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Altering Gating and Analysis through Integration of High Speed, High Resolution Imaging with Flow Cytometry Data

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INTRODUCTION

Subjectivity in flow cytometry data analysis decision points has long been a challenge for our industry. Instrument and reagent innovations continue to increase the range of information captured at the single cell level, yet fundamental data quality concerns still plague many flow cytometry experiments. The aim of this work was to leverage recent advancements in high speed camera capabilities in-line with flow cytometry data in the Invitrogen™ Attune™ CytPix™ Flow Cytometer. By simultaneously evaluating fluorescence data with the wealth of event morphology information present in brightfield images, we have been able to improve upon our gating and data analysis strategies, leading to more robust outcomes for downstream research conclusions.

MATERIALS AND METHODS

All data acquired on a CytPix™ cytometer with default BRV6Y filter configurations.

To determine an appropriate aggregate plot for cryopreserved human PBMCs (AllCells), cells were thawed per supplier's direction. After recording on the CytPix cytometer, aggregate images (~50) were backgated onto exclusion plots (Figure 1).

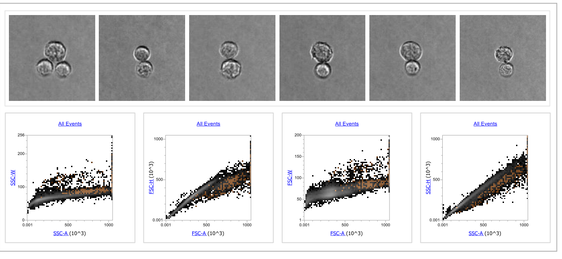
We utilized extracted image parameters (Figure 2) to remove debris from minimally handled mouse whole lung homogenate (University of Oregon). As the goal of this work was to increase yield of target cells, tissue was prepared with minimal handling (Dounce homogenization without gradient separation). Cells were stained with Invitrogen™ SYTOX™ AADvanced™ Ready Flow™ Reagent and Hoechst 33342 prior to analysis.

Acute toxicity of the fungicide carbendazim (CBZ, Sigma) was explored with *Saccharomyces cerevisiae* (ATCC) incubated with 0, 25, 50, 100, 200, 400 or 800 µM CBZ in Gibco™ YPD Broth for 18 hours 27ºC with 150 rpm agitation in the dark. Samples were analyzed on the CytPix cytometer and Invitrogen™ CytKick™ Max Autosampler.

Whole peripheral blood (AllCells) was aged (~36 hrs) and separated in SepMate™ (STEMCELL Technologies) without recommended secondary rinses. Cells were stained with Invitrogen™ eBioscience™ eFluor™ 450:CD14, eFluor™ 506:CD45, Super Bright™ 600:CD56, PE-Cyanine 5.5:CD4, PE:CD19, PE-Cyanine 7:CD8, APC:CD16 and APC-eFluor™ 780:CD3 and gated as presented in Figure 4.

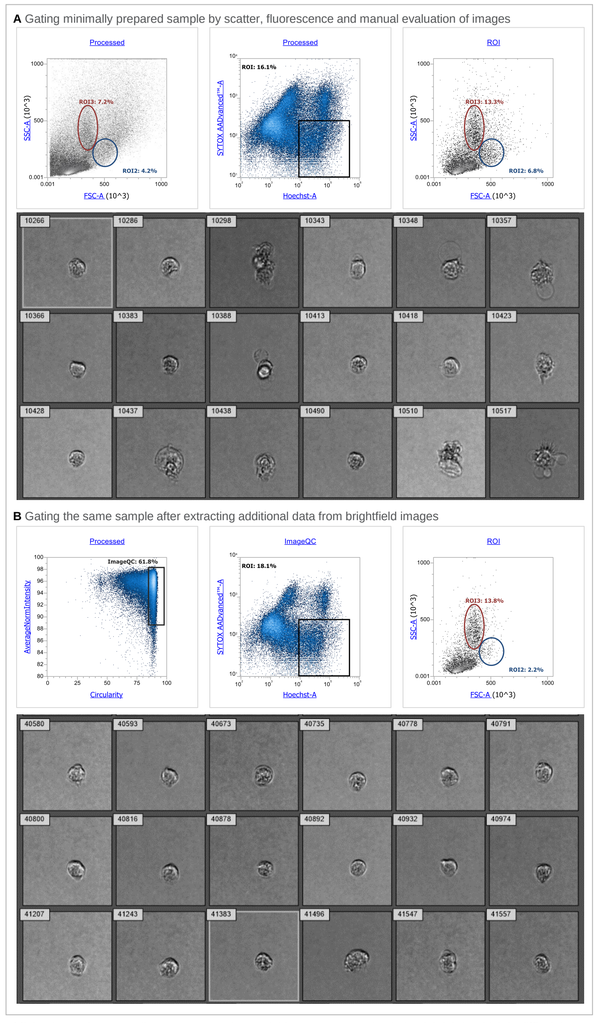
RESULTS

Figure 1. Doublet exclusion using aggregate image backgating makes appropriate singlet gate parameters readily apparent



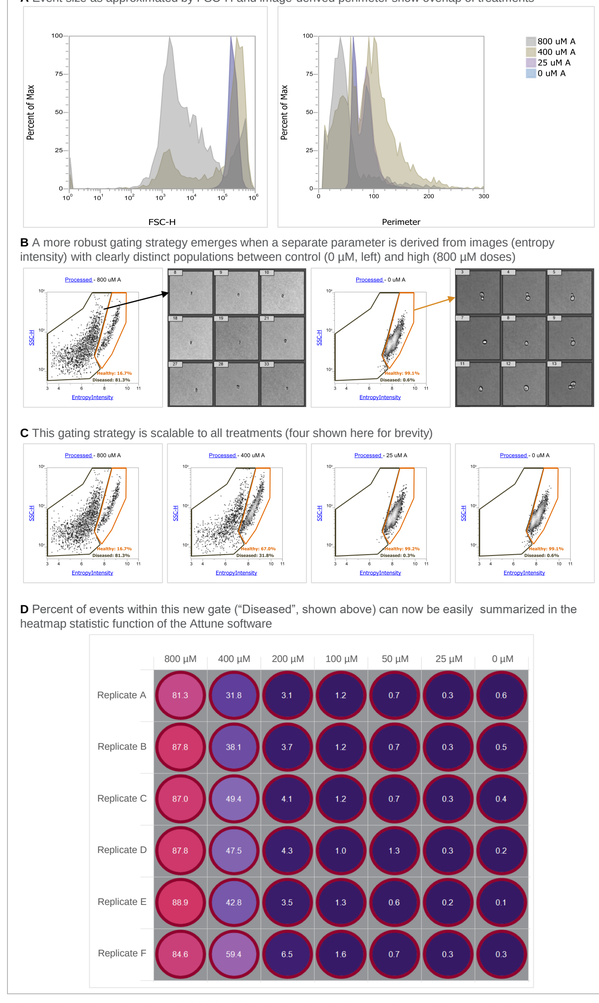
While several aggregate exclusion parameters are often employed in routine flow cytometry data analysis, our work has shown that for most samples only a SSC-A vs. SSC-H plot can remove the majority of aggregate events. Above, ~50 doublet/aggregate hPBMCs were backgated from the CytPix image view gallery onto four common doublet exclusion plots (orange dots with representative images shown).

Figure 2. Improving data quality directly through morphological features



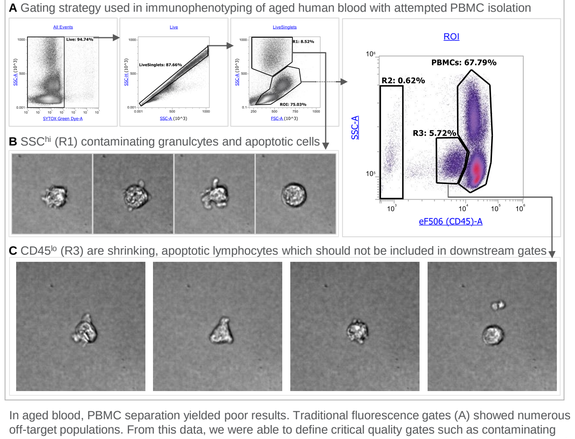
Minimal sample preparation ensures maximum yield, yet can present substantial debris which is difficult to remove even with image assistance (A). Here, whole mouse lung homogenate data is dramatically improved by gating on an extracted image parameter (event circularity and background normalized intensity) (B). Each gallery representative of final "ROI3" gate.

Figure 3. Robust treatment outcomes characterized by brightfield image data



Incubation with varying doses of CBZ for 18 hr. had profound effects on *S. cerevisiae* morphology which we were unable to capture with fluorescent staining or scatter, or even derived size (A). These perturbations in cell shape and proliferation were rapidly characterized with extracted image parameters (B, C, D). Doses of 0, 25, 400 and 800 µM CBZ shown in plots for brevity; all doses and biological replicates presented in heatmap view.

Figure 4. Confirm long-standing suspicions in immune cell gating



In aged blood, PBMC separation yielded poor results. Traditional fluorescence gates (A) showed numerous off-target populations. From this data, we were able to define critical quality gates such as contaminating granulocytes and high SSC apoptotic cells (B, gate R1) and shrinking and apoptotic PBMCs which showed characteristically dim CD45 expression and low/intermediate SSC (C, R3).

CONCLUSIONS

Including high speed image collection and the data from these images enhanced our team's efforts to improve upon our existing flow cytometry protocols and gating strategies. Not only were we able to better identify events of interest, but we were also able to characterize new outcomes which were previously unavailable.

Here we have highlighted several of these efforts, including the utilization of images to refine aggregate exclusion gates and to remove debris from samples with minimal upstream preparation. Furthermore, image evaluation and data extraction allowed for novel research conclusions to be drawn from CBZ treated yeast samples without additional sample preparation. Off-target events, including aggregates and apoptotic cells, were rapidly identified and excluded from downstream analysis of hPBMC and whole blood samples using their distinct morphology.

These results demonstrate the value of integrated brightfield imaging in refining and redefining flow cytometry gating and data analysis.

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TRADEMARKS/LICENSING

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