Published in final edited form as:

Methods. 2017 January 01; 112: 9–17. doi:10.1016/j.ymeth.2016.07.013.

Masks in Imaging Flow Cytometry

Venina Dominical, Leigh Samsel, and J. Philip McCoy Jr.

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

Abstract

Data analysis in imaging flow cytometry incorporates elements of flow cytometry together with other aspects of morphological analysis of images. A crucial early step in this analysis is the creation of a mask to distinguish the portion of the image upon which further examination of specified features can be performed. Default masks are provided by the manufacturer of the imaging flow cytometer but additional custom masks can be created by the individual user for specific applications. Flawed or inaccurate masks can have a substantial negative impact on the overall analysis of a sample, thus great care must be taken to ensure the accuracy of masks. Here we discuss various types of masks and cite examples of their use. Furthermore we provide our insight for how to approach selecting and assessing the optimal mask for a specific analysis.

Keywords

Imaging flow cytometry; mask; features; data analysis; ImageStream

Introduction

Over the past half a century, flow cytometry has emerged as a valuable technology for the study of cells and other particles. Traditionally, flow cytometry is based on the light excitation of cells labeled with fluorescent stains with collection of the corresponding fluorescent emissions. Using tightly controlled fluidics, cells are analyzed one at a time in a high throughput manner. The data collected are generally the intensities of fluorescence and/or light scattering signals on a per cells basis. Multiple fluorescent stains (20 or more) can be measured simultaneously. While flow cytometry has proven to be a crucial and powerful tool for biologists, the bane of this technology has been the lack of an ability to visualize the spatial distribution of fluorescent staining on each cell. Imaging flow cytometry (IFC) is a powerful technique that dramatically extends the utility of flow cytometric analyses by adding the ability to simultaneously examine aspects of cellular morphology on a cell-by-cell basis. Morphology includes brightfield (BF) images of cells and also patterns of fluorescence staining in all of the channels specified, thus permitting interpretation of the spatial distribution of the fluorescent staining in the context of brightfield images. To date, the ImageStream and FlowSight (EMD Millipore) are the primary commercially available

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

imaging flow cytometers on the market [1]. The addition of imaging to flow cytometry dictates that conventional approaches used to analyze flow cytometry data must be augmented by methods to assess morphological features such as staining patterns within each cell vis a vis the flow cytometric phenotyping data. The measurements obtained from an image are called “features” and they are object-based measurements like size, shape, intensity, circularity, and texture. While it is possible to examine a robust number of features based on morphology using IFC, it is necessary to analyze these features only on a specific cell, or compartment of a cell, that is of specific interest. To accomplish this, ‘masks’ are used to spatially distinguish the area of a cell of interest and exclude other cellular areas of the image that might confound the analysis. Masks are based on the two dimensional image of each cell and often approximate a defined cellular compartment (e.g. entire cell, nucleus, membrane, cytoplasm). Features are then measured on the information contained within the mask of an object. By contrast, gates are based on one or two dimensional plots of scatter or fluorescence signals rather than single cell images. Whereas gates are used to distinguish a cell or populations of cells from other populations, image masks are used to assure that the appropriate portion of each individual cell is being examined during analysis. For example, when the goal is to analyze markers within nuclei, a mask can be set using a nuclear stain to delineate the nucleus from the remaining parts of a cell and thus permit analysis of those signals only within the nuclear mask. Since IFC encompasses elements of both microscopy and traditional flow cytometry, masks can be used in conjunction with gates to analyze samples. Therefore, masks are crucial elements in analyzing IFC data, and faulty or suboptimal masks may have substantial deleterious effects on the overall analysis.

Masks can be created based on BF, side scatter, or fluorescence images, and for a variety of sub-cellular components. In addition, masks can be combined in a Boolean manner, and can be custom created for specific samples or analysis. Naturally, the quality of masks is related to the consistency and quality of cell preparation and staining. Establishing optimal masks for a particular analysis can be straightforward in some studies, but can be difficult in others that require novel, complex approaches to analysis. In this manuscript we will examine nuances associated with designing and using effective, optimal masks. Examples of potentially problematic scenarios for creating masks will be presented along with approaches to mitigate these problems. We will also demonstrate masks created for a variety of applications with the hope of conveying to the reader practical pathways and solutions for complex analyses.

The masks and features described in this manuscript are from IDEAS software (ImageStream Data Exploration and Analysis Software, EMD Millipore, the same vendor of ImageStream and FlowSight Imaging Flow Cytometers). The user guide of the IDEAS software can be found online at the EMD Millipore - Amnis customer portal, by creating an account at <http://www.emdmillipore.com/US/en/life-science-research/cell-analysis/amnis-imaging-flow-cytometers/amnis-customer-portal/MM2b.qB.WXEAAFL47op.zHv.nav> and downloading the manual under the Subject Area section in the portal: Data Analysis for all instruments Step 4.

There are also other software programs available which can be used for IFC data analysis: CellProfiler (<http://cellprofiler.org/> - Broad Institute), and FCSExpress (<https://>

www.denovosoftware.com/ - De Novo Software™). Masking can be performed in CellProfiler, but the number of customizable options is not as robust as in IDEAS, and may be better suited for analyzing data from IDEAS and other high content analyzers which have already had masks and gates applied. Feature values, however, can be generated in both CellProfiler and FCSExpress, though the number of features may be more limited in CellProfiler. FCSExpress is widely used for traditional flow cytometric analysis, and has incorporated the ability to do image analysis. Only recently, however, has FCSExpress been usable for imagery from IDEAS. More information about how to use CellProfiler and FCSExpress for image data analysis can be found at their respective internet pages.

1. Types of mask

Essentially, there are three types of masks: Default masks, Function masks, and Combined Masks. The default masks are generated by IDEAS software when the raw image file is opened and these cannot be changed by the user. The software uses algorithms based on pixel intensity and variation in an object image frame. Default masks may be sufficient to resolve the population of interest in some cases, such as calculating contrast or differences with the background for example. Often, the default mask of the BF is used to identify cells in focus and single cells by plotting the Gradient RMS and the Area BF Aspect Ratio, respectively, and for these purposes the default masks may work very well. When the mean fluorescence intensity of objects is desired, intensity features are often used with the Default mask of the channel of fluorescence.

On the other hand, Function, also called custom, masks are masks created by the user through the Mask Manager using the existing function masks in the analysis software. Currently, there are 19 different types of function masks and each one has a specific purpose. These masks can be adjusted to better fit the region of interest, making them customizable according to the user needs. For example, the Erode mask can be adjusted in pixels of erosion from all edges, while in a Threshold mask the intensity of pixels to be excluded can be chosen, or yet, the area covered by the mask can be selected in Range mask. Some types of function masks are: Dilate, Erode (those can be adjusted in pixels from all edges), Morphology, Intensity, Peak, and Spot masks. An Object function mask is going to be tightened to the object of interest and not mask pixels outside of the main object. A better version of Erode mask, called Adaptive Erode, erodes pixels adapting to the morphology of the object and can be applied for a variety of analysis including shape change. To analyze the bacterial burden in mammalian cells by IFC, Jenner and colleagues [2] made use of the Spot mask to identify the number of bacteria per cell, evaluating bright spots in the referenced channel of the bacteria stain in the cell. Because they were evaluating internalization of two different types of bacteria with distinct staining pattern, they decided to refine the Spot mask, using a Threshold mask with different cut-offs from the background to be able to identify each type. In a different study by Henery and colleagues [3] a Threshold mask was applied to the Default mask of the nuclear imagery in order to mask only the pixels with intensity values in the brightest 30% to evaluate nuclear morphology. A study by Lampe and coworkers [4] of the recognition by NK cells of target cells is a prime example of the use of an Interface mask. Examples of a Morphology mask and of an Erode mask can be seen in publications by McGrath et al., [5] and George et al., [6], respectively.

A new version of IDEAS software (version 6.2) offers a Component mask, which can be used when an input mask contains multiple pieces and it is desired to categorize each piece as a component and rank it based on a feature value. For example, multiple spots masked can be sorted based on size (using area feature) or brightness (using bright detail intensity feature). No publications using this mask were found by the time of this article, but useful applications include: finding the largest or brightest endosome per cell, measuring asymmetric cell division, nuclear ploidy, etc. More information about cited masks and other ones not mentioned here can be found in the IDEAS software manual.

Combined masks use Boolean logic to create a mask combination by subtracting or adding masks. For example, to make a cytoplasmic mask, which could be applied when discrimination of whether a probe is located in the nuclear or cytoplasmic region of a cell is desired, a two-step approach works well. A mask of the intracellular component can be made by eroding in few pixels from the outermost pixels of Brightfield or a surface staining image mask. A nuclear mask, generated by using a nuclear dye, is necessary. Then, by using Boolean logic of the intracellular mask and not a nucleus mask, one would get only the cytosol excluding the nuclear and membrane region (Figure 1). The literature is replete with examples of Boolean logic applied to masks for IFC. For example, Jenner et al., [2] used a combination of an intracellular and spot counting masks to enumerate intracellular bacteria. In their study of radiation damage to lymphocytes, Durdik and coworkers [7] used custom masks which combined Spot, Peak, and Intensity features. Studies by McGrath et al and by Katz and colleagues also show excellent examples of using mask combinations [5,8].

Some masks seem better suited for specific applications. For example, the Morphology mask may best fit the nuclear shape when aiming to mask nuclei. When one wants to measure the expression of protein in the region of contact between cells (synapse) two masks work well for that: Valley or Interface. A Valley mask (Figure 2 A) makes a rectangular region in the dimmer portion between two bright signals in a starting mask, identifying the intersection between two objects. By contrast, the Interface mask (Figure 2 B) identifies pixels in an object where there are points of contact between this object and another object. The mask conforms to the shape of the synapse in the object of interest and requires two input masks: one being the mask of one of the objects (cell of interest), and the second covering the entire conjugate (e.g. BF). In both masks, the width of the masking region can be adjusted. Ahmed et al., [9] used Valley and Interface masks in their study to assess the recruitment of the Lck protein in the immune synapse between a T-cell and an antigen-presenting cell (APC). The authors discussed the use of Valley over an Interface mask to evaluate the synapse between objects since the Valley mask considers expressions from both cells in the conjugate, while the Interface locates only proteins derived from one of the two cells of the conjugate (“object of interest” input mask), as can be observed on Figure 2 A and B. The use of an Adaptive Erode mask (a recently developed mask) is of great use to distinguish variations in cell shape. The functionality of this mask is to erode from all edges according to the morphology of the object. The coefficient of erosion is adjustable and can be used to more accurately distinguish cells based on their shape, as shown below in Figure 3.

Masks can be helpful in determining whether a subcellular particle is inside the cell. One simple approach would be to create an internal mask, and calculate the internalization

feature within that mask. If the particle is well within the inside of the mask, this may work well enough. However, it is often very difficult to visually verify whether a particle is actually internalized, or rather, just on the surface, “in front of” or “behind” the cell. Smirnov et al [10] developed a method to address this issue by incubating an antibody which recognizes the particle, with non-permeabilized cells, resulting in antibody binding only to extracellular particles. Intracellular particles were only single positive, whereas extracellular particles were double positive.

2. Mask validation

To confirm whether a mask (Default, Custom or Combined) is working properly, it is important to visually inspect the mask and make sure the area covered is appropriate before calculating features from the mask and starting further analysis. To verify accuracies of masks, use the software to show a mask (show/hide mask button) and visually inspect numerous images of cells to look for possible flaws of the mask. Flaws could include whether the mask is covering more than the area of interest or missing essential parts of the image (hint: pointing the cursor to the channel name shows the mask which is being displayed in that channel). In many cases, even after this validation is done, it can still be found that the default masks will fail to accurately identify the region of interest due to default masks being intentionally permissive to capture all of the fluorescence for a complete intensity feature.

To illustrate the importance of determining whether a default mask is sufficient for an analysis, or whether a custom mask may improve the analysis, we will examine a scenario for assessing cell shape using default and custom masks, and how the results were affected. van Beers, et al. [11], used custom masking to improve shape ratio feature values and improve the accuracy of classifying red blood cells as sickled or round. Figure 4 highlights how a Brightfield default mask may not be optimal for assessing shape, since this mask fills in the area inside of the “sickle” making it appear and result in shape ratio values characteristic of round cells. Low shape ratio feature values (on a scale of 0 – 1) correspond with elongated images, and high shape ratio features correspond to rounder images. By customizing and removing a small particle from the Brightfield mask (in this case the custom mask was “Range(System80(300))”), the shape ratio feature values of cells which previously contained a small particle in the default mask (Figure 5, panel B, left column) went from a value characteristic of sickled cells (a cut off of below 0.5 according to van Beers, Figure 5, panel A) to values characteristic of round cells (Figure 5, panel B right column and panel C). Had these small particles not been removed from the mask, round cells would have been erroneously classified as falling within the sickled shape ratio value range. Figures 4 and 5 also emphasize the importance of visually inspecting masks to determine whether a default or chosen custom mask is appropriate, as the default Brightfield mask clearly distorts the shape of the masked object. In taking visual inspection of the mask into account along with the differences in shape ratio feature values from the two masks (Panel B left column versus right column), this is an excellent example of how custom masks can affect and improve results.

Intensity based features can be more forgiving as to the accuracy of the mask fit, while size and shape features such as area, shape ratio, or diameter for example, may have more deleterious effects if the mask is not accurately depicting the true shape of the cell.

Mask validation is made in order to determine which mask is best suited for a particular analysis, i.e. fitting more accurately the region of interest. One easy approach to assess the appropriateness of a mask is to create a custom view of the new masks and to perform a visual validation of them. There are two main ways in which masks can be viewed for validation: displayed on a black background so that only the mask is seen (as in Figure 1 B, C, and D), or overlaid on top of the cell image (either BF or fluorescence, as seen in Figure 2). Views can be created to display any combination of these. In order to do this, one must go to Image Properties and create a new view. To create the black background, empty channels of fluorescence (not being used for BF, SSC, or any fluorescent marker in the experiment) can be added to the view as columns according to the number of masks requiring verification (Hint: the same empty channel can be used for each mask to be viewed). For each column in the new view, a different mask can be selected to be displayed, as demonstrated in Figure 6. By viewing potential masks side by side, the most appropriate mask can be more easily visually determined. To display masks overlaid on top of a BF or fluorescence image as demonstrated in Figure 2, create a new view, but instead of selecting empty channels, select the desired image channel, followed by selecting the mask to be displayed in each column. (Hint: the software uses as a default the cyan color to represent the mask, however this color can be changed by going to options, applications defaults, and on the Mask tab this can be modified.) After this new view is generated, it can be displayed in the image gallery. Visually verify the various masks on numerous objects by scrolling down the cursor in the image gallery and observe whether the mask (cyan color) is placed precisely on the region of interest and does not include areas that should not be masked. For example, to check which mask would be good for evaluation of internalization of a probe, the best mask is the one that uniquely represents the internal portion of the cell. It should not include external membrane components or extracellular objects (e.g. debris or objects attached to the cell of interest), and at the same time should not be so tight that it would miss a considerable part of the cytoplasm.

Another example of validating masks, comparing default versus custom masks, and assessing how results were affected involves measuring the amount of “Protein A” in lysosomes. Cells were stained with Lamp1 to identify the lysosomes and an antibody for “Protein A”. Panel A in Figure 7 shows the effects of deriving the Area feature values from the default combined masks. A combined mask was created by using the AND operator to create a new mask of the overlap between the default Lamp1 mask “M12” and the default Protein A mask “M11”, resulting in mask “M11 AND M12”, which can be seen in the far right column of Panel A. The Area feature values were then calculated on the “M11 AND M12” mask, and these values can be seen on the far right mask image of Panel A. Panel B in Figure 7, on the other hand, shows the effects of deriving the Area feature values from the combined custom masks. First, custom masks were made for the Lamp1 and Protein A staining, separately, such that the custom masks only covered pixels of a specified Intensity range to reduce spotty background and mask pixels resulting from true positivity (in this case, the custom masks used each had an intensity range of 100 – 4095 pixels). These masks

can be seen in columns 2 and 4 of panel B, respectively. A combined custom mask was then created by using the AND operator to create a new mask of the overlap between the custom Lamp1 mask “Intensity(M12,Lamp1,100-4095)” and the custom Protein A mask “Intensity(M11,ProteinA,100-4095)” resulting in mask “Intensity(M12,Lamp1,100-4095) AND Intensity(M11,ProteinA,100-4095)”, which can be seen in the far right column of Panel B. The Area feature values were then calculated on the “Intensity(M12,Lamp1,100-4095) AND Intensity(M11,ProteinA,100-4095)” combined mask, and these values can be seen on the far right mask image of Panel B.

Not only does the custom masking result in more accurate feature values for calculating the amount of Protein A in the lysosomes than the default masking, visual inspection of the default and custom masks in Figure 7 demonstrates the importance of verifying a chosen mask. It can be seen that the default masks cover much more than the true positive staining, thus resulting in Area feature values which are greater than that of the custom masks, and that the custom masks much more accurately represent the true staining.

Another approach to validate masks is by creating features for the desired analysis using all the different masks made and then applying the feature finder wizard to validate these. To verify nuclear translocation for example, make similarity features using all the different nuclear masks created and then apply the feature finder wizard using those new features. Verify where the new features fall into the rank and which one gives you the best Rd discrimination score (Fisher’s discriminatory ratio). The de la Calle group [12] demonstrated a good example of how features are ranked. Identifying a good mask is an essential step for getting reliable data.

3. Troubleshooting masks

Different approaches to the same type of analysis can be used, and sometimes, the data resulting from each can be equally good. However, validation of the masks and features is essential for consistency and reliability of your data. For some applications in IFC, the analysis procedure is already established in the literature, translocation of NFkB for example. Using one of the wizard options in IDEAS software to do the analysis greatly facilitates the analysis, but it also relies upon the sample type, conditions, and the final goal to define the need of a customizable analysis. Identifying good and bad masks or which feature is the most appropriate for the analysis is a huge part of the success of the experiment. In this section we will demonstrate how to troubleshoot a mask in some scenarios and give some tips and tricks for getting good data out of your IFC experiments.

In the example of Figure 8, it is shown how a mask can affect the results. The image in letter A highlighted in red (top-left side of Figure 8) shows a mask too permissive that includes a small particle in the same frame with the object of interest. In this case because both cell and particle are considered one object, measurements like shape or Aspect Ratio are distorted, and the data generated are not accurate. Each object in Figure 8 has a different shape and size, and should thus result in varying Area, Aspect Ratio, and Shape Ratio feature values. However, because the mask in panel A is inappropriate, the feature values result in the cell in panel A having the same Aspect Ratio as the object in panel B, the same Area as the cell in

panel C, and the same Shape Ratio as the cell in panel D. Because the mask in A is not the most appropriate, it can be difficult to distinguish cells which were captured sideways in the frame from doublets or even single cells based on shape. To optimize the mask, choose to use a function mask that is tighter to the object of interest to eliminate small particles in the same frame, reducing erroneous results in the analysis. The use of a fluorescent marker to generate a mask might also improve masking the object of interest. It may help to eliminate particles in the same frame from the analysis, as often the particle will not be positive for the fluorescent marker.

To identify spots in a cell, the use of Spot mask might be the best option [13, 14, 15]. This mask extracts bright or dark regions of an object, and can be further refined with Peak and/or Range masks. Peak mask [16] can be used to separate connected spots in a Spot mask, while to select spots within a certain range of pixel values, Range mask is an option. Sometimes, when the fluorescence background of an image is high and appears spotty (especially when the Extended Depth of Field – EDF function is activated during file acquisition), it may result in many more spots masked, causing erroneously high spot counts. To reduce the number of spots being masked due to spotty background, it can be helpful to exclude “false positive spots”. One way to do this is use the Raw Max Pixel feature to exclude those events close to the background and only analyze cells truly positive for the spot-signal to be counted. Jenner and colleagues [2] present excellent examples of good and poor masks used for spot counting in their study of intracellular *Burkholderia thailandensis* infection.

In situations where there is a discrete difference in shape among the cells to be analyzed, it can be tricky to fine tune a mask. In the next example described, it was necessary to discriminate human erythrocytes between two different morphologies: the ones resembling a star, called here as “star-shape”, represented in Figure 9 A, and the ones with the typical shape of erythrocytes (resembling a donut shape) in the Figure 9 B, called here as “round-normal” cells. Cells in focus, debris, and aggregates were excluded from the analysis followed by cells in a side position on the frame were subsequently eliminated. To distinguish between the two shapes cited above, only cells “front-faced” on the image were considered. Because both shapes (normal and star) had the same size and area when front-faced, using a mask of size or morphology was not enough to differentiate them. Adaptive Erode mask in this case was not very efficient to discriminate them as the more the coefficient of erosion of the edges was applied in this mask, the more the mask would fill in the space between the tips of the star-shaped cells, making them round instead. When we tried to create a membrane mask by Boolean logic, the input masks used to create it were not accurate enough to point to the software the differences. Yet, cells in the star-shape seem to have more contrast compared to the background, suggesting that maybe a texture mask would be an option, but still a considerable number of star-shaped cells were showing low contrast, falling into the round-normal cells “low contrast” gate, providing false results. For these cases involving complex brightfield images, using a LevelSet mask can be extremely helpful. LevelSet mask is an extension of the Morphology mask that identifies pixels in non-homogeneous regions into three different levels: dim, middle and bright, masking the respective areas of an object. There is also the combination of all the three masks (combined option) to get a closely fitting mask in lieu of object or morphology (Figure 10 A). Using the LevelSet Bright option (Figure 10 A, red rectangle) which masks the bright areas of an

object, it was possible to discriminate between the two shapes of the cells. The varying levels of “peaks and valleys” of the star-shaped cells resulted in many small bright spots being masked (peaks, Figure 10 A bottom 3 images), while in the normal round cells, only one large spot (similar to the ring of the donut) was masked, appearing as one constant bright spot (Figure 10 A top 3 images). By creating the Spot Area Min feature on this mask, it was possible to discriminate the normal round cells from the star-shaped cells, as the masked spots in the star-shaped cells each have an area of around zero counts while the masked ring shaped spot of the round normal cells have a larger area, as shown on Figure 10 B. We also made a combined mask using the Boolean logic of LevelSet dim mask and not Adaptive Erode mask of 60 coefficient of erosion (the latter to mask the internal region) to create a more accurate membrane mask, and then other features could be further calculated to complement the dot plot graph and better distinguish/identify the cells (Figure 10 C).

4. Summary and conclusions

In contrast to traditional flow cytometry, data generated by IFC and the spatial/morphometric measurements provided requires a meticulous mask and feature selection. Since features are measurements originating from a specified mask within the image, the ability of a feature to resolve one population from another is greatly dependent of the quality of the input mask. As such, mask optimization becomes a crucial step in order to get reliable and reproducible data in IFC.

The ability of IFC to discriminate populations based on size, shape, texture, location of protein, as well as fluorescent and BF information is continually evolving along with new masks and analysis approaches. Therefore, the development of protocols and guidelines about choosing the right mask is of great interest [17] and consequently, the feature to be utilized for a given application. Identification of poorly performing masks can help eliminate false positive results and can define the success of an experiment. When reporting a mask used in a scientific journal publication it is important that the authors provide sufficient details to allow the reader to comprehend and reproduce this step of analysis. For example, mentioning the type of mask, the referenced channel used for creation of it, number of pixels eroded or dilated from, range or threshold values, and/or other specific details in order to give as much information as possible to guide the readers through the analysis made. Filby and Davies [17], suggest to include images for each masking adaption and to include the software version used for the analysis. They also indicate that the use of morphometrically relevant biological controls may help in the design of a mask and point out the importance of utilizing controls such fluorescence minus one (FMO) and isotypes and in obtaining optimal results – just as one would do with conventional flow cytometry analysis. Further, because IFC enables the analyses of large numbers of images, the statistical robustness obtained with conventional flow cytometry is conveyed to the quantitative image analysis, along with less bias. It is very important to note that depending on the sample, cell type, conditions, staining, and some other factors, a mask which worked well for a specific analysis may not be the most suitable for a subsequent analysis, and thus, the importance of validating masks not only for each new analysis, but also for on-going analysis with established masks and features. For example, sample preparation variability and donor to donor variability can affect the quality of the mask chosen. Trying to keep the data and procedures consistent, and

having a good positive control to validate the mask are good practices to keep the work as reproducible and reliable as possible.

There are a number of resources for inexperienced users who may require training or assistance in mask creation and data analysis, including regional imaging flow cytometry user's group meetings hosted by Amnis, and workshops at regional and national conferences such as CYTO. An excellent resource for both inexperienced and more advanced users is the Methods in Molecular Biology protocol book on Imaging Flow Cytometry which has recently been published [18]. Additional information and discussion about data analysis for IFC can be found at the Purdue Flow Cytometry list group and ISAC websites.

In summary, selecting the best mask is a fundamental step when performing IFC assays. The selection process includes both defining what seems to be a good mask for the experiment and validating it in order to achieve optimal results and reproducible data.

Acknowledgments

This work was funded by the Division of Intramural Research, National Heart, Lung, and Blood Institute, NIH.

References

1. Basiji DA, Ortyn WE, Liang L, Venkatachalam V, Morrissey P. Cellular image analysis and imaging by flow cytometry. Clin. Lab. Med. 2007; 27(3):653–670. [PubMed: 17658411]
2. Jenner D, Ducker C, Clark G, Prior J, Rowland CA. Using multispectral imaging flow cytometry to assess an in vitro intracellular Burkholderia thailandensis infection model. Cytometry A. 2016; 89:328–337. [PubMed: 26841315]
3. Henery S, George T, Hall B, Basiji D, Ortyn W, Morrissey P. Quantitative image based apoptotic index measurement using multispectral imaging flow cytometry: a comparison with standard photometric methods. Apoptosis. 2008; 13(8):1054–1063. [PubMed: 18543109]
4. Lampe K, Endale M, Cashman S, Fang H, Mattner J, Hildeman D, Hoebe K. SIp-76 is a critical determinant of NK-cell mediated recognition of missing-self targets. Eur. J. Immunol. 2015; 45(7): 2072–2083. [PubMed: 25929249]
5. McGrath KE, Bushnell TP, Palis J. Multispectral imaging of hematopoietic cells: where flow meets morphology. J. Immunol. Methods. 2008; 336(2):91–97. [PubMed: 18539294]
6. George TC, Fanning SL, Fitzgerald-Bocarsly P, Medeiros RB, Highfill S, Shimizu Y, Hall BE, Frost K, Basiji D, Ortyn WE, Morrissey PJ, Lynch DH. Quantitative measurement of nuclear translocation events using similarity analysis of multispectral cellular images obtained in flow. J. Immunol. Methods. 2006; 311:117–129. [PubMed: 16563425]
7. Durdik M, Kosik P, Gursky J, Vokalova L, Markova E, Belyaev I. Imaging flow cytometry as a sensitive tool to detect low-dose-induced DNA damage by analyzing 53BP1 and γ H2AX foci in human lymphocytes. Cytometry A. 2015; 87:1070–1078. [PubMed: 26243567]
8. Katz JD, Ondr JK, Opoka RJ, Garcia Z, Janssen EM. Cutting edge: merocytic dendritic cells break T cell tolerance to beta cell antigens in nonobese diabetic mouse diabetes. J. Immunol. 2010; 185(4):1999–2003. [PubMed: 20644171]
9. Ahmed F, Friend S, George TC, Barteneva N, Lieberman J. Numbers matter: Quantitative and dynamic analysis of the formation of an immunological synapse using imaging flow cytometry. J. Immunol. Methods. 2009; 347:79–86. [PubMed: 19524586]
10. Smirnov A, Solga MD, Lannigan J, Criss AK. An improved method for differentiating cell-bound from internalized particles by imaging flow cytometry. J Immunol Methods. 2015; 423:60–69. [PubMed: 25967947]
11. van Beers EJ, Samsel L, Mendelsohn L, Saiyed R, Fertrin KY, Brantner CA, Daniels MP, Nichols J, McCoy JP, Kato GJ. Imaging flow cytometry for automated detection of hypoxia-induced

- erythrocyte shape change in sickle cell disease. *Am. J. Hematol.* 2014; 89(6):598–603. [PubMed: 24585634]
12. de la Calle C, Joubert PE, Law HK, Hasan M, Albert ML. Simultaneous assessment of autophagy and apoptosis using multispectral imaging cytometry. *Autophagy.* 2011; 7(9):1045–1051. [PubMed: 21606680]
13. Beaton LA, Ferrarotto C, Kutzner BC, McNamee JP, Bellier PV, Wilkins RC. Analysis of chromosome damage for biodosimetry using imaging flow cytometry. *Mutat. Res.* 2013; 756(1–2): 192–195. [PubMed: 23618924]
14. Franzen CA, Simms PE, Van Huis AF, Foreman KE, Kuo PC, Gupta GN. Characterization of uptake and internalization of exosomes by bladder cancer cells. *Biomed. Res. Int.* 2014;ID: 619829.
15. Souwer Y, Griekspoor A, de Wit J, Martinoli C, Zagato E, Janssen H, Jorritsma T, Bar-Ephraim YE, Rescigno M, Neefjes J, van Ham SM. Selective infection of antigen-specific B lymphocytes by *Salmonella* mediates bacterial survival and systemic spreading of infection. *PLoS One.* 2012; 7(11):e50667. [PubMed: 23209805]
16. Messer JS, Murphy SF, Logsdon MF, Lodolce JP, Grimm WA, Bartulis SJ, Vogel TP, Burn M, Boone DL. The Crohn's disease: associated ATG16L1 variant and *Salmonella* invasion. *BMJ Open.* 2013; 3(6)
17. Filby A, Davies D. Reporting imaging flow cytometry data for publication: Why mask the detail? *Cytometry A.* 2012; 81(8):637–642. [PubMed: 22760920]
18. Barteneva, NS.; Vorobjev, IA. *Imaging Flow Cytometry: Methods and Protocols (Methods in Molecular Biology)*. first. Humana Press; 2016.

Figure 1.

Representative image of a cell and its respective masks. **a)** Brightfield and nuclear image (purple color) of an endothelial cell acquired by Amnis ImageStream in 60× magnification. Scale bar in the bottom left corner; **b)** Cytoplasm mask: respective cytoplasmic mask created of the BF image, eroded in 2 pixels from all edges; **c)** Nuclear mask: respective nuclear morphology mask generated from the nuclear staining; **d)** Cytoplasm and not nucleus mask: cytoplasmic and not nuclear mask generated by subtracting the nuclear mask from the cytoplasm mask. Masks are shown in cyan color.

Figure 2.

Representative images of cell conjugates and their respective masks. **a) i.** Brightfield and nuclear image (purple color) of a conjugate of Antigen Presenting Cell (APC - cell on the top) and T-cell (cell on the bottom part of the conjugate), and **ii.** respective nuclear image showing Valley mask in cyan placed between the two nuclei. The size of Valley mask chosen for this example was 2; **b) i.** Brightfield and orange fluorescence image of a conjugate (cell of interest is indicated by the orange color and arrows in this example), **ii.** Respective BF image and Interface masks **iii.** Fluorescent images of the cell of interest (input mask) with the interface mask displayed in cyan color. The width index used in this example for the interface mask was of 3 pixels.

Figure 3.

Representative BF images of a cell and its respective masks (cyan color) generated based on the BF image. **a)** BF (brightfield image) with no mask; **b)** Object tight - Object Tight mask; **c)** Erode 2 pixels - Erode mask eroded in 2 pixels from all edges; **d)** Adaptive erode - Adaptive Erode mask of BF with 80 erosion coefficient; **(b), (c) and (d)** images with BF as background; and **e)** Same mask applied in **(d)** without BF as background (showing only the mask).

default mask

custom mask

Figure 4.

Representative brightfield images of sickled human erythrocytes with the respective masks overlaid (cyan color). On the top, default masks generated by IDEAS software and on the bottom, examples of custom masks created by the user to increase the accuracy of masking. White arrows point the main differences between masks in the same object.

Figure 5.

Shape ratio feature values using Default Brightfield mask versus the custom Brightfield mask. Shape ratio feature values calculated from each respective mask are shown on the imagery, with the Default mask shown on the left image of each pair, and the custom mask shown in the right image. Panel A shows the shape ratio feature values of sickled cells, panel B shows the feature values of cells containing a small particle in the default mask and the values once the custom mask removed the small particle, and panel C shows the feature values of round cells. Shape ratio feature values are shown in the upper right of each image.

Figure 6. Screen view of Image Gallery Properties of the IDEAS software demonstrating how to make a customized view. On the Views tab, red arrows show how to generate a new view. On the View Definition area (underlined in green) an example can be seen of how to add the columns (image) and respective masks to be displayed (blue arrows indicate these commands) in this customized view.

Figure 7. Area feature values of Protein A in Lysosomes using Default Combined Lamp1:Protein A mask versus the Custom Combined Lamp1:Protein A mask. Panel A shows (from left to right) imagery of Lamp1 lysosomal staining, the Lamp1 channel default mask, Protein A staining, the Protein A channel default mask, and the mask made by combining the Default Lamp1 mask AND Default Protein A mask. Feature values of the area of the combined Default Lamp1 mask AND Default Protein A mask are shown in the upper right of the combined mask image. Panel B shows (from left to right) imagery of Lamp1 lysosomal staining, the Lamp1 channel custom mask, Protein A staining, the Protein A channel custom mask, and the mask made by combining the custom Lamp1 mask AND custom Protein A mask. Feature values of the area of the combined custom Lamp1 mask AND Default Protein A mask are shown in the upper right of the combined mask image.

Figure 8.

Representative BF images and corresponding image masked on the right side represented in cyan, showing the effect of a mask can have in the results. **a)** Red blood cell captured sideways in the frame and a small particle in the same frame being included in the mask (right side in cyan); **b)** Two overlapping red blood cells having aspect ratio of 0.756 similar to the masked object in letter (a); **c)** RBC front-sided that has the same area of 63 obtained as the object in letter (a); **d)** Distorted RBC with the same shape ratio (0.266) of letter (a).

Figure 9.
Representative BF images of human erythrocytes. A) Examples of deformed erythrocytes assuming a star shape; B) Examples of the normal shape of human erythrocytes. Magnification of 60×, acquired by ImageStream.

Figure 10.

Representative images of LevelSet masks. **a)** From left to the right: Brightfield images of human erythrocytes and respective LevelSet masks: dim, middle, bright and combined (combination of all three masks) mask in cyan color. Bright mask highlighted in a red rectangle; **b)** In the center, dot-plot graph of Spot Area Min feature of LevelSet Bright 5 mask of the brightfield on the X axis. Numbers representing the area value of the smallest spot in each object. On the Y axis, the diameter feature of the combined mask represented in letter C. Star-shaped cells fall in the white gate represented on the left, with area of the

smallest spot around zero. Round-normal cells fall into the pink gate on the right and some BF images of objects inside this gate. The red arrows point representative BF images of the respective population; **c)** Demonstrative images of the BF of two cells and respective masks. From the left to the right: A= LevelSet Dim mask (masking only dim areas of the image), B=Adaptive Erode mask 60 (eroding the BF mask using a coefficient of erosion of 60), and MC = mask combined using Boolean logic of A mask and not B mask.