Quantifying Autophagy: Measuring LC3 Puncta and Autolysosome Formation in Cells Using Multispectral Imaging Flow Cytometry

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Abstract

The use of multispectral imaging flow cytometry has been gaining popularity due to its quantitative power, high throughput capabilities, multiplexing potential and its ability to acquire images of every cell. Autophagy is a process in which dysfunctional organelles and cellular components that accumulate during growth and differentiation are degraded via the lysosome and recycled. During autophagy, cytoplasmic LC3 is processed and recruited to the autophagosomal membranes; the autophagosome then fuses with the lysosome to form the autolysosome. Therefore, cells undergoing autophagy can be identified by visualizing fluorescently labeled LC3 puncta and/or the co-localization of fluorescently labeled LC3 and lysosomal markers. Multispectral imaging flow cytometry is able to collect imagery of large numbers of cells and assess autophagy in an objective, quantitative, and statistically robust manner. This review will examine the four predominant methods that have been used to measure autophagy via multispectral imaging flow cytometry.
Conflict of interest statement: I am employed by EMD Millipore the maker of the Amnis brand ImageStream that was used in this review.

**Keywords:** Autophagy; Multispectral Imaging Flow Cytometry (MIFC); ImageStream; autophagosome; autolysosome; LC3

**Abbreviations:** Multispectral Imaging Flow Cytometry (MIFC); Chloroquine (CQ); Bright Detail Intensity (BDI); Bright Detail Similarity (BDS)

1. Introduction

Macroautophagy, hereafter referred to as autophagy, is a catabolic pathway in which long-lived proteins and organelles that accumulate during growth and differentiation are degraded via the lysosome.[1] Autophagy is also a survival mechanism that reallocates nutrients from unnecessary processes to more vital processes in the cell.[2] Basal levels of autophagy are generally low but can be upregulated by various stimuli including nutrient starvation, physiological stress, pharmacological agents and infections.[3] Autophagy is a dynamic multi-step process that involves the formation of autophagosomes, fusion of the autophagosome with the lysosome to form the autolysosome, and finally the degradation of the contents in the autolysosome.[4]

The key biological marker to identify autophagy in mammalian systems is the microtubule associated protein 1A/1B-light chain 3 (LC3). During autophagy, cytosolic LC3-1 is conjugated to phosphatidylethanolamine to form LC3-II. LC3-II is recruited and incorporated into the autophagosomal membrane.[5] While there is no single "gold standard" to measure autophagy,[4, 6] methods that have traditionally been used to
measure LC3 include Western blot analysis and fluorescence microscopy. In recent years, measurement of LC3 by flow cytometry and multispectral imaging flow cytometry (MIFC) has been gaining popularity due to their quantitative power, high throughput capabilities and multiplexing potential. A recent Methods review article by Warnes demonstrates that traditional flow cytometry can be used to measure various aspects of the autophagic process including the accumulation of LC3 upregulation and corresponding cell cycle distribution. Phadwal et al. used MIFC to multiplex immunophenotyping, measuring LC3 (autophagosome) and LYSO-ID (lysosome) co-localization as well as to measure the levels of γH2AX. Neither of these examples would have been possible using traditional methods. Furthermore, the experiment done by Phadwal et al. could not have been accomplished using traditional flow cytometry because the spatial information obtained using MIFC is required to measure co-localization.

The ImageStream® (EMD Millipore) is a MIFC that combines the speed and statistical power of flow cytometry with the information content of fluorescent microscopy. Unlike traditional flow cytometers that use photomultiplier tubes to collect fluorescence intensities, the ImageStream® uses a charge-coupled device camera to collect multiple high-resolution images of every cell in flow, including brightfield, darkfield (SSC), and up to 10 fluorescent markers. Measuring signal intensity alone by traditional flow cytometry can work well with reporter cell lines to distinguish the control population from the treated population. However, fluorescent dye background and non-specific staining may complicate analysis. MIFC is better able to cope with these staining issues as well as troubleshooting experimental details since images of the cells
can be examined; and therefore puncta can be observed and measured. In addition, an increase in LC3 signal alone does not provide a complete picture of what is happening in the cells and recent publications emphasize the need to examine concurrent formation of the autolysosome.\[4, 9-11\] MIFC is uniquely able to measure this formation by the co-localization of the autophagosomes and lysosomes which is not possible using traditional flow cytometry.

An examination of publications reveals four principal methods using MIFC to assess autophagy have been published: Bright Detail Intensity, Spot Count, Bright Detail Similarity and a combined method incorporating Bright Detail Similarity and Spot Count. This review describes the specific techniques that others have previously reported for thorough evaluation of autophagic events. This review will take a critical look at each method and how it performs on a single sample set. The image analysis techniques highlighted in this review are not a full list of all possible techniques that could be done by MIFC but is a comprehensive review of the most used methods to assess autophagy using MIFC.

2. Materials and Methods
2.1 Cell Culture

Jurkat human acute T cell leukemia cells were grown in RPMI (EMD Millipore) supplemented with 5% fetal bovine serum (Thermo Scientific HyClone), 1X non-essential amino acids (EMD Millipore) and 1X sodium pyruvate (EMD Millipore) at 37°C and 5% CO₂.
2.2 Treatment and Labeling

Jurkat cells in the exponential growth phase were washed with Hank’s Balanced Salt Solution (HBSS) without Ca\(^{++}\) and Mg\(^{++}\) (Invitrogen) and given fresh media or Earle’s Balanced Salt Solution (EBSS; Sigma-Aldrich) for 2 hours at 37\(^{\circ}\)C and 5% CO\(_2\). For the chloroquine (CQ) samples, both the fresh media and EBSS were supplemented with 100µM chloroquine (Sigma-Aldrich) a lysosomal degradation inhibitor. After a 2 hour incubation the cells were washed with HBSS. Cells were fixed in 4% formalin (Polysciences) for 10 minutes. After fixation as well as in between each of the staining steps the samples were washed with wash buffer (PBS (Thermo Scientific HyClone)/0.09% sodium azide (Ricca Chemical Company)/2% FBS (Thermo Scientific HyClone)). Antibody working solutions were in permeabilization buffer (0.1% triton X-100 (EMD Millipore)/2% FBS/0.09% sodium azide/PBS). The samples were first stained with 1:100 mouse anti-LC3 (Medical & Biological Laboratories, M152-3) for 30 minutes and stained with 1:100 Alexa Fluor 647 Donkey anti-mouse (Invitrogen) for 30 minutes. Next, the samples were stained with 1:20 anti-human LAMP1-PE (Biolegend, 328607). The samples were resuspended in 1% formalin (Polysciences). For nuclear staining DAPI (Molecular Probes) was added to the samples at 1µg/mL.

2.3 Image Acquisition

In each experiment 5,000 events for each sample were acquired using a 12 channel Amnis\textsuperscript{\textregistered} brand ImageStream\textsuperscript{\textsuperscript{X}} Mark II (EMD Millipore) imaging flow cytometer equipped with the 405 nm, 488 nm and 642 nm lasers. Samples were acquired at 40x magnification. Single color compensation controls were also acquired. The integrated software INSPIRE\textsuperscript{\textregistered} (EMD Millipore) was used for data collection.
2.4 Image Analysis Using IDEAS®

Image analysis was completed using image-based algorithms in the
ImageStream Data Exploration and Analysis Software (IDEAS® 6.2, EMD Millipore).
The two core concepts needed to understand and analyze data in IDEAS® are Masks
and Features. Masks are a set of pixels that contain the region of interest. The Mask
defines a specific area of an image to use for Feature value calculations. There are
three types of Masks: Default, Combined and Function masks. Default Masks are
created in INSPIRE® (instrument collection software) during acquisition or in IDEAS®
when a file is opened for the first time. Default Masks are quite generous since they
contain all pixels that are detected as different from the background. Combined Masks
are created using Boolean logic to combine or subtract Masks. Function Masks are
created with user input or in analysis Wizards in the IDEAS® software. The many
available Function Masks include: AdaptiveErode, Component, Dilate, Erode, Fill,
Inspire, Intensity, Interface, LevelSet, Morphology, Threshold, Spot, System, Object,
Peak, Range, Skeleton, Valley and Watershed.[12] This review will describe in detail
Spot, Peak and Range masks and how they can be used to evaluate LC3 puncta.

A Feature is a mathematical expression that contains quantitative and positional
information about the image. Features are created in IDEAS® using base feature
algorithms. A Feature is applied to specific locations of an image by using a Mask that
identifies the pixels within the region of interest in the image. There are eight Feature
categories: Size, Location, Shape, Texture, Signal Strength, Comparison, System and
Combined.[12] This review will address Features from the Texture and Comparison
categories.
The IDEAS® analysis was performed as follows. Single color controls were used to calculate a spectral crosstalk matrix that was applied to each of the files for spectral compensations in the detection channels. The resulting compensated data files were analyzed using image-based algorithms available in the IDEAS® statistical analysis software package. Single cells were separated from debris and doublets using a bivariate plot of aspect ratio vs area of the Brightfield image. Next cells in best focus were identified using Gradient RMS of the Brightfield image. This is followed by gating on positive events for DAPI, LAMP1 and LC3. Finally apoptotic cells are gated out and Bright Detail Intensity, Spot Count, and Bright Detail Similarity values were calculated from the positive non-apoptotic cells (Supplemental Figure 1 and 2). A more detailed description of the analysis is given in the following sections.

3. Bright Detail Intensity

The first method described here was originally published in 2007 by Lee et al.[11] At that time, Bright Detail Intensity R7 was called “small-spot total intensity”. In this study MIFC was used to quantify the LC3-GFP autophagosomes in pDCs obtained from LC3-GFP transgenic mice. A few years later and with the updated name Bright Detail Intensity (BDI) de la Calle et al. and Kovaleav et al. found that BDI R3 accurately separates cells with high levels of LC3 puncta.[13, 14] De la Calle et al. screened over 40 texture features before choosing BDI R3 as the feature that best distinguished the cells with low vs high LC3 puncta. De la Calle et al. found that there is considerable heterogeneity of LC3 puncta in size, shape and intensity; and that counting spots accurately might be challenging, especially if the autophagosome/lysosome fusion is inhibited resulting in massive LC3 accumulation.[13] Furthermore, LC3 staining is often
a combination of LC3-II puncta and diffuse cytosolic LC3-I staining, in addition to non-specific binding of primary or secondary antibodies. The diffuse LC3-I staining and non-specific staining can lead to a significant increase in the intensity value. This overall intensity increase is not due to autophagy, BDI removes the diffuse signal and calculates the intensity on only the bright LC3 puncta.

The IDEAS® software has two versions of BDI, BDI R3 and BDI R7. Both features compute the intensity of localized bright spots within the masked area in the image. BDI R3 computes the intensity of bright spots that are 3 pixels in radius or less, while BDI R7 computes the intensity of bright spots in the image that are 7 pixels in radius or less. In each case, the local background surrounding the spots is removed before the intensity computation.[12] Figure 1 shows graphical representation of how the BDI R3 feature is calculated. The image is processed using a top-hat transform to produce a bright detail image, after which the total intensity of the bright detail image is then calculated. BDI increases both with the increase in the intensity of individual LC3 puncta as well as with an increase in the number of puncta in a cell.

To demonstrate BDI. Figure 2A shows histograms of BDI R3 LC3-AF647 for Basal + CQ (blue) and Starved + CQ (red) Jurkat cells. There is a clear shift in BDI from the Basal + CQ (mean BDI=2195) to the Starved + CQ (mean BDI=4055) Jurkat cells. Representative images for Basal + CQ and Starved + CQ Jurkat cells are shown in Figure 2B and 2C, respectively.

BDI is very useful when there is high background staining or a large amount of LC3-I. However, if there is little background, LC3-I, or non-specific staining a measurement of the LC3 puncta intensity may be sufficient. Although Intensity can be
measured with a traditional flow cytometer, verification of LC3 puncta as the source of signal cannot be accomplished without the images provided by MIFC.

4. Spot counting

The second and more widely used method to assess autophagy using MIFC is spot counting of the LC3 puncta or autophagosomes. Spot counting has been used for both LC3 reporter cell lines [15-22] and anti-LC3 antibody labeled cells [10, 23] using MIFC. Spot counting for autophagy can be problematic due to the large range of size and intensity between the spots. There are two methods for creating a Feature to count spots in the IDEAS® software. The Spot Wizard in IDEAS® will create an analysis template, Mask, and Spot Count Feature on ‘truth populations’ identified by the user. Alternatively, one can use the Mask and Feature Managers to manually make Masks and Features. Critical to effective spot counting is finding the right Mask to identify the spots. One method to determine if you have an appropriate Mask is to add the Mask to the image gallery view and see how well the Mask identifies the LC3 spots.

There are three Masks used to create the Spot Count Feature. The Spot Mask obtains bright regions from an image regardless of the intensity differences amongst spots. The bright objects are extracted by eroding the image and leaving only the bright areas. The spot to cell background ratio (the spot pixel value divided by the background in the bright detail image) along with the minimum and maximum radii can be specified by the user or determined by the Spot Wizard. The Peak Mask identifies intensity areas from an image that have local maxima (bright) or minima (dark). Initially, the Peak Mask will identify all peaks in the image. To select peaks which have certain brightness, the spot to cell background ratio is used. This is the ratio between the spot pixel value to the
mean camera background value in the original image. As with other mask settings, this ratio can be selected by the user in the Mask manager or by the Spot Wizard. The Range Mask provides the capability to select components in an image within a selected size and/or aspect ratio by setting a minimum and maximum area and aspect ratio, or is determined in the Spot Wizard.[12] A combination of Spot, Peak and Range may be used as well. Once a Mask is obtained, the user can create a Spot Count Feature to count the spots the Mask identified.

The Spot Wizard in the IDEAS® software can create an algorithm to calculate the number of LC3 spots. In the wizard two ‘truth populations’ need to be created (e.g. populations hand selected by the user). The first, a positive population of cells, which have a high level of autophagosomes (many spots); the second, a negative population of cells, which have low levels of autophagosomes (few spots). The wizard uses Fisher’s discriminant ratio (Rd) to determine the spot count Feature/Mask by finding the best statistical separation (largest Rd) based on these user defined ‘truth populations’.

Fisher’s discriminant ratio (Rd):

\[
Rd = \frac{|\text{Mean}_1 - \text{Mean}_2|}{(\text{Std.Dev.}_1 + \text{Std.Dev.}_2)}
\]

where 1 and 2 refer to the two ‘truth populations’.

When using the spot count wizard, selection of exemplary ‘truth populations’ is critical. The Spot Count Wizard can assist the user in determining which Mask works best with the data. To get a good robust mask it might take a few rounds of the Wizard to get an appropriate feature. This may mean re-evaluating and refining the ‘truth populations.’
To demonstrate how spot count is used to measure autophagy, the same population of cells were used as described for the BDI example. The Peak Mask was determined to be the most appropriate for assessment of autophagy in this data set. For further evaluation of Mask selection, see Supplemental Figure 3. The Peak Mask used was the AF647 channel with the Bright ratio of 4. Therefore, the Spot Count Feature for this data set was Spot Count_Peak(M11, Ch11-LC3-AF647, Bright,4); meaning the Spot Count Feature counted the spots that the Peak Mask identified within the Default channel 11 Mask (M11) on Channel 11 (Ch11-LC3-AF647) with a spot to cell background ratio of 4 (Bright, 4). To demonstrate the applicability of the Spot Count Feature for this data set, Figure 3 shows images of Jurkat cells labeled with anti-LC3-AF647 collected on the ImageStream® MKII. For each cell the spot count is indicated along with brightfield, LC3-AF647 (white), Peak Mask only (cyan), and LC3-AF647 with the Peak Mask applied (white and cyan). The same display settings in IDEAS® are used for each example cell; this shows the large range of image intensity between spots. Figure 4A shows the LC3 Spot Count histograms for the Basal + CQ (blue) and Starved + CQ (red) Jurkat cells using the Spot Wizard in the IDEAS® software. Mean LC3 spot counts for the Basal + CQ and Starved + CQ Jurkat cells were found to be 0.61 and 2.82 spots, respectively. Brightfield (BF), LC3-AF647, DAPI nuclear dye and a composite of LC3-AF647 and DAPI images of representative cells for the mean spot counts are shown, Figure 4B and 4C respectively.

The size/shape/brightness of LC3 puncta can vary drastically between cells. This fact was emphasized by de la Calle et al. and cited as a rationale for choosing the Texture Feature BDI.[13] Furthermore, no Spot Count Feature will be perfect due to
this large variety in size/shape/brightness of LC3 puncta but a good Spot Count Feature will work most of the time and should have the appropriate trend. Background staining and reproducibility of the experiment are other potential pitfalls with Spot Count; this could result in the same Feature for Spot Count producing differential results amongst data sets of varying quality. For large studies in particular, it is important to ensure the Spot Count Feature being used is robust and produces consistent quality amongst multiple data sets.

5. Bright Detail Similarity

Autophagy is a highly dynamic, multi-step process and the mere detection of the number of autophagosomes or measurement of LC3 puncta is insufficient for a comprehensive evaluation of the entire autophagic process. It has been well documented that the formation of the autophagosome as well as an increase in the lysosomal content are hallmarks of autophagy.[6] The term autophagic flux was coined to represent the dynamic process of autophagy and refers to the entire process of autophagy; including autophagosome formation, maturation, fusion with the lysosome, subsequent breakdown and the release of macromolecules into the cytosol.[4] MIFC can enhance measurement of autophagic flux by imaging and quantifying autophagosome/lysosome fusion in thousands of cells per population. Furthermore, an increase in autophagosomes does not necessarily mean an increase in autophagy as it could also represent a blockade in the process. LC3-II turnover is a useful parameter for measuring autophagic flux and can be achieved by analyzing cells in the presence and absence of a lysosomal degradation inhibitor like CQ.[4] which inhibits the fusion of the autophagosomes with the lysosomes. CQ permits quantitation of the
autophagosome formation step as a measure of the degree of autophagy by arresting autophagic flux before lysosomal degradation can occur.[8]

Over the last 5 years there have been several studies assessing autophagic flux by examination of the co-localization of autophagosomes and lysosomes using MIFC.[9, 10, 22-27] These papers used the Bright Detail Similarity R3 (BDS) Feature in the IDEAS® software. One of the first reports of using BDS was by Phadwal et al. Phadwal et al. found that counting LC3 puncta was not a reliable measurement of autophagy in primary cells and that measuring comprehensive autophagic flux was a better, more reliable measurement to study autophagy in primary cells. This study measured the co-localization of LC3 and lysosomes to quantify the delivery of LC3 into the lysosome in T and B cells from human PBMCs. By using this feature, Phadwal et al. found that BDS avoids measuring artifactual signals generated from individual markers.[9]

The BDS Feature is designed specifically to compare the small bright image detail of two images and can be used to quantify the co-localization of two probes. In this case, BDS measures the co-localization of fluorescently labeled autophagosome and lysosome markers. BDS is the log transformed Pearson’s correlation coefficient of the localized bright spots with a radius of 3 pixels or less within the masked area in the two input images. In other words, BDS calculates the degree of overlapping pixels from two different fluorescent channels. Since the bright spots in the two images are either correlated (in the same spatial location) or uncorrelated (in different spatial locations), the correlation coefficient varies between 0 (uncorrelated) and 1 (perfect correlation). The coefficient is log transformed to increase the dynamic range to between zero and infinity (0, inf). For BDS to be accurate, it is essential to gate on cells that are bright for
both fluorescent markers of interest.[9] Gating on positive events is needed to prevent
measuring BDS on non-specific binding, imaging artifacts or noise. This is a crucial
step since BDS can potentially amplify these artifacts that are not true signals. Since all
the cells need to have bright signal for both LAMP1-PE and LC3-AF647 the gate
thresholds are different than what was used for the BDI and Spot Count examples,
Supplemental Figure 2.

To demonstrate BDS and how it can be used to evaluate autophagic flux, co-
localization of LC3-AF647 (autophagosomal marker) and Lamp1-PE (lysosomal marker)
was measured. Figure 5A shows histograms of Basal + CQ (blue) and Starved + CQ
(red) Jurkat cells. BDS score increases directly with co-localization of LC3-AF647 and
Lamp1-PE. The mean BDS score for the Basal + CQ Jurkat cells was 1.47 and Starved
+ CQ Jurkat cells was 1.77. Representative images of cells with mean BDS scores are
shown for both the Basal + CQ and Starved + CQ Jurkat cells in Figure 5B and 5C,
respectively. There is a shift from the Basal + CQ to the Starved + CQ sample;
however, it is not as dramatic of a shift compared to other methods show in this review.
The images from the means of both the Basal + CQ and Starved + CQ samples show a
greater difference than the histograms may lead you to believe; highlighting the fact that
BDS does not take into account the number of autophagosomes and lysosomes that
are co-localizing.

6. Bright Detail Similarity and Spot Count

The final method reviewed is the combined use of BDS-LC3/LAMP1 and LC3
Spot Count. There are several reports of using both BDS and Spot Count in the same
study.[10, 11, 22, 23] However, there is only one publication that added dimension by
using a bivariate plot of Spot Count and BDS to assess autophagic flux. Rajan et al. found that the measurement of BDS alone is not always sufficient and that using only BDS could lead to false positive or false negative results. Although BDS evaluates autophagosome and lysosome co-localization it does not take into account the number of autophagy organelles. To account for the number of autophagosomes, Rajan et al. included spot counting of the LC3 puncta. Rajan et al. found that when autophagosome degradation is inhibited there is an accumulation of autophagy organelles; and in cells with low BDS scores they were not able to distinguish between cells with and without accumulation of autophagosomes. Furthermore, CQ increased the number of autophagosomes, but had little impact on the number of lysosomes. Therefore Rajan et al. combined BDS of the LC3 (autophagosome marker) and LAMP1 (lysosomal marker) with LC3 spot counting. Using a bivariate scatter plot of LC3 Spot Count vs BDS two populations were first identified, one with high LC3 spots and one with low LC3 spots. The high LC3 spot population was further classified into those with low co-localization (accumulation of autophagosomes) and those with high co-localization (accumulation of autolysosomes). By separating the bivariate into three populations, Rajan et al. was able to distinguish between cells with very few autophagosomes versus cells with an accumulation of autophagosomes or autolysosomes.

To demonstrate the benefit of combining BDS-LC3/LAMP1 and LC3 Spot Count to assess autophagic flux, Jurkat cells in basal conditions or amino acid starved conditions treated with and without CQ were used (Figure 6A) and evaluated using the bivariate analysis method laid out by Rajan et al. As previously stated, BDS is performed on only LC3-AF647 and LAMP1-PE bright positive cells; therefore, the
populations used for the bivariate plot are also performed only on the LC3-AF647 and LAMP1-PE bright positive cells. As a result, the LC3 Spot Count mean values are different than what is shown in the LC3 Spot Count only example (section 4 of this review) since cells that did not have bright signal were removed from the population. It should also be noted that cells might be bright enough for positive staining with BDS but might not reach the threshold set by the Peak Mask to be considered a spot.

The Basal Control sample used to set the gating strategy for the three population demonstrated 97% of cells with 1 or fewer spots using the feature of Spot Count_Peak(M11, Ch11-LC3-AF647, Bright, 4); therefore, the Low Spots population was set to cells with 1 or fewer spots. The boundary between the High Spots, Low BDS and High Spots, High BDS was set at a BDS score of 2 because the 98% of the basal control cells had a BDS score less than 2. As expected, the Basal Control sample had fewer cells in the bivariate plot and the Starved + CQ treated sample had the most cells in the bivariate plot due to the accumulation of the autophagosomes and autolysosomes. As shown in Figure 6A, LC3 Spot Count vs BDS-LC3/LAMP1 bivariate plots are shown for Basal Control, Basal + CQ, Starved and Starved + CQ conditions. Sample images from the three regions of the Starved + CQ sample are shown in Figure 6B. Table 1 summarizes the bivariate results for the different experimental conditions. This experiment led to a similar conclusion as that derived by Rajan et al. Under basal conditions the number of autophagosomes was low and few cells were found with high spots, while the addition of CQ increased the number of LC3 puncta. Since the lysosome is unable to break down the autophagosome, this leads to an increase in the co-localization of the autophagosomes and lysosomes. This effect is amplified under
nutrient starvation which induces autophagy; however, without the addition of CQ there is not a significant increase in the number of autophagosomes, likely due to an increase in the rate of autophagic turnover. When the cells are starved in the presence of CQ there is an increase in the co-localization and number of autophagosomes, supporting the conclusion that starvation increases autophagic flux.

7. Discussion and Conclusions

In this review 4 methods were presented to measure autophagy using MIFC. However, the question still remains what method does the best job to represent autophagy and does MIFC add additional information that is not obtained by traditional flow cytometry (i.e. measuring Intensity of LC3-AF647 only). To address this Table 2 has the mean and standard deviation values of Intensity-LC3, BDI-LC3, LC3 Spot Count and BDS-LC3/LAMP1 for the Basal Control, Basal + CQ, Starved and Starved + CQ samples. The histograms for Intensity, BDI and Spot Count of LC3-AF647 for each of the samples are in Supplemental Figure 4. One way to compare how well a Feature measures autophagy would be to use Fisher’s discriminant ratio (Rd). As previously stated, Fisher’s discriminant ratio (Rd) measures the separation between two sample populations; the larger the RD, the larger the separation between the two sample populations. There are 4 combinations to investigate in the experiment presented: Basal Control vs Basal + CQ; Basal Control vs Starved; Starved vs Starved + CQ; and Basal + CQ vs Starved + CQ. Table 3 summarizes the Rd values for the different sample combinations for the Features. Analyzing the table there is not a clear winner; for example, Intensity-LC3 of Basal + CQ vs Starved + CQ has the highest Rd, but if you look at Basal Control vs Basal + CQ BDI-LC3 has the highest Rd. BDS-
LC3/LAMP1 constantly had one of the lower Rd values but BDS gives co-localization data that cannot be obtained by traditional flow cytometry, it is performed on only bright positive events; therefore, removing cells that are negative for either LC3 or Lamp1. These negative cells are not removed from BDI, Spot Count or Intensity. In addition, BDS does not take into account the number of autophagosomes and lysosomes that are co-localizing. These two factors are reflected in the Rd values for BDS-LC3/LAMP1. While there are differences between the sample populations BDS alone does not do a good job measuring the breadth of the range of LC3 signal as well as other methods for this experimental data set.

Rd also penalizes sample/Features that have a large range of values. Fisher’s discriminant ratio is the differences of the means divided by the sums of the standard deviation for two samples or populations; therefore, the larger the standard deviation the lower the Rd. It might be that a particular Feature/method does a better job separating out the range of LC3 signal in the sample better than another Feature/method but because of the higher standard deviation it has a lower Rd. I believe this to be the case when looking at BDI-LC3 compared to Intensity-LC3; the Basal + CQ vs Starved + CQ samples have significantly different mean values for both Intensity and BDI but BDI has more variation in it values resulting in a lower Rd. From analysis of the cell images BDI does a more accurate job showing the range of LC3-AF647 puncta compared to Intensity. Upon further analysis of the LC3-AF647 cell images many cells with a high Intensity value do not have bright puncta so the BDI score is low. A low BDI score is appropriate in this case as there is not an accumulation of LC3-AF647 puncta. Example
cell images, Intensity and BDI values along with a bivariate plot of Intensity-LC3 vs BDI-LC3 from the Starved + CQ sample are shown in Supplemental Figure 5.

Autophagy is a dynamic process that can have a large range of LC3 puncta. BDS alone did not do a good job demonstrating the variation in the number of autophagy organelles in a sample; however, I believe the bivariate plot of LC3 Spot Count vs BDS-LC3/LAMP1 does a superior job measuring autophagy and autophagic flux. The co-localization of the autophagosome and lysosome to form the autolysosome is an important step in the autophagic process and the measuring of LC3 puncta alone is not always sufficient to quantify autophagy. In addition, LC3 Spot Count vs BDS-LC3/LAMP1 is the only method presented in this review that can distinguish between autophagosomes and autolysosomes accumulation. However, it needs to be noted that Spot Count vs BDS will be excluding cells that are not positive for LC3 and/or LAMP1; at basal levels this can be a significant portion of the sample.

For the model system shown in this review I believe LC3 Spot Count vs BDS-LC3/LAMP1 is the most appropriate method to quantify autophagy/autophagic flux. However, there is not one single correct method that will work for all systems. Each researcher needs to examine their data and the question they are trying to answer to determine which method is most appropriate for their data set. For simple LC3 puncta accumulation purposes, BDI or Spot Count should suffice. BDS will enhance comprehensive assessment of autophagic flux by measuring the autophagosome/lysosome fusion but if there is a large difference in the number of autophagy organelles it is likely that BDS alone might not be sufficient and LC3 Spot
Count vs BDS-LC3/LAMP1 might be required to measure the full range of the autophagy organelles in the sample.

Autophagy is a complex process that has been implicated in a broad spectrum of mammalian diseases including cancer, neurodegenerative disorders and inflammation. There is an increasing need to accurately detect/measure autophagy because it is critical to improve the understanding of this fundamental cellular process and its link to disease. Furthermore, MIFC has the potential for use in pharmaceutical development due to its capacity for high throughput screening of drug targets that modulate autophagy.

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**Figure 1:** Graphical representation of how the BDI R3 feature is calculated. (A) the original image (B) bright detail image that is used to calculate the BDI R3.

**Figure 2:** (A) Histograms of BDI-LC3 for Basal + CQ (blue) and Starved + CQ (red) Jurkat cells labeled with LC3-AF647. The mean BDI for the Basal +CQ and Starved + CQ cells were 2195 and 4055 respectively. (B) Representative images from the mean BDI for Basal + CQ and (C) Starved + CQ cells are shown.

**Figure 3:** LC3-AF647 labeled Jurkat cells analyzed using the Spot Count Feature: Spot Count_Peak(M11, Ch11-LC3-AF647, Bright,4). For each cell shown the Spot Count is indicated along with brightfield, LC3-AF647(white), Peak Mask only (cyan), and LC3-AF647 with the Peak Mask applied (white and cyan).

**Figure 4:** Using the same Spot Count Feature as Figure 3; (A) LC3-AF647 Spot Count histograms for the Basal + CQ (blue) and Starved + CQ (red) Jurkat cells. The mean spot counts for the Basal + CQ and Starved + C Q Jurkat cells were found to be 0.61 and 2.82 spots, respectively. Brightfield (BF), LC3-AF647, DAPI nuclear dye and a composite of LC3-AF647 and DAPI images of representative cells for the mean Spot Counts are shown for Basal + C Q (B) and Starved + C Q (C).

**Figure 5:** (A) BDS-LC3/LAMP1 histograms of Basal + C Q (blue) and Starved + C Q (red) treated Jurkat cells. The mean BDS score for the Basal + C Q Jurkat cells was 1.47 and Starved + C Q Jurkat cells was 1.77. Representative images of cells with a mean BDS scores are shown for both the (B) Basal + C Q and (C) Starved + C Q Jurkat cells.

**Figure 6:** (A) Bivariate plots of LC3 Spot Count vs BDS-LC3/LAMP1 for Basal Control, Starved, Basal + C Q and Starved + C Q Jurkat cells. (B) Brightfield, LAMP1, LC3 and a composite of LAMP1 and LC3 example images from the three regions: Low Spots; High Spots, Low BDS and High Spots, High BDS are shown for the Starved + C Q sample.
Table 1: Summary of Jurkat LC3 Spot Count vs BDS bivariate plot experiment.

|                | count | BDS mean | LC3+ Spot Count Mean | % Low Spot | % High Spot Low BDS | % High Spots High BDS |
|----------------|-------|----------|----------------------|------------|---------------------|----------------------|
| Basal Control  | 442   | 1.27     | 0.09                 | 97.7       | 2.3                 | 0.0                  |
| Basal + CQ     | 1449  | 1.47     | 1.16                 | 68.3       | 27.3                | 4.4                  |
| Starved        | 1208  | 1.46     | 0.09                 | 98.6       | 0.7                 | 0.7                  |
| Starved + CQ   | 1801  | 1.77     | 3.05                 | 30.4       | 45.7                | 23.9                 |

Table 2: Intensity-LC3, BDI-LC3, LC3 Spot count, and BDS-LC3 /LAMP1 Mean and Standard Deviation values for Jurkat cells in basal conditions or amino acid starved conditions treated with and without CQ. Note: BDS requires cells to be brightly positive for LC3-AF647 and LAMP1-PE; therefore, there is a different cell count/population compared to Intensity, BDI and Spot Count.

|                | Count | Intensity Mean | Intensity Std. Dev. | BDI Mean | BDI Std. Dev. | LC3 Spot Count Mean | LC3 Spot Count Std. Dev. | LC3+ Lamp1+ Count (BDS) | BDS Mean | BDS Std. Dev. |
|----------------|-------|----------------|---------------------|----------|---------------|---------------------|--------------------------|--------------------------|----------|---------------|
| Basal Control  | 3312  | 7173           | 2966                | 1395     | 498           | 0.01                | 0.19                     | 442                      | 1.27     | 0.31          |
| Basal + CQ     | 3331  | 10136          | 4704                | 2195     | 1144          | 0.61                | 1.06                     | 1449                     | 1.47     | 0.44          |
| Starved        | 2239  | 10965          | 4025                | 1568     | 554           | 0.05                | 0.35                     | 1208                     | 1.46     | 0.35          |
| Starved + CQ   | 1963  | 20449          | 8462                | 4055     | 2048          | 2.82                | 2.45                     | 1801                     | 1.77     | 0.53          |

Table 3: Rd values for Intensity-LC3, BDI-LC3, LC3 Spot count, and BDS-LC3 /LAMP1 for the 4 different sample combinations Basal Control vs Basal + CQ; Basal Control vs Starved; Starved vs Starved + CQ; and Basal + CQ vs Starved + CQ.

|                | Intensity | BDI | LC3 Spot Count | BDS |
|----------------|-----------|-----|----------------|-----|
| Basal Control vs Basal + CQ | 0.39 | 0.49 | 0.48 | 0.26 |
| Basal vs Starved | 0.54 | 0.16 | 0.08 | 0.28 |
| Starved vs Starved + CQ | 0.76 | 0.96 | 0.99 | 0.36 |
| Basal + CQ vs Starved + CQ | 0.78 | 0.58 | 0.63 | 0.32 |
Supplemental Figure 1: Gating strategy for Intensity-LC3, Bright Detail Intensity (BDI) LC3 and LC3 Spot Count. Single cells were separated from debris and doublets using a bivariate plot of aspect ratio vs area of the Brightfield image. Next cells in best focus were identified using Gradient RMS of the Brightfield image. Followed by gating on positive events for DAPI, LAMP1 and LC3. This is followed by gating on non-apoptotic cells “Cells” population in Area_Threshold(DAPI, 50%)_DAPI vs Contrast_M01_BF plot.

Supplemental Figure 2: Gating strategy for Bright Detail Similarity (BDS) LC3/LAMP1 and bivariate plot BDS-LC3/LAMP1 and LC3 Spot Count. Single cells were separated from debris and doublets using a bivariate plot of aspect ratio vs area of the Brightfield image. Next cells in best focus were identified using Gradient RMS of the Brightfield image. Followed by gating on positive events for DAPI, LAMP1 and LC3. This is followed by gating on non-apoptotic cells “Cells” population in Area_Threshold(DAPI, 50%)_DAPI vs Contrast_M01_BF plot. Note: BDS requires cells to be brightly positive for LC3-AF647 and LAMP1-PE; therefore, the positive gate for LAMP1 and LC3 starts at 10,000 (not 1,000 as in Supplemental Figure 1).

Supplemental Figure 3: Jurkat Cell images and Masks used to create the Spot Count Feature. Shown are Brightfield; LC3-AF647; Peak(M11, Ch11-LC3-AF647, Bright,2); Peak(M11, Ch11-LC3-AF647, Bright,4); Peak(M11, Ch11-LC3-AF647, Bright,5); Spot(M11, Ch11-LC3-AF647, Bright,6,2,1); and Spot(M11, Ch11-LC3-AF647, Bright,5,3,1). An appropriate Range Mask for this data set was not obtained. The Mask that worked the best for all cells shown was Peak(M11, Ch11-LC3-AF647, Bright,4). This is not a full list of Mask that were tried on the data set but rather a sampling of Masks.

Supplemental Figure 4: Histograms of Intensity-LC3, BDI-LC3, and LC3 Spot count for the Basal Control, Basal + CQ, Starved and Starved + CQ samples.

Supplemental Figure 5: Intensity-LC3 vs BDI-LC3 bivariate plot with example cells tagged. Tagged cells from the bivariate plot are shown in BF and LC3-AF647 images along with Intensity-LC3 and BDI-LC3 values. This figure shows that similar Intensity values can show a wide range of BDI values.
Figure 1
### Figure 3

| Spot Count | BF | LC3 | Mask | LC3/Mask |
|------------|----|-----|------|----------|
| 1          | ![BF Image] | ![LC3 Image] | ![Mask Image] | ![LC3/Mask Image] |
| 2          | ![BF Image] | ![LC3 Image] | ![Mask Image] | ![LC3/Mask Image] |
| 4          | ![BF Image] | ![LC3 Image] | ![Mask Image] | ![LC3/Mask Image] |
| 5          | ![BF Image] | ![LC3 Image] | ![Mask Image] | ![LC3/Mask Image] |
| 6          | ![BF Image] | ![LC3 Image] | ![Mask Image] | ![LC3/Mask Image] |
| 12         | ![BF Image] | ![LC3 Image] | ![Mask Image] | ![LC3/Mask Image] |
Figure 4

(A) Basal + CQ

(B) Basal + CQ

(C) Starved + CQ
Quantifying Autophagy: Measuring LC3 Puncta and Autolysosome Formation in Cells Using Multispectral Imaging Flow Cytometry

Highlights:

- A detailed review examining the four predominant methods that have been previously reported to measure autophagy via multispectral imaging flow cytometry. The four methods examined in this review are: Bright Detail Intensity, Spot Count, Bright Detail Similarity and a combined method incorporating Bright Detail Similarity and Spot Count.
- Bright Detail Intensity and Spot Count work well for measuring LC3 puncta accumulation.
- Bright Detail Similarity can measure the formation of the autolysosome (LC3 and LAMP1 co-localization) but does not take into account the number of autophagy organelles; therefore, might not be sufficient to measure autophagy.
- A bivariate plot of LC3 Spot Count vs Bright Detail Similarity of LC3 and LAMP1 provides the most comprehensive information on autophagy, since it measures both the number of autophagy organelles as well as the formation of the autolysosome.
- There is not a single correct method for measuring autophagy using multispectral imaging flow cytometry; rather, each researcher needs to examine their data and the question they are trying to answer to determine which method is most appropriate for their data set.