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Flow cytometry

Automated image analysis reduces user-to-user variability in flow cytometry gating strategies

Introduction

The need for high-throughput sample acquisition and automation in flow cytometry creates demand for new methods for analytical standardization and for reducing sources of variability. Sources of variation in flow cytometry experiments include differences in sample preparation, user uncertainty, differences in biological samples, cell analyzer performance, and instrument acquisition settings [1]. Additionally, differences in how users gate populations and analyze the same data within an experiment are major sources of variability. Researchers often debate about the best gating strategies, and variations in such strategies may often lead to poor assay standardization. Standardization is crucial for obtaining reproducible results and ensuring that experimental data support research conclusions.

Flow cytometry provides statistical analysis of populations at the single-cell level and is thus quantitative by design. However, the analyses can be subject to individual gating differences by relying on user assumptions to draw gates on dot plots and histograms that may lack clear and distinct boundaries between populations [2]. The emergence of imaging-enhanced flow cytometers enables users to visualize their samples during acquisition, verify the accuracy of their gating strategies, and support their results with reliable documentation for publication purposes. Direct visual observation of events from images in a population can reduce ambiguity and variability in data attributed to differences between users.

Attune CytPix Flow Cytometer

The Invitrogen™ Attune™ NxT Flow Cytometer is a clog-resistant, workhorse cell analyzer that can be configured with up to four spatially separated lasers for analysis of up to 14 fluorescence parameters. Its unique acoustic-assisted hydrodynamic focusing feature enables sample flow rates up to 1,000 µL/min and acquisition speeds up to 35,000 events/sec, making it one of the fastest benchtop cell analyzers on the market. The Invitrogen™

Attune™ CytPix™ Flow Cytometer comes equipped with all the features of the Attune NxT Flow Cytometer. In addition, it has a high-speed bright-field camera that records image events as they pass through the flow cell to verify that cell populations consist of single cells and to establish cell morphology. The Attune CytPix Flow Cytometer captures up to 6,000 high-resolution images/second and particles as small as 0.8 µm. A variety of particles and cell types can be reliably imaged, analyzed, and correlated to their respective events on bivariate plots, providing flexibility for developing a wealth of biological applications.

Invitrogen™ Attune™ Cytometric Software v6.0 empowers users of the Attune CytPix Flow Cytometer with automated image analysis capabilities. Using this tool, the image data are translated into digital data that captures measurements of particles or cells. Users can correlate spatial and cell morphological characteristics with cell phenotypes and augment their conventional flow cytometry data with image-derived datasets. These datasets allow users to better understand their samples. Attune Cytometric Software v6.0 automates data extraction from images, providing label-free quantitative parameters for profiling size measurements, population-level statistics, population identification, and particle enumeration. These image parameters can then be plotted against conventional flow parameters and back-gated with any image from a single event onto the flow cytometry standard (FCS) data.

invitrogen

Automated image analysis for flow cytometry

The high-speed camera in the Attune CytPix Flow Cytometer has a 10x effective magnification capability, enabling the capture of hundreds of 16-bit bright-field images up to 248 x 248 pixels in microseconds for high-throughput pixel data extraction. Machine learning algorithms are used to rapidly identify an object of interest within each of those images and perform image segmentation for classifying each pixel, resulting in a binary image overlay known as a mask [3]. Objects in the image field of view are segmented to classify pixels as part of an object or not (Figure 1A). Masks are used to annotate every pixel in an image and distinguish features within an object. They provide visual confirmation that an image was processed for particle characterization. In the software, a mask appears as an outline around the particle, denoting the edge of the object with the background, while the number of centroids within a mask is proportional to the number of objects in the field of view (Figure 1B). During software development, our subject matter experts manually annotated images to provide ground-truth data to ensure the prediction models provide accurate quantitative measurements using the masks.

Image processing is an operation that can identify specific patterns in an image and transform image data into digital data that capture the measurements about the identified patterns and objects. The digital data are then compiled for each image to generate derived parameters that can then be correlated with the associated FCS file event data. A broad array of image-processing features provide particle or cell morphology measurements that are displayed as parameters for histograms and dot plots, similar to scatter and fluorescence parameters. The light and dark pixels in an image are assigned intensity values and compared to the normalized background intensity and thresholding values to create image-based measurements [4].

The image-processing features enable accurate label-free analysis from images and quantitative image-based measurements to automate size measurements, population-level statistics, population identification, and particle enumeration.

For example, the software can recognize and count the number of beads or cells within an image to inform more accurate singlet gating. Image-derived statistics are generated for any specified population and can be correlated with marker statistics at the population level to further characterize and define cell populations. Users no longer need to make assumptions about cell morphology because the image analysis software can calculate measurements relaying size, shape, and intracellular complexity from individual full-resolution images. The images can also be used to inform and verify gating accuracy for users of any experience level. Automated image analysis eliminates manual annotation of images and reduces user analysis bias when quantifying morphology.

How to use the image-derived parameters

The image processing parameters enable users to verify image processing, set up image data for downstream analysis, and find metrics that resolve objects within populations of interest. Divided into five feature categories—system, object, intensity, pixel, and shape—the parameters provide quality control for image-based analysis and morphology measurements from each event in an image (Table 1). In the image analysis workflow, several parameters can be used to set up an experiment to ensure quality control and accurate analysis after the images are processed. The "IsProcessed" and "IsOnBorder" parameters ensure that all events are captured by the Attune CytPix camera and within the image field of view (Figure 1C). Gates drawn in the "PseudoDiameterMicrons" parameter display statistics based on the diameter of each particle or cell in that population. Similar to conventional flow cytometry gating, the "PseudoDiameterMicrons" enables users to exclude suspected debris, dead cells, or events of dissimilar size at the population level. "ParticleCount" parameter provides an exact readout of the number of particles or cells in each event, removing the potentially erroneous subjective nature of gating out doublets using conventional approaches of doublet discrimination such as side scatter area (SSC-A) and height (Figure 1D).
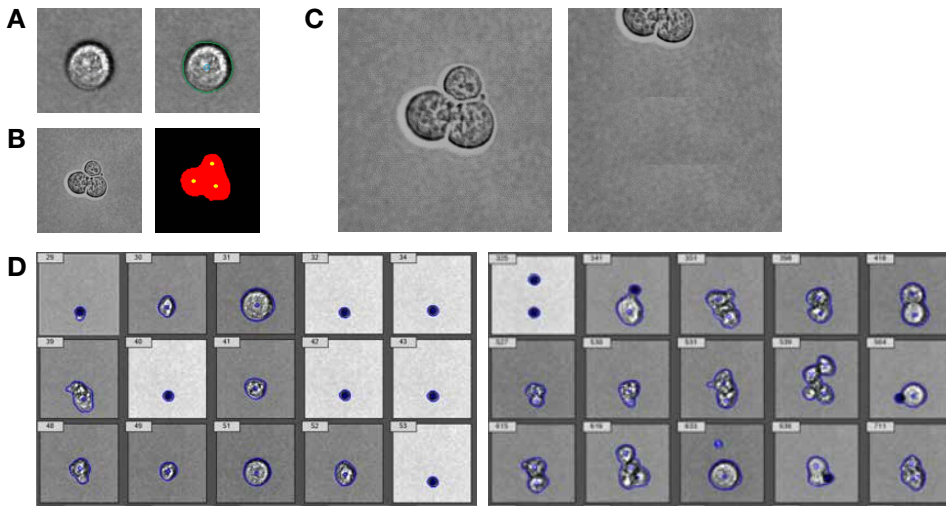
Figure 1. Image-processing elements and parameters. (A) Objects in images analyzed by the software are displayed with masks on the borders and centroids to determine the centers of the circular objects. (B) Centroids in circular objects assist in determining number of particles in an image. (C) Quality control features allow exclusion of objects not in the field of view prior to analysis. Users will acknowledge image analysis for objects located within the borders (left) and exclude images with objects located on the border (right). (D) Image-derived parameters can be used to gate singlet from aggregate and doublet events in images.

Table 1. Examples of image-derived parameters.

Display name	Description	Notes
System features		
IsOnBorder	On border	Indicates that one or more objects intersects with the field of view of the image.
IsProcessed	Processed	Indicates that the image was processed. This is generated when the image processing FCS file is loaded and merged with the raw FCS file data.
Pixel features		
Pixel count	Number pixels	Provides pixel count.
Object features		
ParticleCount	Number of cells / particles within the identified object	Used for gating singlets from aggregates or doublets.
Shape features		
CircularityPercent	Measure of roundness as determined by the ratio of the perimeter to area of the object Circularity percent = 100 / PerimeterToArea	A perfectly circular object = 100. For round cells, values falling below 80 often start to indicate dead or dying cells.
PseudoDiameterMicrons	Diameter of circle with area equal to area of the object $D = 2 * sqrt(Object.Area\_um2/pi)$	This value is calculated by comparing the diameter of the object to the diameter of a circle with the same area. Can be used to calculate the area of the object; 1 pixel = 0.3 µm.
EccentricityPercent	Eccentricity of an ellipse = $100 * sqrt (1-ShortAxisMicrons ^2 / LongAxisMicrons^2)$	Eccentricity of a perfect circle = 0; eccentricity of a parabola = 1; beads have lower eccentricity than cells.
Intensity features		
EntropyIntensity	Entropy of intensity distribution of all pixels within an object	Healthy cells: ~6–7; sick/dead/dying cells: ~8. The highest values tend to indicate shriveled, dead cells. Healthy "ruffled" cells with texture may have higher values. Low entropy events are also not typically cells.
SkewnessIntensity	Skewness of intensity distribution of all pixels within an object	Unhealthy and dead cells tend to be either very high or very low on the range. Textured cells often have higher skewness intensity.
KurtosisIntensity	Peakedness of intensity distribution of all pixels within an object	Healthy cells: ~0. Useful when attempting to differentiate cells attached to beads from just beads or just cells.
CVNormIntensity	100 * StandardDeviationIntensity / AverageIntensity	Indicates increased pixel intensity compared to the background.

To demonstrate how the Attune CytPix Flow Cytometer can be used to reduce gating variability, a group of Attune CytPix Flow Cytometer users were asked to gate cells in an apoptosis experiment based on size and single cells with and without the image-derived parameters. Camptothecin-treated Jurkat cells were dual-stained with Annexin V (AV) and propidium iodide (PI) to positively distinguish apoptotic populations. The Attune CytPix cytometer images enable users to visualize viable, apoptotic, and dead cells, while the addition of image processing automates population identification prior to user gating. The potential of these new capabilities suggest that gating standardization in a multiuser study is possible. Previous multiuser studies have reported that diagrammatical protocols may reduce user analysis variation, so detailed protocols were provided to the focus group to improve standardization of how the analysis was performed [2].

Materials and methods

Log-stage Jurkat cells (T cell lymphoma) were treated with 10 µM camptothecin (MilliporeSigma) for 4 hours. Following this treatment, cells were stained with the Invitrogen™ Dead Cell Apoptosis Kits with Annexin V for Flow Cytometry, with Invitrogen™ Alexa Fluor 488™ Anti-Annexin V (AV) and propidium iodide (PI) (Cat. No. V13241) and Invitrogen™ Hoechst 33342 nucleic acid stain. Cell data were acquired from a 96-well U-bottom plate using the Invitrogen™ Attune™ CytPix Flow Cytometer instrument and the Invitrogen™ CytKick™ Max Autosampler at a sample injection rate of 100 µL/minute. For each sample, 30,000 events were collected in "All Events" mode and 7,000 images were photographed. Processing of all images was completed using the "Cells Full Resolution" mode on Attune Cytometric Software.

Focus group study

First method: Gating without image analysis

1. Nucleated cells/debris exclusion gate

   a. Parent population: Processed

   b. To remove free apoptotic bodies, use either a debris exclusion gate or a nucleated cell gate (using Hoechst stain, in VL1 channel).

2. Single cells gate

   a. Parent population: Nucleated cells (drawn in step 1, above), or processed

   b. This plot is pre-populated; adjust gate as needed or create a new gate (as shown in Figure 2A).

3. AV/PI bivariate plot

   a. Parent population: Singlet gate (drawn in step 2, above)

   b. This plot is already populated; adjust gate position.

Second method: Gating with image analysis

1. Size exclusion gate of cells within an 8–25 µm range

   a. Parent population: Processed

   b. A histogram plot using the "PseudoDiameterMicrons" parameter is present (as shown in Figure 2B). Adjust the gate minimum to 8 µm and the gate maximum to 25 µm. It is recommended to use the customized option by right-clicking on the gate and choosing "customize" to set this numerically instead of "by eye".

2. Single cell gating where "ParticleCount" = 1

   a. Parent population: The 8–25 µm size exclusion gate (adjusted in step 1)

   b. Adjust the pre-populated histogram for this parameter.

3. AV/PI bivariate plot

   a. Parent population: Singlet gate (drawn in step 2)

   b. This plot is already populated; adjust gate position.

Results

A focus group study consisting of eight flow cytometry users with various experience levels was conducted to challenge the learning model's robustness for standardizing gating strategy. Half of the participants were either inexperienced in using the Attune Cytometric Software or non-experienced flow cytometry users.

Participants were provided with two sets of identical files (with each file containing three samples) and written instructions for creating gating hierarchies using conventional flow parameters and image-derived parameters.

To set up the analysis in each workflow, the participants created a hierarchy starting with size exclusion and singlet gating. To ensure that the same data would be used for both analysis methods, the image files for both methods were processed. All events were gated on the "IsOnBorder"- and "IsProcessed"-derived parameters as 0 and 1, respectively. These parameters ensure that only events from processed

images appear in daughter gates. For the first analysis method, the participants used conventional parameters where they could use manually drawn gates to remove apoptotic bodies and refine the gate to include nucleated cells that appear high for SSC-A and Hoechst stain (Figure 2A). After setting the single cells gate and quadrant gating, they could verify their gating accuracy using representative images from the Attune CytPix Flow Cytometer.

For the image analysis–assisted method, the "PseudoDiameterMicrons" parameter was used to exclude apoptotic bodies. With predictions from the learning model, the "PseudoDiameterMicrons" parameter represents a more accurate measurement compared to scatter. A simple gate shown in Figure 2B excludes events smaller than 8 µm and larger than 25 µm. For the "ParticleCount" parameter, participants set a histogram gate around value "1" to denote that only images with single cells would be included in the analysis, resulting in exclusion of events most likely to be debris or apoptotic bodies.
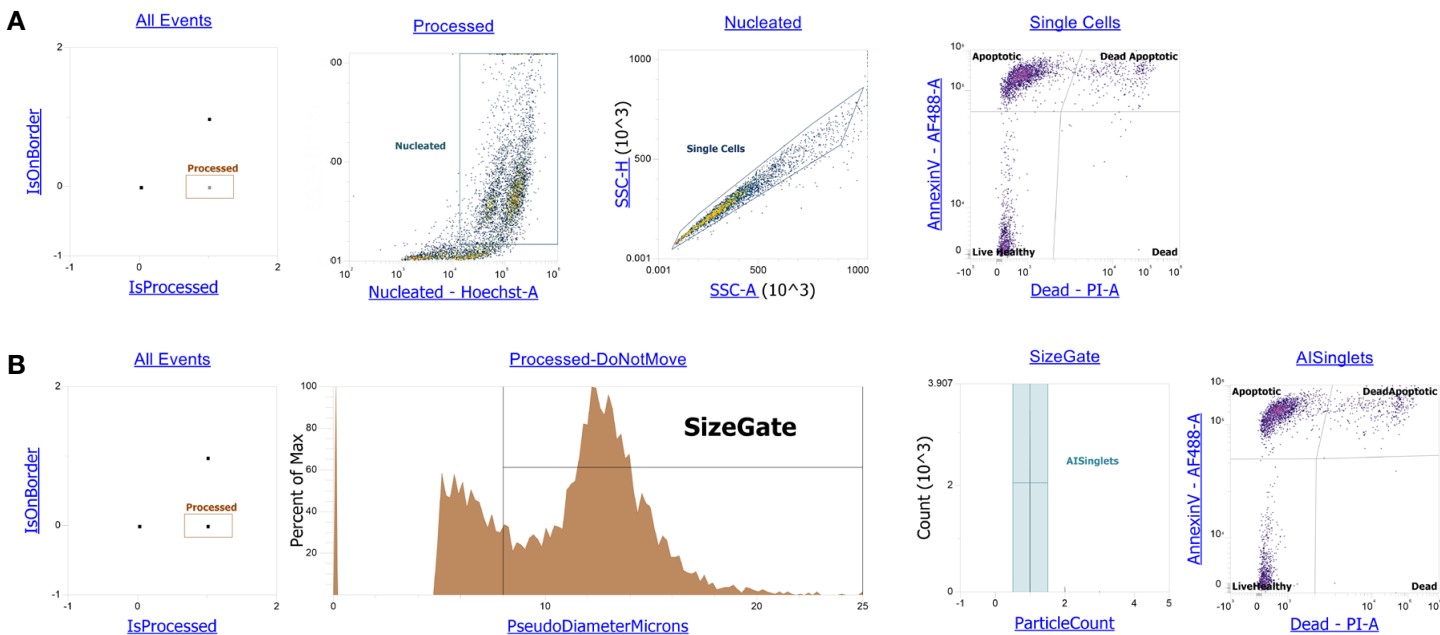

Figure 2. Gating strategy using automated image analysis requires less user subjectivity. Conventional flow cytometry scatter methods were used for excluding small events and doublets/aggregates. **(A)** Camptothecin-induced apoptotic Jurkat cells were stained with Annexin V using the Dead Cell Apoptosis kit and with Hoechst stain. All image events were processed by the software and within the borders. Users excluded low SSC-A events, suspected apobodies and debris, by creating a rectangular nucleated gate for Hoechst dye–stained cells. Suspected doublets were chosen from the nucleated gate with SSC-H/SSC-A bivariate plot to calculate statistics for apoptotic populations. **(B)** All image events were processed by the software and within the borders. Users excluded small events likely to be apobodies and debris using gate customization settings to create a SizeGate for events greater than 8 µm. The users drew a gate around "1" to denote singlets. The AISinglets gate allowed users to calculate statistics for apoptotic cell. For both **(A)** and **(B)** apoptotic cells (Annexin V⁺/PI⁻), dead apoptotic (Annexin V⁺/PI⁺), live healthy (Annexin V⁻/PI⁻) and dead (Annexin V⁻/PI⁺) populations were delineated using Annexin V, Alexa Fluor 488 dye, and propidium iodide staining for dead cells.

The consistency in large diameter gate (Figure 3B) suggests that automated image analysis provides accurate detection of singlet and intact cells compared to scatter and fluorescence properties used to draw the nucleated gate (Figure 3A). Even though the pseudodiameter gate does not specify singlet detection particles that are not contiguous, they are most likely excluded. The fluorescence parameter employing Hoechst stain was not used for the image analysis–assisted method and demonstrates that this method does not require fluorescence, suggesting this channel could be utilized for other stains. Using the image-derived parameters for size exclusion and single cells enables more consistency in gating decisions across users (Figure 4A) and reduces average variation within the experiment

(Figure 4B). The image analysis method reduced the average coefficient of variation (CV) by at least 78% for the populations in upstream gating. The representative images from nucleated and single cell populations acquired using conventional flow cytometry parameters suggest that debris and doublets were gated out (Figure 4C). However, the average variation across users and samples remained higher compared to image-derived parameters for the downstream gating hierarchies (Figure 4D). Unsurprisingly, the results suggest that there was user variation across all populations of interest, even when analyzing identical files. Images for each quadrant population were highly representative of apoptotic, dead, dead apoptotic, and live healthy cells (Figures 4E and 4F).

A Apoptotic bodies

Processed

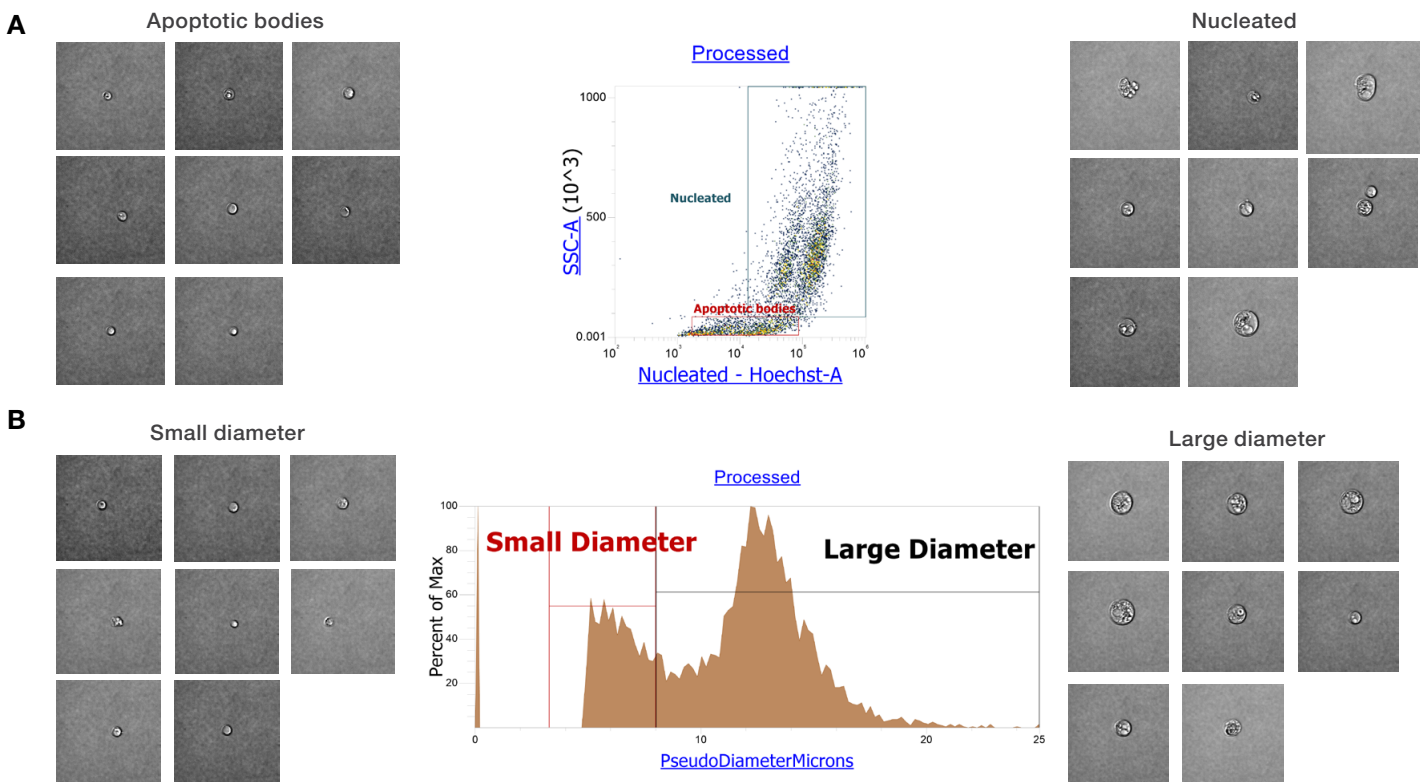Nucleated

B Small diameter

Large diameter

Processed

Figure 3. Automated image feature provides consistent and relevant gating options. (A) Size exclusion gating excludes apparently smaller events as low SSC-A with Hoechst stain events were distributed. The nucleated population represents more diverse events including dead cells, doublets, and debris, as shown in images. (B) Size gate for automated image analysis–assisted gating strategy provides distinction between populations based on diameter. "Small diameter" gate denotes particles consistently smaller than 8 μm; "Large diameter" population is larger than 8 μm and represents a variety of Jurkat cell sizes.
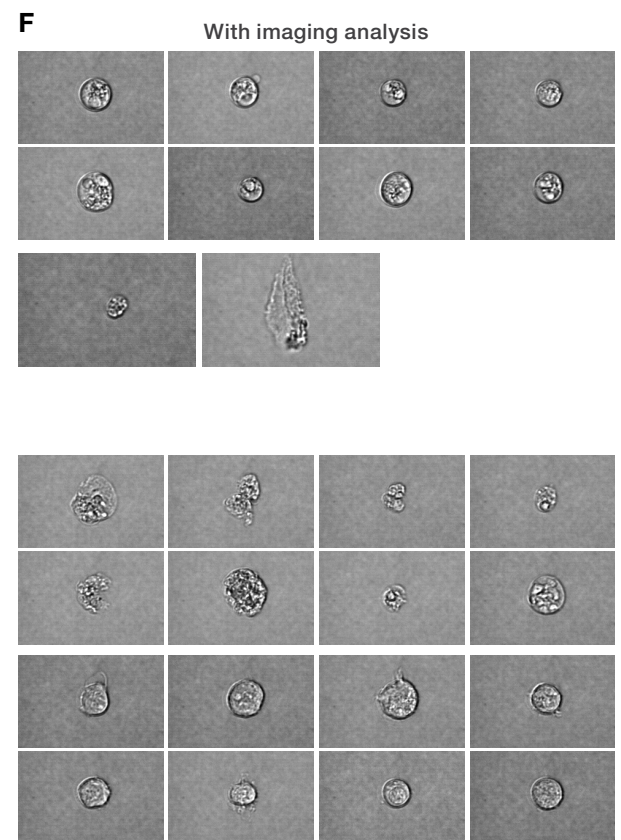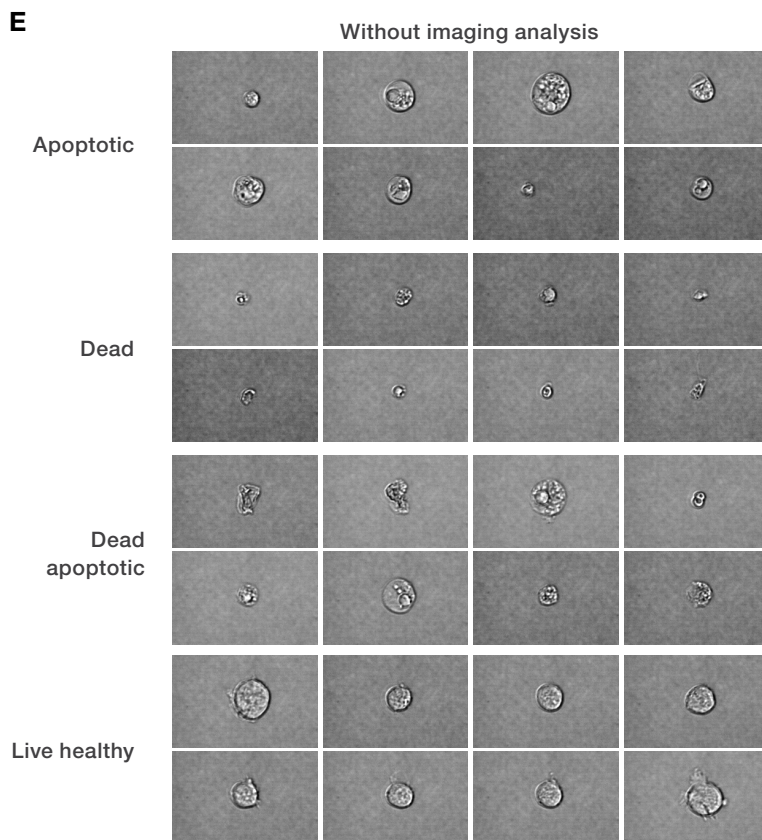
A

	1	2	3	4	5	6	7	8
Nucleated	58.73	62.66	66.00	54.52	57.70	60.19	62.04	57.88
SizeGate – IA	68.02	68.21	67.93	68.21	68.21	65.72	68.21	68.21
Single cells	94.19	81.42	88.55	99.52	90.43	92.60	97.54	99.37
AISinglets – IA	96.68	96.69	96.67	99.57	96.69	94.95	96.69	96.69

Users

B — Average variation in upstream gating

5.92 / 1.28 (Size exclusion); 7.66 / 1.43 (Singlet gating)

Without image analysis / With image analysis

C

	1	2	3	4	5	6	7	8
Apoptotic	62.94	68.89	66.48	66.34	69.58	67.71	66.27	64.84
Apoptotic – IA	64.64	65.43	64.60	66.66	64.55	65.95	64.85	63.79
Dead apoptotic	11.51	9.94	10.90	6.43	9.53	9.93	8.06	8.67
Dead apoptotic – IA	10.72	10.02	10.73	9.10	11.57	10.46	10.52	11.81
Live healthy	25.54	21.08	22.52	27.15	20.82	22.33	25.44	26.32
Live healthy – IA	24.57	24.44	24.59	24.16	23.81	23.51	24.53	24.33

Users

D — Average variation in downstream gating

3.52 / 1.60 (Apoptotic); 17.30 / 7.91 (Dead apoptotic); 11.09 / 2.00 (Live healthy)

Without image analysis / With image analysis

E Without imaging analysis — Apoptotic, Dead, Dead apoptotic, Live healthy
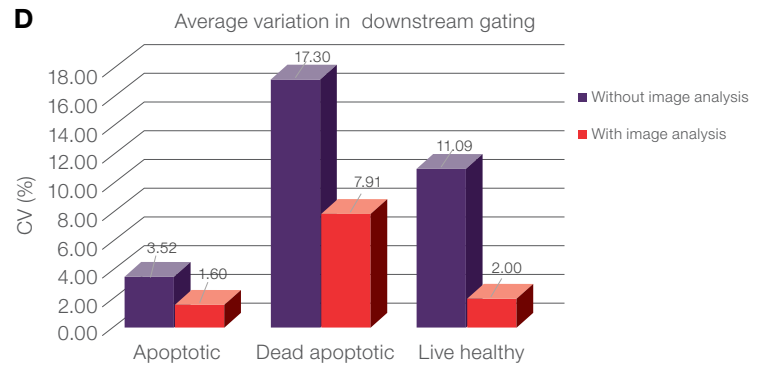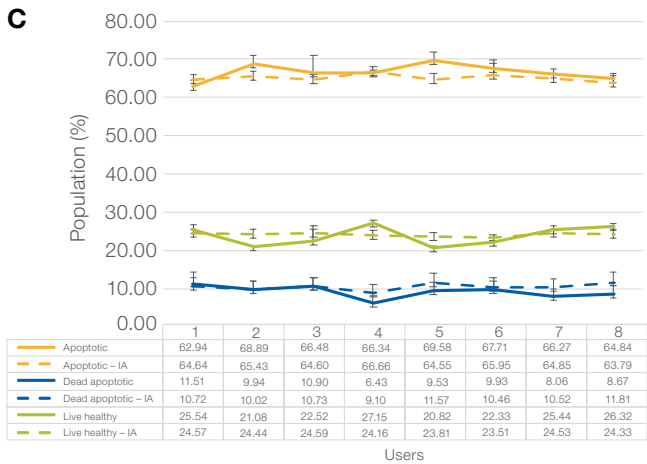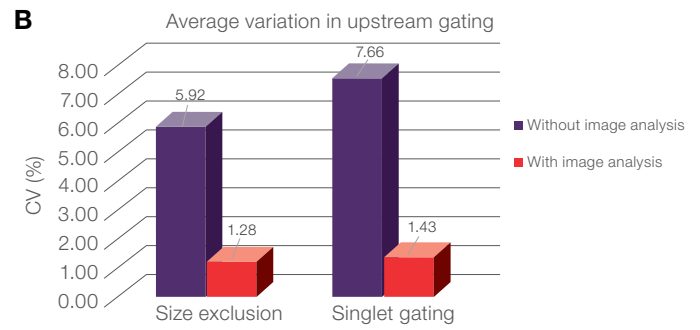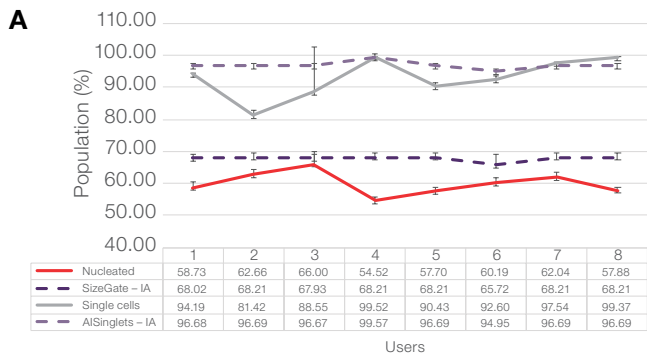
F With imaging analysis

Figure 4. Automated image analysis–assisted gating reduces variability among users or downstream cell population. (A) Automated image analysis (IA) reduces variance for size exclusion and singlet gating across users. Standard error and deviation for populations data was lower using image analysis. **(B)** The average variation (CV) for size exclusion and singlet gating improves using image analysis for gating. **(C)** Automatic image analysis slightly improves consistency for downstream population statistics across users. **(D)** With automated image analysis CV improves for apoptotic, dead apoptotic, and live healthy Jurkat cells. Dead cells were excluded because of low numbers. **(E–F)** Images provide population-level view to visually distinguish populations and characterize cell behavior.

Conclusions

When performed on the Attune CytPix Flow Cytometer, automated image analysis combined with conventional flow cytometry provides a robust quantitative method for standardizing flow cytometry data analysis. Our results demonstrate that automated image-assisted analysis enables more accurate gating of true singlet populations and exclusion of unwanted events. This has potential to modify experimental conclusions. In addition, our results suggest that this analysis method could be used to reduce inter-user and intra-user variability within an experiment and across samples. Users can gain distinct quantitative measurements using the images in a high-throughput manner, acquiring more data faster than is possible with manual annotation. The image analysis method could be used to assess sample quality, optimize assay protocols, and verify rare events. With automated image analysis and flow cytometry, users can visualize populations and perform stain-free population statistics from a single sample. The result is a streamlined workflow that empowers flow cytometry users with unbiased, data-driven cell analysis to reduce user variability and improve accuracy, while delivering confidence in results.

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