) were FACS sorted from total bone marrow cells isolated from the femurs, tibiae and pelvic bones of Bl/6 mice, as previously described[4]. Sorted cells were kept on ice before use in experiments.

Rabbit monoclonal (9G7) PU.1 antibody was purchased from Cell Signaling Technology, Danvers, MA (PN 2258), and mouse monoclonal (2G7D4) β-tubulin was purchased from GenScript, Piscataway, NJ (PN A01717-40). Alexa Fluor® secondary antibodies were purchased from ThermoFisher Scientific, Carlsbad, CA, and include the Alexa Fluor 647-conjugated donkey anti-rabbit IgG (PN A-31573) and the Alexa Fluor 555-conjugated donkey anti-mouse IgG (PN A-31570). The flow cytometry-validated rabbit monoclonal, Alexa Fluor 647-conjugated PU.1 (9G7) was purchased from Cell Signaling Technology (PN 2240).

We used the Small scWest Kit (PN K500), manufactured by ProteinSimple, San Jose, CA. This kit includes Small scWest chips, 10X Suspension Buffer (SB), 5X Wash Buffer (WB), Antibody Diluent 2 and single-use Run/Lysis Buffer. Sample-containing scWest chips were analyzed using Milo. For standard flow cytometry analysis, we used the BD Transcription Factor Buffer Set from BD Biosciences, Franklin Lakes, NJ (PN 562574), which includes the Fixation/Permeabilization Buffer, Diluent Buffer, and Permeabilization/Wash buffer, to fix and permeabilize cells according to the manufacturer's instructions. Samples were analyzed using the BD FACSAria™ II flow cytometer.

### SAMPLE PREPARATION

Cells were prepared using the same procedure and conditions for running on both Milo and by flow cytometry. Primary GMPs, RAW264.7, HPC7, or HEK293T cells were counted, spun at 300 x g for 5 minutes, and re-suspended individually at 100,000 cells/mL in 1X SB for analysis. For experiments using a mixed population of cells, RAW264.7 and HPC7 cells were combined to form one sample with a mixed population of 50% RAW264.7 and 50% HPC7 following the same counting and centrifugation steps as used in those samples containing the same cell type.

### RUNNING ON MILO

Each respective RAW264.7, HPC7 or HEK293T single-cell suspension containing 100,000 cells/mL of the same cell type or a mixed population of cells was settled onto a small rehydrated scWest chip for 10 minutes, after which unsettled cells were washed away with 1 mL of 1X SB. GMPs were settled for 7 minutes before washing. Single-cell occupancy was scored by visually counting 400 wells on a scWest chip using an inverted microscope. Milo captures single cells based on Poisson distribution statistics that control the rate of doublets. Typically, 1,000 cells per chip is the amount targeted to produce a maximum number of singlets and a minimum number of doublets; however, for the data presented in this application note, the number of cells captured was lowered to minimize or eliminate doublets. The scWest chips with cell line samples were run on Milo using the following parameters: 10-second lysis time, 60-second electrophoretic separation at 240 V, and 4-minute UV capture. The scWest chips with primary GMPs were run on Milo using the following parameters: 0-second lysis time, 60-second electrophoretic separation at 240 V, and 4-minute UV capture. After separation and UV-capture of proteins, scWest chips were probed with rabbit monoclonal (9G7) PU.1 antibody (diluted 1:10 in Antibody Diluent 2) and mouse monoclonal β-tubulin antibody (diluted to 50 µg/mL in Antibody Diluent 2) for 2 hours at room temperature. scWest chips were then washed for three 10-minute washes with 1X WB. Alexa Fluor 647-conjugated donkey anti-rabbit and Alexa Fluor 555-conjugated donkey anti-

mouse IgG secondary antibodies (diluted to 100 µg/mL in Antibody Diluent 2) were applied to scWest chips in the dark for 1 hour at room temperature. Finally, scWest chips were washed three times for 15 minutes with 1X WB, dried and scanned using a GenePix 4400A microarray scanner (Molecular Devices, Sunnyvale, CA). Images were saved as individual TIFF files for quantitative analysis of target protein peak area using Scout Software.

### RUNNING ON A FLOW CYTOMETER

A BD Pharmingen™ Transcription Factor Buffer Set was used in these experiments to fix and permeabilize cells. Following fixation and permeabilization of a 100,000-cells/mL sample containing either the same cell type or the mixed population of cells, each respective single-cell suspension was stained with a flow-validated and pre-conjugated rabbit monoclonal anti-PU.1 Alexa Fluor 647 fluorescent antibody. Anti-PU.1 Alexa Fluor 647, diluted 1:50, was applied directly to the sample and incubated in the dark for 30 minutes at 4 °C. Sample data were recorded using a BD FACSAria II instrument and analyzed using the FlowJo V10 software suite.

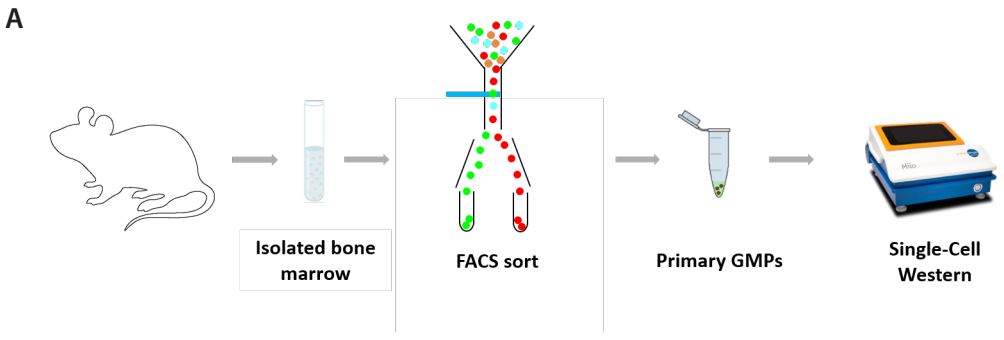
## Ready, Set, Flow: How Does Milo Compare to Flow Cytometry?

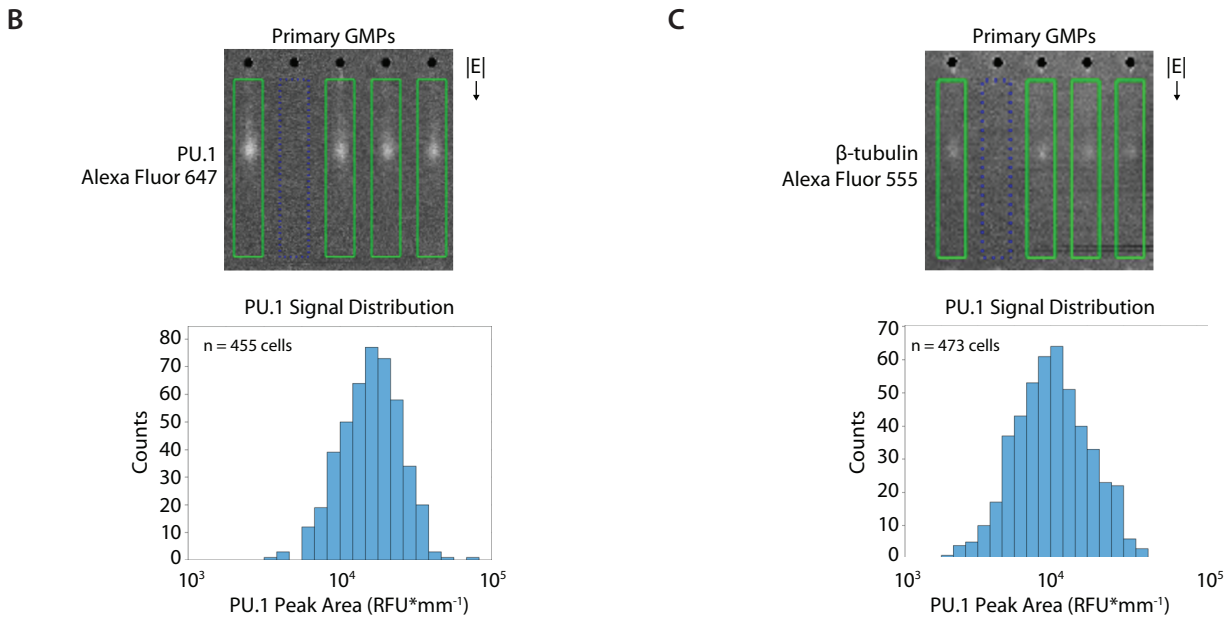
### RESULTS AND DISCUSSION

#### Intracellular PU.1 assay in primary FACS-sorted mouse cells

If you're staining for intracellular targets using traditional techniques such as flow, you're probably fussing with the permeabilization requirement, which entails meeting the following three criteria for success: 1) ability to discern the stained internal target, 2) conservation of surface proteins for differentiation of cell subpopulations of interest and 3) compatibility with the antibody conjugated fluorochromes you plan to use for detection[5]. Milo, however, says goodbye to these struggles with one streamlined workflow irrespective of where your targets are located in or on the cell while generating quantitative results with increased detection specificity. In **Figure 1**, we demonstrate a multiplexed Single-Cell Western PU.1 and β-tubulin (control) assay in 473 primary GMPs. Primary GMPs were FACS sorted from total bone marrow cells isolated from mice and then analyzed on Milo for PU.1 expression (**Figure 1A**). Milo measures PU.1 expression in each β-tubulin-positive single-cell and shows that PU.1 expression varies by approximately 10-fold over the population of single-cells (**Figures 1B and 1C**), matching the range in PU.1 expression measured using flow cytometry (**Figure 2**).

Milo provides quantitative information in two output formats: by the measurement of target abundance (i.e., signal) in every single cell and by the enumeration of target positive cells. Target abundance is measured by integrating the area under the curve for the protein band from your protein target of interest. Target abundance can then be compared across cells analyzed on the chip. Chip-to-chip comparisons of target abundance can also be made to compare samples. The off-target signal can be excluded using Scout software, thereby improving the specificity of your detection. The ability to count cells in your population expressing a certain marker can help you identify and quantitate the presence of cell subpopulations within a sample. Of the total primary GMP population analyzed, 96% of cells expressed PU.1 as measured by Milo (**Figure 1C**), which is validated by the 87% PU.1-positive cell population measured by flow cytometry when using HEK293T cells as a negative control (**Figure 2B**).



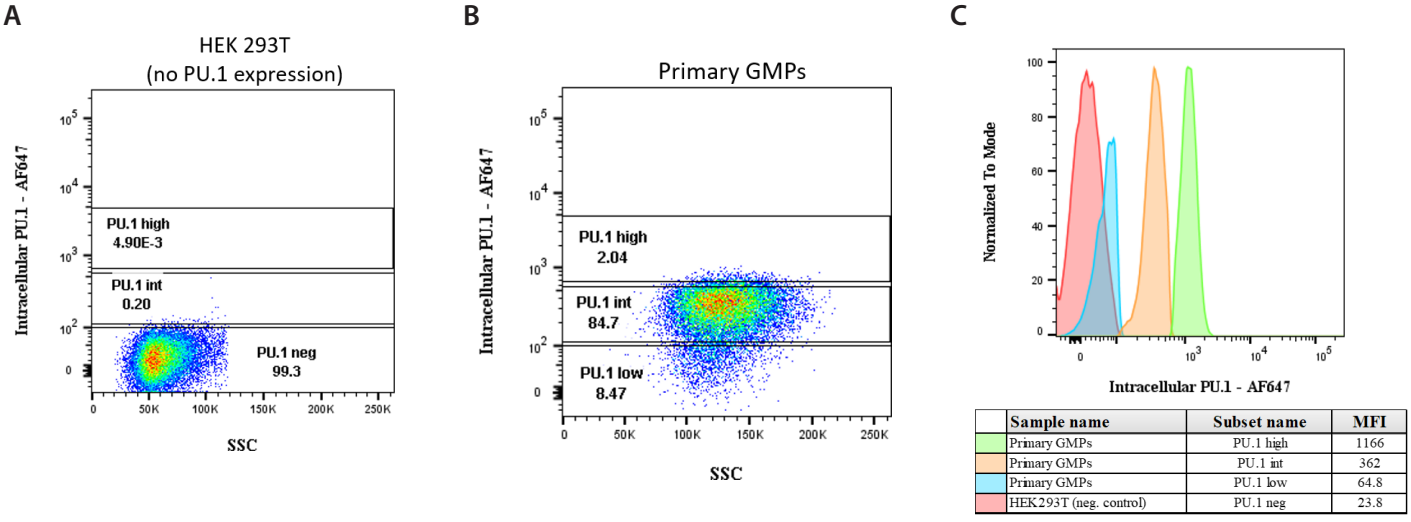



D

| PERCENTAGE | β-TUBULIN+ |
|---|---|
| PU.1+ | 96.2% |
| PU.1- | 3.8% |
| **TOTAL** | **100%** |

| CELL | β-TUBULIN+ |
|---|---|
| PU.1+ | 455 |
| PU.1- | 18 |
| **TOTAL** | **473** |

**FIGURE 1. PU.1 detection in primary GMPs using Milo.** Protocol schematic depicting how Milo was used downstream of FACS-sorted total bone marrow cells isolated from mice (A). Lane view showing representative single-cell separations and a robust PU.1 band. A histogram of area under the curve for each PU.1. band in each single-cell separation shows a normal distribution across all 455 PU.1-positive cells analyzed (B). Lane view of the β-tubulin control for the same lanes shows which lanes contained a cell. β-tubulin control is detected in the distinct Alexa Fluor 555 channel to enable multiplexing with PU.1. A histogram graph of the β-tubulin control peak areas shows the distribution of β-tubulin across all 473 cells analyzed (C). Enumeration of PU.1-positive and PU.1-negative cells (D). scWest chips were incubated with the indicated primary antibodies, and target detection was achieved using the appropriate Alexa Fluor secondary antibodies.

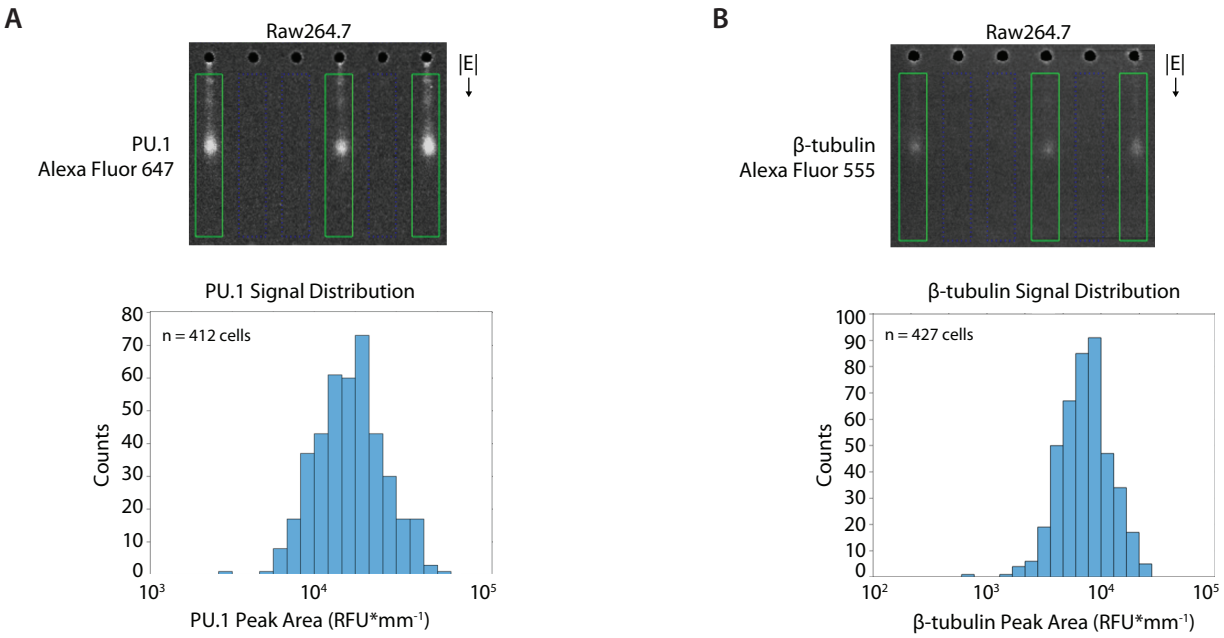



| Sample name | Subset name | MFI |
|---|---|---|
| Primary GMPs | PU.1 high | 1166 |
| Primary GMPs | PU.1 int | 362 |
| Primary GMPs | PU.1 low | 64.8 |
| HEK293T (neg. control) | PU.1 neg | 23.8 |

**FIGURE 2. PU.1 detection in Primary GMPs using flow cytometry.** Density plot of HEK293 cells, which do not express PU.1 and were used as a negative control (A). Density plot showing approximately 10-fold variation in PU.1 expression in primary GMPs (B). Merged data in histogram format showing the various cell subsets detected, compared with the HEK293T negative control (C).

### Different sample types, same workflow

Next, to demonstrate the intracellular PU.1 assay on Milo in another cell line, we assessed PU.1 expression heterogeneity in a RAW264.7 murine macrophage cell line on Milo using the same PU.1 assay conditions and β-tubulin as an internal control. Much like the previous results, we could measure PU.1 expression over a similar 10-fold range of signal intensity using a population size of 427 cells **(Figure 3)**. RAW264.7 cells highly express PU.1, and the enumeration data generated by Scout software depict a sample population where 96% of the total are




C

| PERCENTAGE | β-TUBULIN+ |
|---|---|
| PU.1+ | 96% |
| PU.1- | 4% |
| **TOTAL** | **100%** |

| CELL | β-TUBULIN+ |
|---|---|
| PU.1+ | 412 |
| PU.1- | 15 |
| **TOTAL** | **427** |

**FIGURE 3. PU.1 detection in RAW264.7 cells using Milo.** Lane view showing representative single-cell separations and a robust PU.1 band. A histogram of area under the curve for each PU.1 band in each single-cell separation shows a normal distribution across all 412 PU.1-positive cells analyzed (A). Lane view of the β-tubulin control for the same lanes shows which lanes contained a cell. A histogram graph of the β-tubulin control peak areas shows the distribution of β-tubulin across all 427 cells analyzed. β-tubulin control is detected in the distinct Alexa Fluor 555 channel to enable multiplexing with PU.1 (B). Enumeration of PU.1-positive and PU.1-negative cells (C). scWest chips were incubated with the indicated primary antibodies, and target detection was achieved using the appropriate Alexa Fluor secondary antibodies.

PU.1-positive, and just 4% are negative for the transcription factor **(Figure 3C)**.

### Chip-to-chip comparisons and thresholding target expression levels on Milo

To demonstrate Milo's ability to threshold to resolve distinct populations based on differences in protein expression levels, intracellular PU.1 expression was measured in a mixed sample containing a 50/50 mixture of the low PU.1-expressing early hematopoietic cell line, HPC7, and the high PU.1-expressing RAW264.7 macrophages **(Figure 4)**. As expected, we observed a bimodal distribution of PU.1 accounting for the two cell-type subpopulations on Milo **(Figure 4A)**.

Chip-to-chip comparisons of target abundance can be made to compare target heterogeneity across samples as long as assay conditions are conserved. Comparing the PU.1 expression from the chip run with RAW264.7

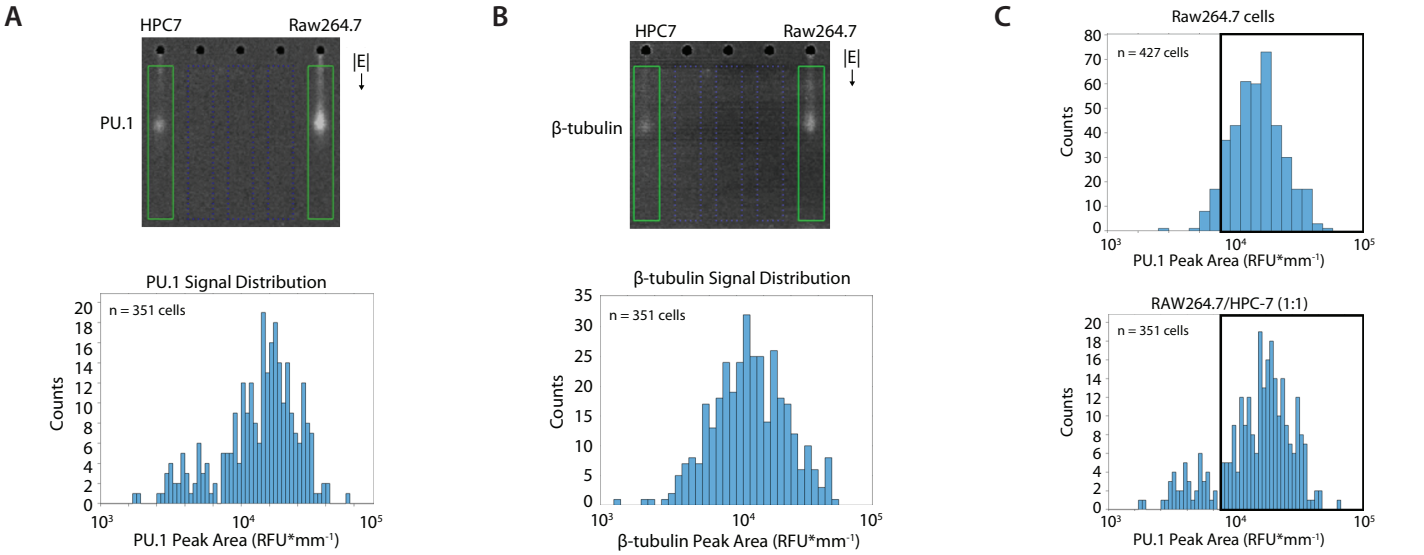
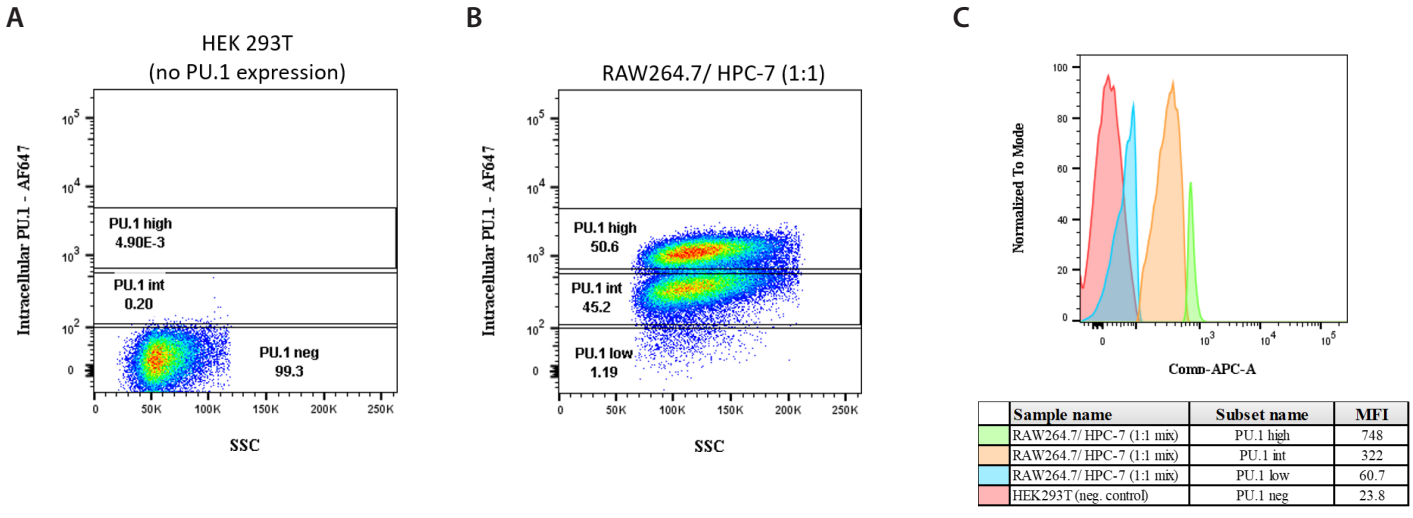



**FIGURE 4. PU.1 detection in a mixed population of RAW264.7 and HPC7 cells using Milo.** Lane view showing representative single-cell separations and a PU.1 signal detected in both HPC7 and RAW264.7 cells corresponding to a bimodal distribution of PU.1 peak areas. Cell separations are labeled HPC7 and RAW264.7 based on PU.1 expression level (A). Lane view of the β-tubulin control for the same lanes shows which lanes contained a cell. β-tubulin control is detected in the distinct Alexa Fluor 555 channel to enable multiplexing with PU.1 (B). Histograms of RAW264.7 cells run alone, and mixed samples show comparability of peak areas for high expression population and ability to threshold to differentiate between the two populations in the mixed sample (C). scWest chips were incubated with the indicated primary antibodies, and target detection was achieved using the appropriate Alexa Fluor secondary antibodies.

macrophages alone and PU.1 expression from the chip run with the mixed cell population, we observe that the high expressing RAW264.7 macrophage population run alone has a very similar quantitative range as the high expressing cell population in the mixed sample. Peak areas from the pure RAW264.7 macrophage sample were used to establish thresholding values to define this population in the mixed sample **(Figure 4C)**. The upper 90% of peak areas in the RAW264.7 macrophage sample was used for thresholding to remove outliers. The thresholding values used from the RAW264.7 sample clearly differentiate between the two populations in the mixed sample.

### Bimodal pattern measured on Milo is validated by flow

To demonstrate the comparability of the Milo results with flow cytometry results, the same 50/50 mixture of RAW264.7 and HPC-7 cells was also run on flow cytometry. As expected, we saw a bimodal distribution of PU.1 accounting for the two cell-type subpopulations on flow **(Figure 5B)**, validating the bimodal distribution




| Sample name | Subset name | MFI |
|---|---|---|
| RAW264.7/ HPC-7 (1:1 mix) | PU.1 high | 748 |
| RAW264.7/ HPC-7 (1:1 mix) | PU.1 int | 322 |
| RAW264.7/ HPC-7 (1:1 mix) | PU.1 low | 60.7 |
| HEK293T (neg. control) | PU.1 neg | 23.8 |

**FIGURE 5. PU.1 detection in a mixed population of RAW264.7 and HPC7 cells using flow cytometry.** Density plot of HEK293 cells which do not express PU.1 and were used as a negative control (A). Density plot showing the presence of a bimodal distribution of cells expressing PU.1 (B). Merged data in histogram format showing the various cell subsets detected with HEK293T as a negative control (C).

measured on Milo **(Figure 4A)**. Milo was able to distinguish PU.1 intensity of expression between the two cell lines with a higher dynamic range compared with flow, spanning almost 2 logs, for this sample **(Figures 4 and 5)**. Furthermore, Milo also displayed greater detection sensitivity in his ability to pick up the PU.1 signal intensity at around just 103 relative fluorescence units, whereas this was the upper limit when the same sample was analyzed using flow cytometry **(Figures 4 and 5)**. Overall, flow cytometry and Milo both detected a similar bimodal distribution but with a slightly diminished sensitivity and range observed by flow cytometry **(Figure 5)**.

## Conclusion

With Milo, you can eliminate the fixation and permeabilization steps required by standard flow/FACS protocols, while efficiently gaining access to intracellular and intranuclear compartments. By lysing your cell sample instead, Milo can better detect challenging proteins like transcription factors using widely available Western blot antibodies. Further, Milo provides molecular weight information in addition to antibody binding information, improving the specificity of your measurement. We demonstrated the application of Milo as an alternative to flow for the sensitive and specific detection of such proteins using the transcription factor PU.1 and various cell lines and primary cells as a proof-of-concept. Milo's ability to discern subpopulations within the same sample type based on thresholding signal abundance and his ability to enumerate distinct populations of cells based on the percentage of target-positive cells makes him an attractive option for the characterization of proteins that are painful to access using traditional flow methods.

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