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Masks in imaging flow cytometry

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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.

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1. 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 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.

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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.WXEAAAFL47op.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.

2. 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 (Fig. 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 (Fig. 2a) 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 (Fig. 2b) 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