Quantitative analysis of protein co-localization on B cells opsonized with rituximab and complement using the ImageStream multispectral imaging flow cytometer

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Abstract

Binding of the chimeric, humanized anti-CD20 mAb Rituximab (RTX) to B lymphocytes activates complement and promotes covalent deposition of C3 fragments (C3b/iC3b) on cells. Previous fluorescence microscopy studies, based on examination of B cell lines and of blood samples from RTX-treated CLL patients, suggest that C3b/iC3b is closely associated with cell-bound RTX. We examined Raji cells opsonized with serum and RTX with the ImageStream imaging flow cytometer. Cells were stained with fluorescently-labeled RTX and mAbs specific for C3b/iC3b fragments or for human IgG, and then imaged using the ImageStream cytometer and analyzed with an algorithm (Similarity Bright Detail Score, SBDS) which tests for co-localization of fluorescent probes. SBDS, calculated on 10,000 cells, verified that the majority of deposited C3b/iC3b is co-localized with bound RTX. In contrast, when cells were first opsonized in serum alone, washed and then reacted with RTX, SBDS confirmed that RTX and C3b/iC3b are poorly co-localized, thus demonstrating that cell-bound RTX directs deposition of C3b. In addition, a sulfhydryl-specific probe, maleimide conjugated to AF488, exhibited substantial co-localization with an anti-C3b/iC3b mAb on Raji cells opsonized with RTX and serum, thus validating maleimide labeling as an alternative for detecting cell-bound C3b/iC3b. The digital imaging method described should have wide applicability for quantitative analysis of co-localization.

Keywords: Multispectral imaging; Rituximab; Complement

1. Introduction

RTX is a chimeric human/mouse anti-CD20 mAb approved by the FDA for the treatment of non-Hodgkin’s lymphoma and rheumatoid arthritis (McLaughlin et al., 1998; Maloney, 1999; Edwards et al., 2004; Cohen et al., 2005). In vitro investigations and analysis of blood samples from RTX-treated patients have demonstrated that binding of RTX to CD20+ cells in the presence of complement results in activation of the...
complement cascade, leading to covalent deposition of fragments of complement component C3 on the cells (Walport, 2001; Kennedy et al., 2003). Fluorescence microscopy studies suggest that the deposited C3 fragments (C3b, iC3b) are very closely associated with cell-bound RTX (Kennedy et al., 2003, 2004).

In order to study the C3 fragment-RTX interaction in greater detail, Raji cells opsonized with fluorescently-labeled RTX in normal human serum (NHS) as a source of complement were probed with fluorescently-labeled mAbs specific for RTX and C3b/iC3b, and then analyzed by digital imaging with the ImageStream imaging flow cytometer (George et al., 2004). The ImageStream is similar to a standard flow cytometer in that thousands of cells per sample that have been stained in suspension can be hydrodynamically focused within a flow cuvette and trans-illuminated along the collection axis by a bright field light source and from the side by a 488-nm laser. However, in the ImageStream, fluorescence, dark field scatter, and transmitted light are collected by an objective lens and relayed to a spectral decomposition element, which divides the image into six spectral bands located side-by-side across a charge-coupled device (CCD) detector with a resolution of 0.25 μm² per pixel. High sensitivity is achieved by operating the CCD in time delay integration (TDI) mode. TDI imaging is a method of electronically panning the detector to track object motion. TDI operation results in signal collection times in the range of milliseconds, orders of magnitude longer than conventional flow cytometry. The ImageStream has a fluorescence sensitivity comparable to that obtained using a conventional flow cytometer (Ortyn et al., 2006). Moreover, because thousands of cells are analyzed in suspension, uncertainties and artifacts associated with fluorescence microscopy are avoided, and quantitative, statistically significant results can be obtained.

In the present study, images acquired with the ImageStream technology have been analyzed by a novel algorithm, the similarity bright detail score (SBDS), which is designed to quantitate the degree of co-localization of two cell surface proteins. The results of this study strengthen and extend our earlier findings (Kennedy et al., 2003, 2004) of co-localization of RTX and C3 fragments and demonstrate the quantitative power of imaging large numbers of cells in flow.

2. Materials and methods

2.1. Cell culture and reagents

Raji cells (American Type Culture Collection, Manasass, VA) were maintained as described (Kennedy et al., 2003). RTX was purchased at the University of Virginia hospital pharmacy. MAb 7C12, specific for C3b/iC3b, and mAb HB43, specific for the Fc region of human IgG, have been described (Tosic et al., 1989; Kennedy et al., 2003, 2004). RTX was labeled with Alexafluor (AF) 488 (Molecular Probes, Eugene, OR) and mAbs HB43 and 7C12 were labeled with AF647 or R-phycoerythrin (PE, Prozyme, San Leandro, CA) according to the manufacturer’s instructions. PE and biotinylated anti-CD45 were obtained from Caltag (Burlingame, CA). AF488 maleimide and AF546 streptavidin were obtained from Molecular Probes.

2.2. Opsonization, maleimide labeling, and probing

Raji cells (5 × 10⁷ cells/ml) were opsonized with 10 μg/ml AF488 RTX or unlabeled RTX for 15 min at 37 °C in either media, 25% normal human serum (NHS), NHS + 10 mM EDTA, or in heat inactivated (HI) NHS (prepared by heating NHS at 56 °C for 30 min). The cells were washed three times with PBS, and then probed, in the presence of 2 mg/ml mouse IgG, with either 10 μg/ml PE anti-CD45, PE mAb 7C12, AF647 mAb 7C12 or PE mAb HB43 for 30 min at room temperature. Cells were washed twice more with PBS and fixed by resuspension in 1% paraformaldehyde. In some experiments, cells were labeled with AF488 maleimide after the opsonization and wash steps, before probing. Labeling was performed using 10 μg/ml AF488 maleimide (prepared by dissolving 1 mg AF488 maleimide in 1 ml dimethylformamide, then diluting 1/10 into PBS, and then diluting 1/10 into the labeling reaction) for 15 min on ice. Cells were then washed three times with PBS and probes as described above.

2.3. Image acquisition and data compensation

Images were acquired on the ImageStream® imaging cytometer (Amnis Corp., Seattle, WA). At least 10,000 images were collected for each sample. Debris was eliminated from the data set by setting a minimum object area in channel 1 (laser scatter channel) of 150 pixels, and approximately 50 mw of laser power was used to excite the fluorophores. The CCD camera was set to 32 stages for scatter and 512 stages for the remaining 5 channels and run at a clock rate of 44 KHz. Cells were collected with the laser scatter image in channel 1, the AF488 image in channel 3 (green), the PE image in channel 4 (orange), and the bright field image in channel 5 (white). In order to create data files for use in spectral compensation, images of unstained cells and
of cells stained with a single antibody/fluorophore combination were acquired without brightfield illumination. Post-acquisition spectral compensation and data analysis was performed using IDEAS® image analysis software package (Amnis Corp.).

The compensation process utilized single color fluorophore control image files. The amount of spectral crosstalk into adjacent channels was determined by plotting intensity for the specific signal channel and the crosstalk channel and then a best-fit line was used to determine the amount of spectral overlap from each fluorescent marker into each channel. The values in the compensation matrix were then applied to each pixel in the image to create a data set that has each signal sequestered into its intended spectral channel. This process is performed transparently by the IDEAS software package. The compensated imagery was validated by verifying that the median fluorescent intensity of the unlabeled cells in the crosstalk channel was similar to the median fluorescent intensity of the single color positive control in the same crosstalk channel.

2.4. Fluorescence microscopy, flow cytometry, and confocal microscopy

Aliquots of samples were also analyzed by fluorescence microscopy, flow cytometry and scanning confocal microscopy. Fluorescence microscopy was performed under oil at high magnification (100×) using a BX40 fluorescent microscope (Olympus, Melville, NY). Images were captured with a digital camera and visualized with Magnafire analysis software. Flow cytometry was performed using a FACScalibur cytometer (BD Biosciences, San Jose, CA) and CellQuest software (BD Biosciences). Mean fluorescence intensities were converted to molecules of equivalent soluble fluorochrome (MESF) using standard fluorescent beads (Spherotech, Libertyville, IL). Confocal microscopy analysis was performed using a Zeiss LSM 5 PASCAL laser scanning confocal microscope.

3. Results

3.1. Flow cytometry and fluorescence microscopy analyses of co-localization

We have previously reported that in NHS, binding of RTX to CD20 on B cells activates the complement cascade, resulting in covalent deposition of C3b/iC3b on the cell surface, and we have used fluorescence microscopy to demonstrate an apparent high degree of co-localization of cell-bound RTX and the deposited C3 fragments (Kennedy et al., 2003, 2004). We have now investigated this co-localization in more detail with a novel quantitative methodology for assessing the co-localization of molecules, in this case, RTX, specific for CD20, and the deposited C3b/iC3b. This method, digital imaging in a flow environment with the ImageStream, captures high resolution images much like a fluorescence microscope or confocal microscope, but, as in flow cytometry, thousands of cells can be analyzed very rapidly post-acquisition, thus providing for high statistical significance in the resulting data.

We first opsonized Raji cells with RTX (unlabeled or AF488-labeled) under conditions which either lead to C3 fragment deposition (NHS) or which preclude such deposition (media, NHS+EDTA, heat-inactivated NHS). Covalent deposition of C3b introduces a free sulfhydryl group onto the cell surface (Shohet et al., 1991, 1993) which should react with maleimide. We then compared the ability of AF488 maleimide and AF647 mAb 7C12 to detect deposited C3b/iC3b. After opsonization, the cells were labeled with AF488 maleimide and subsequently probed with AF647 mAb 7C12, specific for C3b/iC3b. We have previously demonstrated that mAb 7C12 is a reliable marker for C3 fragment deposition (Tosic et al., 1989). The results in Fig. 1 demonstrate that cells opsonized with RTX+NHS have fluorescent intensities (AF647 mAb 7C12 and AF488 maleimide) many times greater than cells opsonized under conditions that do not allow for complement activation and subsequent C3b deposition. This finding validates AF488 maleimide as a reliable probe for C3b deposition.

We next performed co-localization analysis on Raji cells using fluorescent microscopy and scanning confocal microscopy, in order to provide a basis for evaluating the capabilities of the ImageStream. We opsonized Raji cells with unlabeled RTX+NHS, then reacted the cells with AF488 maleimide (to identify deposited C3b/iC3b) and probed with PE mAb 7C12 (or with PE anti-CD45 as a negative control). Alternatively, we opsonized with AF488 RTX+NHS and probed with PE mAb 7C12. Fluorescent micrographs in Fig. 2A, B show that AF488 maleimide co-localizes with PE mAb 7C12, but does not co-localize with PE anti-CD45. This result again validates the use of AF488 maleimide as an alternative reagent to identify cell-bound C3b/iC3b. Fig. 2C, D show scanning confocal microscopy Z sections of cells opsonized with AF488 RTX+NHS and then probed with AF546 mAb 7C12 or biotinylated anti-CD45/AF546 streptavidin. There is a high degree of co-localization of RTX with the anti-C3b/iC3b mAb but much less for RTX with anti-CD45.
3.2. Image analysis and similarity bright detail quantification

Overall, the data shown in Fig. 2 provide strong evidence for co-localization of RTX with C3b/iC3b and of AF488 maleimide with mAb 7C12, specific for C3b/iC3b. However, the data are only qualitative in nature, are subject to individual interpretation, and are therefore limited in these respects. In fact, the cell in the upper left hand corner of Fig. 2D does appear to have some co-localization, thus illustrating the uncertainties associated with use of fluorescence microscopy alone (confocal or conventional) for analysis. The ImageStream technology offers the potential to overcome these limitations by providing quantitative analysis of the degree of co-localization of two fluorophores on a pixel-by-pixel basis. The ImageStream captures digital images of each cell in 6 channels, which include channels for bright-field, AF488 (green), and PE (orange) used in this analysis. The images in the AF488 and PE channels are then compared using the IDEAS software package which utilizes an algorithm developed to calculate the degree of co-localization, the similarity bright detail score (SBDS).

Fig. 3A is a flowchart showing the major analytical stages involved in evaluating the degree of signal co-localization between the two fluorophores: 1) Determination of bright field area and aspect ratio of the cells; 2) Plotting the intensities of fluorophore 1 vs. fluorophore 2; and 3) Calculation of the SBDS. In the initial stage of the analysis, spectral compensation is performed on the data files, and single cells are then identified using a bright field area vs. aspect ratio.

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**Fig. 2.** C3b/iC3b deposited on Raji cells opsonized with RTX in NHS can be detected with AF488 maleimide or with mAb 7C12. A, B. AF488 maleimide co-localizes with PE mAb 7C12, specific for C3b/iC3b, but not with PE anti-CD45 (fluorescence microscopy). C, D. AF488 RTX co-localizes with AF546 mAb 7C12 but not with CD45, as revealed by probing with biotinylated anti-CD45/AF546 streptavidin (confocal fluorescence microscopy).
bivariate plot (Fig. 3B). Single Raji cells are then easily identified because their shape tends to be round, yielding aspect ratios approaching unity. Cells within the single cell region gate are displayed on a logarithmic plot of AF488 intensity vs. PE intensity (Fig. 3C), and double positive cells are identified and analyzed using the similarity bright detail analysis algorithm described below. Similarity bright detail quantifies the degree of similarity between any two channel images on a pixel-by-pixel and cell-by-cell basis. The SBDS is calculated in a three-step process: 1) determination of the opening residue image for each channel image; 2) calculation of a non-mean normalized Pearson’s correlation coefficient; 3) log transformation of the correlation coefficient to give roughly Gaussian distributions, the mean of which is the SBDS. Poorly co-localized events will have SBDS of 1–2 (see below) and well co-localized events will have SBDS values of 3 or greater.

In most cases the vast majority of cells had intensities that fit well within the dynamic range of the instrument. However, in order to insure that evaluation of saturated pixels did not lead to artifacts, peak intensity features were set to exclude cells during analysis if individual pixels had intensities of 1024 or greater, which correspond to saturation at 10 bit resolution.

This process of analysis is illustrated in detail in Fig. 4, which uses sample data obtained during a typical co-localization analysis experiment. In order to enhance bright detail an image processing step (opening residue) is performed on the imagery first. An opening residue consists of three image processing steps (Fig. 4A). The first step is an erosion, the second is a dilation, and the third, a subtraction. The erosion step replaces each grey scale pixel value in an image by the lowest grey scale intensity value from among its neighboring pixels, which are defined as a region around the pixel of interest having a width of seven 0.5 μm pixels. The effect of the erosion is to completely erase any bright features in the image which are narrower than 7 pixels in width, but to leave intact any bright feature with a width greater than 7 pixels. These wider bright features are restored in the dilation step, which replaces the grey scale levels from the encoded image with the brightest value from the same neighborhood of the eroded image. An erosion followed by the complementary dilation is referred to as an opening (Serra, 1982). The opened image is then subtracted from the original image to generate the opening residue, which is an image containing only the bright detail features narrower than seven 0.5 μm pixels. This opening residue image is created for the two channels to be compared, in this instance, channel 3 (e.g. AF488, green) and channel 4 (e.g. PE, orange). The effects of these image processing steps are shown in an example of cell imagery (Fig. 4A). A scatter plot of the actual pixel intensity values of the two images found in Fig. 4A is shown in Fig. 4B. After the opening residue has been applied, the correlation coefficient, r, is calculated using Eq. (1) which is a non-mean normalized version of the Pearson’s correlation coefficient:

$$r = \frac{\sum X \times Y}{\sqrt{\sum X^2 \times \sum Y^2}}$$

where X and Y are the opening residue pixel intensity values of images in the two channels of interest (in this
case, channel 3 is AF488 imagery, and channel 4 is PE imagery) and the sums are taken over pixels for which either $X$ or $Y$ (or both) is greater than a threshold chosen to exclude background noise. The Pearson’s correlation coefficient, $r$, is subjected to a logarithmic transformation to increase the dynamic range of the function. The log transformation to derive the SBDS values is given in Eq. (2):

\[
\text{SBDS} = \ln[(1 + r)/(1-r)]
\]

(2)

The SBDS is calculated for each of \( \sim 10,000 \) cells per data file and averaged, thus allowing for a rigorous and statistically significant analysis of each cell population. Fig. 4C shows examples of SBDS distributions for cell populations with low, high or intermediate similarity.

3.3. SBDS calibration

As a first step in applying the SBDS algorithm to experimental data, we explored the potential range of values for the SBDS in different data sets. To assess the lower limit, Raji cells were opsonized with AF488 RTX, and the SBDS for the AF488 channel image (channel 3) and the laser scatter channel image (channel 1) was calculated. Here the AF488 signal results from the cell surface staining and the scatter image arises from cytoplasmic and nuclear texture and granularity. This comparison gave a SBDS of 1.39±0.08 (\( n=3 \) experiments) (Fig. 5A) and is considered an approximate lower limit defined by the algorithm. To determine an upper limit, images that were identical between the AF488 channel and the PE channel were generated. For this purpose, uncompensated single color control files were evaluated. With no spectral compensation applied to cells labeled with only AF488 RTX, the image in the PE channel (channel 4) would be identical to the AF488 channel (channel 3) because it results from only the AF488 spillover into the PE channel. This gave a SBDS of 4.90±0.45 (\( n=3 \) experiments) (Fig. 5A) and is considered the upper limit attainable. It should be noted that the SBDS values are natural logarithms, and thus the difference between SBDS values of 1 and 5 represents greater than a 50-fold difference between poorly co-localized samples and highly co-localized samples.

3.4. Application of SBDS to RTX and C3b co-localization

We examined Raji cells probed with AF488 RTX and PE anti-CD45 as an example of two signals expected to have nonspecific overlap (Fig. 5B). These images allow for a qualitative inspection of the relative degree of co-localization of the AF488 and PE fluorophores and suggest that the two probes are not co-localized in this sample. Indeed, use of the SBDS algorithm in three separate experiments (>10,000 cells per experiment) yielded an SBDS of 2.07±0.06, consistent with nonspecific overlap. The SBDS values presented in Fig. 5 represent the means of replicate samples from 3–7 individual experiments, and thus the standard deviations account for day-to-day variability in experimental results. For example, Raji cells incubated with NHS alone and, after a wash, probed with AF488 RTX and PE mAb 7C12 gave an SBDS of 2.07±0.13 (Fig. 5C). This result is consistent with our previous observation that opsonization of cells with NHS prior to reaction with RTX leads to very little co-localization of RTX and C3b/iC3b (Kennedy et al., 2003). In contrast, opsonization of Raji cells with AF488 RTX in NHS, followed by probing with PE mAb 7C12, yielded a SBDS value of 3.17±0.09 (\( n=7 \) experiments), representing a high degree of co-localization of the two fluorophores (Fig. 5D), consistent with deposition of C3b on or very near to cell-bound RTX.

In a positive control for co-localization, we found that opsonization of Raji cells with AF488 RTX in media, followed by probing with PE mAb HB43, specific for the Fc region of human IgG, yielded a SBDS value of 3.62±0.31 which was the highest SBDS among the samples analyzed (Fig. 5E). This finding is not unexpected, since mAb HB43 binds to RTX and thus close juxtaposition is to be expected for the AF488 and PE fluorophores in this sample. Opsonization of Raji cells with AF488 RTX in NHS instead of in media, followed by probing with PE mAb HB43, yielded a slightly lower SBDS value of 3.36±0.38, \( n=6 \) experiments (not shown). This slight decrease may be explained based on the ability of mAb HB43 to detect the small amounts of serum IgG which bind nonspecifically to the Raji cells during opsonization.

Finally, we performed the SBDS analysis on Raji cells which were opsonized with NHS and unlabeled RTX, reacted with AF488 maleimide, and then probed with PE mAb 7C12 to determine the ability of maleimide to act as an alternative, independent means of visualizing C3b/iC3b on the cell surface. Raji cells treated in this manner yielded a SBDS value of 3.28±0.20, showing strong co-localization of the two fluorophores (Fig. 5F). By comparison, a SBDS value of 2.05±0.16 (\( n=6 \)) was obtained for Raji cells
opsonized with unlabeled RTX in NHS, reacted with AF488 maleimide, and then probed with PE anti-CD45 (not shown). These quantitative analyses of the degree of co-localization, which were qualitatively examined in Fig. 2A, establish that fluorescently-labeled maleimide can be used as a covalent marker to localize C3b/iC3b.
on the surface of opsonized cells. This chemical labeling could be useful if suitable mAbs specific for C3b are unavailable (e.g. species specific), or in situations where covalent labeling of deposited C3b/iC3b is desired, and also could be used to reinforce results obtained with anti-C3b mAb probes.

**Fig. 5.** Representative images from samples opsonized as indicated and then analyzed on the ImageStream. SBDS values given below the images are mean ± SD (n) for values obtained on ’n’ different replicate samples (at least 10,000 cells/sample) prepared and analyzed over a period of 18 months. Opsonization and probing details are described in Materials and methods.

**Fig. 4.** A. Open residue and the resulting correlation between two images. The raw AF488 and PE images of a cell with low similarity (left panels) and a cell with high similarity (right panels) are displayed on either side of a schematic of a small bright feature with dim background intensity in the first row. The second row shows the erosion (1) and the subsequent dilation (2) that is carried out in the image processing using a 3 pixel structuring element for demonstration purposes. The black pixels in the schematic represent the remainder of the image after the erosion and dilation is carried out with the grey pixels representing the original image. The third row (3) shows the resulting open residue image that is used in calculating the correlation of the AF488 and PE images. Note that the dim background on the inside of the cell has been removed to prevent it from creating an artificially high degree of similarity. The fourth row (4) shows the digital data of the open residue image that is used to create the correlation scatter plots that are displayed in B. B. The scatter plot of a cell with low similarity shows no correlation and the scatter plot of a cell with high similarity shows a high degree of correlation. C. The histogram presents the theoretical distribution of SBDS for populations of cells with low, high or intermediate similarity.
4. Discussion

A method is described for quantitation of protein co-localization in cells using the ImageStream imaging flow cytometer. This method takes advantage of the fact that in the six optical channels of the CCD camera in the ImageStream, the pixels are in spatial registry which allows pixel intensities to be compared between channels with the confidence that they represent the identical area of the cellular image. Here we utilized this feature to investigate the co-localization of proteins on cells. The experimental system chosen was the established system of complement fixation by RTX bound to CD20 on the cell surface of Raji cells. Imagery was generated via conventional means (fluorescence and confocal microscopy, Fig. 2) that indicated a co-localization phenomenon, but that methodology suffers from low throughput and difficulty in quantifying trends in large populations of cells. With the ImageStream imaging cytometer, images of 10,000 cells can be acquired in a few minutes which overcomes the traditional limitations of manual microscopy. The results shown in Fig. 5 demonstrate the utility of the ImageStream technology in concert with SBDS for quantitative analysis of co-localizing signals in a complex biological system.

In order to analyze the cellular imagery acquired by the ImageStream, an image processing step (Figs. 3, 4) was incorporated in order to enhance the small bright detail of the cells which is characteristic of a plasma membrane co-localization event. Preliminary work without incorporating this image processing step resulted in insignificant differences due to the staining background of the cell (not shown). The opening residue step and subsequent subtraction that was developed utilizes an orthogonal 7-pixel structuring element. This results in the elimination of bright staining of an area greater than 7 pixels (3.5 μm) and also eliminates dim background staining. The pixel intensities for the two channels for each cell image are then correlated using the non-mean normalized Pearson’s correlation coefficient (Eq. (1)). The calculated correlation coefficient is then log transformed (Eq. (2)) to provide the SBDS for each cell. These values can be averaged and statistical analysis performed between experimental groups to provide a quantitative statistical assessment of differences between groups. The use of this analytical methodology in the experimental groups described herein results in a distribution of resultant values that correlate well with the expected co-localization. In fact, this methodology allows the determination that maleimide specifically co-localizes to C3b covalently attached to RTX, and justifies the use of maleimide as a unique chemical marker for C3b in this system (Figs. 1, 2, and 3). Also of interest is the observation that the SBDS can reflect situations where experimental conditions decrease specific co-localization. For instance, AF488 RTX labeled Raji cells incubated with or without NHS and then probed with PE anti-huIgG mAb yield SBDS of 3.36±0.38 and 3.62±0.31, respectively. This likely indicates that weak/non-specific binding of serum IgG to the cell membrane is not insignificant and can be demonstrated with this method.

The ImageStream captures up to four fluorescent images from each cell (in addition to bright field imagery and dark field scatter), so additional markers could be used to provide fluorescent imagery in all four channels to allow a more complex set of analyses than is presented here. This analytical method may find additional applications for analyses of proteins co-localized in lipid rafts, caps or immunological synapses, and may also be used to determine sub-cellular co-localization of proteins in endosomes, lysosomes or other cellular organelles. A modified version of the SBDS has been used in quantitation of NF-κB translocation from the cytoplasm to the nucleus and could be used for any translocation event where a signal sequestered in one compartment of the cell translocates to any other fluorescently labeled element or structure (Arechiga et al., 2005; George et al., 2006). Quantification of co-localization may also find many applications in the determination of the mechanism of action of immunotherapeutic mAbs, either approved for use or under investigation.

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