ABSTRACT

Motivation: Recent advances in confocal microscopy have allowed scientists to assess the expression, and to some extent, the interaction/colocalization of multiple molecules within cells and tissues. In some instances, accurately quantifying the colocalization of two or more proteins may be critical. This can require the acquisition of multiple Z plane images (Z stacks) throughout a specimen and, as such, we report here the successful development of a freeware, open-source image analysis tool, IMAJIN_COLOC, developed in PERL (v. 5.8, build 806), using the PERLMagick libraries (Image-Magick). Using a pixel-by-pixel analysis algorithm, IMAJIN_COLOC can analyze images for antigen expression (any number of colors) and can measure all possible combinations of colocalization for up to three colors by analyzing a Z stack gallery acquired for each sample. The simultaneous (i.e. in a single pass) analysis of three-color colocalization, and batch analysis capabilities are distinctive features of this program.

Results: A control image, containing known individual and colocalized pixel counts, was used to validate the accuracy of IMAJIN_COLOC. As further validation, pixel counts and colocalization values from the control image were compared to those obtained with the software packaged with the Zeiss laser-scanning microscope (LSM AIM, version 3.2). The values from both programs were found to be identical. To demonstrate the applicability of this program in addressing novel biological questions, we examined the role of neurons in eliciting an immune reaction in response to viral infection. Specifically, we successfully examined expression of the chemokine RANTES in measles virus (MV) infected hippocampal neurons and quantified changes in RANTES production throughout the disease period. The resultant quantitative data were also evaluated visually, using a gif image created during the analysis.

Availability: PERL (ActivePerl, version 5.8) is available at activestate.com; the PERLMagick libraries are available at imagemagick.org, and IMAJIN_COLOC, the source code and user documentation can be downloaded from http://www.fda.gov/cber/research/imaging/imagemanalysis.htm

Contact: Malik@cber.fda.gov

INTRODUCTION

Over the past decade, advances in confocal laser scanning microscopy (CLSM) as well as the development of new synthetic fluorescent probes have advanced the role of image analysis in the biological sciences (Halbhuber and Konig, 2003; Sheppard and Shotton, 1997; Paddock, 1999). Additionally, the recent introduction of the META CLSM system by Carl Zeiss, Inc. (Thorowood, NY) (Dickinson et al., 2003) now allows researchers to utilize any number of fluorophores simultaneously, thus allowing for the analyses of complex cellular mechanisms.

However, despite advances in CLSM (Halbhuber and Konig, 2003; Sheppard and Shotton, 1997; Paddock, 1999) and image acquisition software (Demandolx and Davoust, 1997; Holmvall and Szekely, 1999; Oberholzer et al., 1996), it remains difficult to simultaneously quantify the colocalization of three fluorescent markers and hence, the interaction between three proteins. Further, a common difficulty in the analyses of confocal images is ‘artifactual’ colocalization caused by the acquisition of Z stacks with thick (e.g. 2–3 μm) optical sections (Carrington et al., 1990). To reduce this potential error, Z stacks comprised of micrometer thick optical slices taken at optimum intervals should be acquired from biological specimens.

Numerous commercial packages, including Imaris (Saint Paul, MN) (http://www.bitplane.com/products/imaris/imaris_product.shtml) and the Zeiss LSM software (Version 3.2) (Zeiss, 2003), are available; and while these programs can accurately count pixels in a given image, simultaneous quantitative colocalization analysis is limited to two colors at a time. Other programs such as Universal Imaging Corporation’s (Downtowning, PA) Metamorph (http://www.image1.com/products/metamorph/), also limited to colocalization analyses of two fluorescently labeled molecules at a time, are often used in conjunction with a charged coupled device (CCD) camera for image capture. The images taken with CCD cameras can be contaminated with out-of-focus signals, and while there are several algorithms designed to remove the out-of-focus blur (i.e. deconvolution) (Scalaett et al., 1996; Chomik et al., 1997), they vary in efficiency and length of processor time.

In order to facilitate the analyses of 8-bit confocal images, we developed an open-source image analysis program, IMAJIN_COLOC, written in PERL (http://www.activestate.com/) using the Image-Magick libraries (http://www.imagemagick.org/). Each optical slice...
of a Z stack, was analyzed for pixels over a minimum intensity (i.e. over background noise intensity, the value of which can be either directly entered by the user, or automatically determined from a control image [e.g. an image acquired under identical confocal settings of a sample stained with secondary antibodies only]) for each color as well as colocalized colors in all desired combinations from triple-labeled confocal images. The resulting pixel counts were output to a text file, and a corresponding gif image containing only counted pixels was generated for visual comparison with the original (tif) image.

SYSTEM AND METHODS

Preparation of specimens for image analysis

(A) Antigen detection  Serial sagittal mouse brain sections (12 µm), dissected from the cryopreserved brains of mock and MV (laboratory passaged Edmonston strain) infected interferon alpha/beta receptor inactivated/CD46 (Ifnar®-CD46Ge) transgenic mice (kindly provided by Dr Cattaneo, Mayo Clinic, Rochester, MN) (Mrkic et al., 1998), were incubated with anti-measles HA mouse IgG (Chemicon, Temecula, CA; 1:200) to detect MV, biotin conjugated anti-RANTES IgG (R&D Systems, Minneapolis, MN, 1:50) to detect the chemokine RANTES and anti-NSE rabbit IgG (Chemicon; 1:200) to detect neurons. The secondary antibodies anti-mouse IgG Cy-3 (Chemicon; 1:50) to detect the chemokine RANTES and anti-rabbit IgG Cy-5 (Chemicon; 1:400), streptavidin-FITC (Chemicon; 1:200) and anti-rabbit IgG Cy-5 (Chemicon; 1:200) were used to detect the MV, RANTES and neuronal primary antibodies, respectively. Tissue sections stained with secondary antibodies only, served as controls for image analysis (data not shown).

(B) Image collection  A Zeiss Pascal confocal microscope configured with three lasers (Argon 488 nm/514 nm/543 nm, HeNe 563 nm and HeNe 633 nm) and a 40x Plan-Neofluar 1.4 NA objective lens was calibrated using fluorescent beads by a certified Zeiss microscope technician, prior to the collection of 8-bit triple labeled Z stack images which were 1024 × 1024 pixels with a pixel size of 0.22 µm. Pinhole diameters, detector gain, and laser powers were optimized such that images had a full range of pixel intensities (0–255), with very little saturation (<2%) at either end. The fluorophores FITC, Cy-3 and Cy-5 were excited by the 488, 543 and 633 nm laser lines, respectively, followed by emission collection with a 505–530 nm band pass, a 560–615 nm band pass and a 650 nm long pass filter, respectively. The thickness of each optical slice was ~1.6 µm, and the optimal interval between slices was ~0.8 µm.

(C) Image export and analysis  Following image collection, the resultant Z stacks were exported from the Zeiss LSM AIM software (Zeiss, 2003) as uncompressed 8-bit tif images in gallery view. The graphical user interface (GUI) of IMAJIN_COLOC allows users with relatively little prior experience in image analysis to analyze confocal images. The user can specify the number of colors, as well as the type of analysis desired (i.e. pixel counting and/or colocalization). To facilitate optimal signal-to-noise ratio, when determining individual and colocalized pixel counts, the program does not specify minimal threshold intensities for each color. Instead, a minimum threshold intensity value can be entered by the user based on past image analyses, or predetermined with a calibration tool that analyzes control images (i.e. images acquired from samples stained with secondary antibody only). This initialization/configuration needs to be conducted only once for a given dataset of images.

(D) Theoretical Z stack  A control image containing nine squares/slices (Fig. 1) was created in Adobe Photoshop 6.0 to assess the ability of IMAJIN_COLOC to accurately count individual and colocalized pixels within each square. As discussed above, to ensure that only pixels above background noise are assessed, IMAJIN_COLOC does not assign a global threshold value to images. An arbitrary threshold value of 100 was assigned to assess this image. The control image Z stack represents seven 3D diamonds, with slice 1 the top, slice 5 the center, and slice 9 the bottom of each diamond. In the center slice of each diamond (slice 5), intensities range from 255 (center 13 pixels) to 25 (192 pixel outer perimeter), diminishing five intensity intervals (2%) for each color every x or y pixel. Additionally, from the center slice (slice 5), every subsequent slice (above and below) diminishes 51 intensity intervals (20%). Diamonds comprised of individual component colors [red (r), green (g) and blue (b)] are in the top row, diamonds representing double colocalization [red and green (RG), red and blue (RB), green and blue (GB), left to right] are in the bottom row, and a diamond representing triple colocalization (white, RGB) is in the center.

ALGORITHM

Simultaneous colocalization analysis using currently available image analysis software is limited to two colors. IMAJIN_COLOC can be...
Fig. 2. An application of IMAJIN_COLOC to triple labeled Z stack slices obtained from day 6 post-inoculated (mock and MV) mouse brain hippocampal sections. (A) The original tif image showing punctate RANTES (green) expression in non-infected neurons (blue). (B) After analysis with IMAJIN_COLOC, all pixels above threshold values were counted, and exported to a gif image and a tabulated text file (Table 2). (C) The original tif image showing localized expression of RANTES (green) in and adjacent to MV (red)-infected neurons (blue). (D) After analysis with IMAJIN_COLOC, all pixels above threshold values were counted, and exported to a gif image and a tabulated text file (Table 2). Note, that the double-colocalization of RANTES in non-infected hippocampal neurons is observed as teal colored pixels, while the triple-colocalization of RANTES in MV-infected neurons is observed as white (RGB) pixels in the gif images.

configured to (1) measure antigen expression (for any number of colors) and (2) determine colocalization in a single pass for up to three colors.

For Z-stack galleries (Figs 1A, 2A and C), a white line is used to separate optical slices. IMAJIN_COLOC determines the height and width of the image, identifies the location of the separating lines using an ‘if’ statement with the criterion of a saturated white pixel. With the boundaries determined, the following algorithm is applied through the height and width of each square:

- Using ‘if’ statements, each pixel \((x, y)\) coordinate is assessed for red, green and/or blue intensities over the minimum intensities specified.
  - If the intensity of red, for example, meets the threshold criterion, a counter for red pixels is incremented, a red pixel is drawn in the gif image, and the intensities for green and blue are subsequently assessed.
  - If the intensity of green also meets its minimum threshold, then a red–green colocalized counter is incremented, and a red–green (yellow) pixel is drawn in the gif image.
  - If the intensity of blue also meets its minimum threshold, then a red–green–blue colocalized counter is incremented, and a red–green–blue (white) pixel is drawn to the gif image.
  - If the intensity of red does not meet the threshold criterion, then the intensities of green and blue are assessed, colocalization determined, counted and drawn accordingly.
  - If the intensities of red, green and blue do not meet the threshold criterion, then a black pixel is drawn to the gif image.

- The algorithm is repeated throughout the entire height and width of each square.

- When complete, the resulting pixel counts (individual red, green, blue counts and all possible combinations of colocalization) are printed to a tabulated text file. This file can be imported into any database and/or statistics program for further analysis (e.g. to determine % colocalization), if necessary.
Proceeding along the x-axis, the maximum intensity of the 13 pixels in the center of each diamond is 152. The central diamond contained 13 pixels that met the minimum intensity criteria. Hence, a total of 26 double-colocalized pixels were counted. The seven diamonds each contained 13 double-colocalized pixels of a given type (RG, RB and/or GB) that met the minimum intensity criteria. Thus, a total of 52 pixels for each color were counted. Two of the seven diamonds each contained 13 double-colocalized pixels of a given type (RG, RB and/or GB) that met the minimum intensity criteria. Hence, a total of 26 double-colocalized pixels were counted. The central diamond contained 13 pixels that met the minimum intensity criteria for triple-colocalized pixels; (2) in slice 2, there were four diamonds each with 13 red, green and/or blue pixels that met the minimum intensity criteria. Thus, a total of 52 pixels for each color were counted. Two of the seven diamonds each contained 13 double-colocalized pixels of a given type (RG, RB and/or GB) that met the minimum intensity criteria. Hence, a total of 26 double-colocalized pixels were counted. The central diamond contained 13 pixels that met the minimum intensity criteria for triple-colocalized pixels; (3) in slice 3, the maximum intensity of the 13 pixels in the center of each diamond is 152. Proceeding along the x or y axes, the intensities diminish by five intensity intervals, i.e. 147, 142, 137, etc., each forming a perimeter diamond around the central 13 pixels. The equation for the perimeter is \((2 + n) \times 4\), where \(n\) is the number of pixels away from the central diamond, beginning with 1. Thus, there are \((2 + 1) \times 4\) or 12 pixels at intensity 147, \((2 + 2) \times 4\) or 16 pixels at intensity 142, \((2 + 3) \times 4\) or 20 pixels at intensity 137, etc. Summating pixel counts at all intensities through 102 (the last intensity met by the minimum criteria) results in 313 pixels. Again, since four of the seven diamonds each contained red, green and/or blue pixels that met the minimum intensity criteria, a total of 313 \(\times 4\) = 1252 pixels were counted for each color. Two of the seven diamonds each contained double-colocalized pixels of a given type (RG, RB and/or GB) that met the minimum intensity criteria. Hence a total of 313 \(\times 2\) = 626 double-colocalized pixels were counted. The central diamond contained 313 pixels that met the minimum intensity criteria for triple-colocalized pixels. Individual and colocalized pixel counts were similarly determined in the remaining slices. Note, that since the algorithm used in assessing pixel counts is not exclusive, a single pixel can be counted more than once; i.e. a white pixel is counted as an red, green, blue, RG, RB and GB pixel. In order to further validate the data derived from the pixel counting algorithm used by IMAJIN_COLOC, the control image was analyzed with the Zeiss LSM AIM software (version 3.2). This software is packaged with the Zeiss laser scanning microscope. The resultant data was exported to and analyzed in Microsoft Excel. As shown in Table 1, identical pixel counts (individual and double-colocalized) were achieved from the control image with both IMAJIN_COLOC and the Zeiss LSM software. The Zeiss software is unable to simultaneously (i.e. in a single pass) assess triple-colocalized (white, RGB) pixels, and hence these are not presented in Table 1.

### Table 1. Resultant pixel counts from the nine squares/Z stack slices of the control image described in Figure 1, following analysis with IMAJIN_COLOC and the Zeiss LSM AIM software

<table>
<thead>
<tr>
<th>IMAJIN_COLOC and the Zeiss LSM AIM software</th>
<th>Square</th>
<th>Red</th>
<th>Green</th>
<th>Blue</th>
<th>RG (yellow)</th>
<th>RB (purple)</th>
<th>GB (cyan)</th>
<th>IMAJIN ONLY</th>
<th>White</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>52</td>
<td>52</td>
<td>52</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>1252</td>
<td>1252</td>
<td>1252</td>
<td>626</td>
<td>626</td>
<td>626</td>
<td>626</td>
<td>313</td>
<td>313</td>
</tr>
<tr>
<td>4</td>
<td>4052</td>
<td>4052</td>
<td>4052</td>
<td>2026</td>
<td>2026</td>
<td>2026</td>
<td>2026</td>
<td>1013</td>
<td>1013</td>
</tr>
<tr>
<td>5</td>
<td>8452</td>
<td>8452</td>
<td>8452</td>
<td>4226</td>
<td>4226</td>
<td>4226</td>
<td>4226</td>
<td>2113</td>
<td>2113</td>
</tr>
<tr>
<td>6</td>
<td>4052</td>
<td>4052</td>
<td>4052</td>
<td>2026</td>
<td>2026</td>
<td>2026</td>
<td>2026</td>
<td>1013</td>
<td>1013</td>
</tr>
<tr>
<td>7</td>
<td>1252</td>
<td>1252</td>
<td>1252</td>
<td>626</td>
<td>626</td>
<td>626</td>
<td>626</td>
<td>313</td>
<td>313</td>
</tr>
<tr>
<td>8</td>
<td>52</td>
<td>52</td>
<td>52</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The Zeiss LSM AIM software is unable to simultaneously assess triple-colocalized (white, RGB) pixels.

### IMPLEMENTATION

#### Analysis of a control image

A theoretical Z stack was used to assess the program’s ability to count red, green and blue pixels, as well as all possible combinations of colocalization (RG, RB and GB) (Fig. 1). With the minimum intensity arbitrarily set to 100 (as described above, minimum intensity values can be entered by the user or be predetermined with a calibration tool that analyzes control images (i.e. images acquired from samples stained with secondary antibody only)) for each color in this particular image, expected individual and colocalized pixel counts were achieved (Table 1). For example: (1) zero pixels were counted in slices 1 and 9, because no pixels met the minimum intensity criteria; (2) in slice 2, there were four diamonds each with 13 red, green and/or blue pixels that met the minimum intensity criteria. Thus, a total of 52 pixels for each color were counted. Two of the seven diamonds each contained 13 double-colocalized pixels of a given type (RG, RB and GB) that met the minimum intensity criteria. Hence, a total of 26 double-colocalized pixels were counted. The central diamond contained 13 pixels that met the minimum intensity criteria for triple-colocalized pixels; (3) in slice 3, the maximum intensity of the 13 pixels in the center of each diamond is 152. Proceeding along the x or y axes, the intensities diminish by five intensity intervals, i.e. 147, 142, 137, etc., each forming a perimeter diamond around the central 13 pixels. The equation for the perimeter is \((2 + n) \times 4\), where \(n\) is the number of pixels away from the central diamond, beginning with 1. Thus, there are \((2 + 1) \times 4\) or 12 pixels at intensity 147, \((2 + 2) \times 4\) or 16 pixels at intensity 142, \((2 + 3) \times 4\) or 20 pixels at intensity 137, etc. Summating pixel counts at all intensities through 102 (the last intensity met by the minimum criteria) results in 313 pixels. Again, since four of the seven diamonds each contained red, green and/or blue pixels that met the minimum intensity criteria, a total of 313 \(\times 4\) = 1252 pixels were counted for each color. Two of the seven diamonds each contained double-colocalized pixels of a given type (RG, RB and/or GB) that met the minimum intensity criteria. Hence a total of 313 \(\times 2\) = 626 double-colocalized pixels were counted. The central diamond contained 313 pixels that met the minimum intensity criteria for triple-colocalized pixels. Individual and colocalized pixel counts were similarly determined in the remaining slices. Note, that since the algorithm used in assessing pixel counts is not exclusive, a single pixel can be counted more than once; i.e. a white pixel is counted as an red, green, blue, RG, RB and RGB pixel.

#### A novel biological application of IMAJIN_COLOC

The chemokine RANTES can chemoattract T cells and monocytes (Schall et al., 1988, 1990) and has been implicated in regulating the host response to many infections including MV (Manchester et al., 1999). The induction of RANTES in MV-infected primary neurons has only recently been described (Patterson et al., 2003). The role of neurons in inhibiting the spread of agents such as MV provides a novel area of research for further understanding the pathogenesis of this virus. Current commercially available colocalization software is unable to simultaneously (i.e. in one pass) determine the colocalization of three-labeled molecules. Hence it has not been possible to simultaneously correlate cell types infected, viral protein expression and immune mediator induction in vivo. Here, we have utilized IMAJIN_COLOC’s novel quantitative triple-colocalization tool to determine if the levels of RANTES expression (i.e. activation of the immune response) can be correlated with the progression of MV infection in the central nervous system (CNS), over time. Since the optimal number of Z stack slices depends on the thickness of the particular specimen being imaged, IMAJIN_COLOC can examine any number of slices. We used six Z stack slices to examine sections showing measles infection of the mouse CNS. Figure 2 shows the six representative Z stack slices from day 6 post-inoculated mouse brain (hippocampus, CA3) sections. Table 2 shows the resultant pixel counts for each slice shown in Figure 2. We found that ∼18% of the total RANTES being expressed was colocalized in MV-infected neurons of the hippocampus. Additionally, we observed an increased expression/activation of RANTES in neurons (GB = 41.36%) adjacent to the site of infection. In contrast, non-infected CNS tissue showed lower levels of punctate staining for RANTES (GB = 27.25%) in hippocampal neurons. Using the image analysis data, Figure 3 was generated to correlate RANTES expression with MV spread in hippocampal neurons, over time. RANTES expression in MV-infected neurons was found to increase incrementally until day 7 post-inoculation, but by day 8 post-inoculation had dropped to approximately one-seventh

---

**Confocal image analysis**

- If specified, the gif image containing colored pixels drawn at a single intensity (representing true signal) can be assessed for visual verification of the counted pixels. The tif to gif conversion of the input image is a requirement/limitation of the ImageMagick plug-in used in this program. However, the dimensions and resolution of thegif image are the same as the original input tif image.
Table 2. Pixel counts from each optical slice of the mock and measles virus infected neonatal mouse brain hippocampal sections (day 6 post-inoculation) shown in Figure 2

<table>
<thead>
<tr>
<th>Square</th>
<th>Red (measles)</th>
<th>Green (RANTES)</th>
<th>Blue (neurons)</th>
<th>RG</th>
<th>RB</th>
<th>GB</th>
<th>White (RGB)</th>
<th>GB/G *100</th>
<th>White/G *100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pixel counts from Figure 2A (mock tissue)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>ND</td>
<td>762</td>
<td>40,852</td>
<td>ND</td>
<td>ND</td>
<td>262</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
<td>654</td>
<td>37,648</td>
<td>ND</td>
<td>ND</td>
<td>201</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
<td>450</td>
<td>34,594</td>
<td>ND</td>
<td>ND</td>
<td>127</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
<td>429</td>
<td>29,492</td>
<td>ND</td>
<td>ND</td>
<td>97</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>ND</td>
<td>415</td>
<td>24,687</td>
<td>ND</td>
<td>ND</td>
<td>83</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>ND</td>
<td>409</td>
<td>19,834</td>
<td>ND</td>
<td>ND</td>
<td>80</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Total</td>
<td>ND</td>
<td>3119</td>
<td>18,7107</td>
<td>ND</td>
<td>ND</td>
<td>850</td>
<td>ND</td>
<td>27.25</td>
<td>ND</td>
</tr>
</tbody>
</table>

Pixel counts from Figure 2C (MV infected tissue)

| 1      | 18,389        | 1468           | 15,958         | ND | ND | 487 | 306         |
| 2      | 24,219        | 2392           | 26,334         | ND | ND | 979 | 565         |
| 3      | 27,054        | 3026           | 32,785         | ND | ND | 1303| 616         |
| 4      | 27,808        | 3762           | 36,391         | ND | ND | 1691| 726         |
| 5      | 28,072        | 4050           | 36,935         | ND | ND | 1766| 713         |
| 6      | 27,421        | 4102           | 31,987         | ND | ND | 1549| 504         |
| Total  | 112,650       | 18,800         | 180,390        | ND | ND | 7775| 41.36       |

The last line of the table contains summated data for the pixels counted from the six slices, as well as the ratios (percentages) of colocalization. Notice (1) that infected neurons account for >18% of the total RANTES (i.e. triple-colocalized expression [white pixels]) present and (2) the 1.5-fold increase in RANTES expression in non-infected neurons adjacent to the site of infection. ND, not determined.

Fig. 3. A time-course study of the expression of RANTES in MV-infected hippocampal neurons. Using IMAJIN_COLOC’s batch-processing function and novel triple-colocalization tool, multiple (6) images, similar to those shown in Figure 2 were analyzed for expression at each time point and colocalization counts determined. Brains from three animals were used per time point for this study.

The levels of day 7. This decrease in RANTES expression could be correlated to MV-infected neuronal cell destruction and the death of the animals by day 9 post-inoculation (data not shown).

Artificial colocalization

To demonstrate the importance of analyzing Z stack images with optimal Z slice thickness, three slices from the MV-infected tissue section, shown in Figure 2C, were projected into one slice (resulting in a 3.2 µm thick optical section; Fig. 4) and analyzed with IMAJIN_COLOC. Table 3 shows the resulting pixel counts and the percentages of colocalization. Notice that the colocalization of RANTES in the projected image is higher both in (2.0-fold ↑) and adjacent (1.6-fold ↑) to the MV infected neurons. The three slices were also analyzed without projection using IMAJIN_COLOC. Ratios of colocalization, in this case, were determined to be 43% for blue and green colocalization and 21% for triple (white) colocalization (data not shown). The higher colocalization values with three unprojected optical slices from the middle of a tissue section versus slices taken throughout the entire thickness of the tissue emphasizes the importance of using optimal numbers of slices with optimum thickness to obtain accurate data.

DISCUSSION

Recent advances in confocal microscopy have allowed researchers to analyze multiple interactions in cells using bio-fluorescence and/or immunofluorescent labeling of proteins (Halbhuber and Konig, 2003; Sheppard and Shotton, 1997; Paddock, 1999). However, image analysis of confocal data remains complicated. Previously, the output of colocalization data was largely centered on the pixel histogram (Pratt, 1978; Feller, 1971; Oberholzer et al., 1996). This method of analysis requires comparison and identification of overlap across all grayscale values for each color. The resultant scatter plot describes the occurrences of colocalized pixels within the image using color-coding (Demandolx and Davoust, 1997). However, pictures with low background and high true colocalization produce distribution patterns (e.g. clustering around the diagonals of the diagram) that appear similar to those that have high non-specific background without significant colocalization. Although most current programs now also output actual definitive overlap values in a tabular format using
Confocal image analysis

Fig. 4. Analysis of a 3.2 µm optical section of MV-infected tissue. Using three slices from the Z stack projected into one slice (resulting in a 3.2 µm thick optical section), the tif picture (A) was created, and image analysis performed to determine pixel counts and colocalization data. (B) represents the resulting gif image. Both images are shown at a magnification of 200% relative to each square of Figure 2.

Table 3. Pixel counts from a 3.2 µm optical section

<table>
<thead>
<tr>
<th>Square</th>
<th>Red (measles)</th>
<th>Green (RANTES)</th>
<th>Blue (neurons)</th>
<th>RG</th>
<th>RB</th>
<th>GB</th>
<th>White (RGB)</th>
<th>GB/G*100</th>
<th>White/G*100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z proj.tif</td>
<td>38</td>
<td>8283</td>
<td>54266</td>
<td>ND</td>
<td>ND</td>
<td>5362</td>
<td>3098</td>
<td>64.73</td>
<td>37.40</td>
</tr>
</tbody>
</table>

Sections obtained by projecting three slices into one slice from the Z stack image of the MV-infected tissue section. The colocalization of RG, RB, GB and white pixels represents the interaction of fluorescent proteins. ND = not determined.

third party software (e.g., Microsoft Excel), colocalization analysis is still limited to two colors at a time. Further, the lack of a batch-processing function in most programs makes the task of analyzing multiple Z stack images (in a folder containing images captured using the same settings with the confocal microscope) repetitive and time consuming.

Here we have described IMAJIN_COLOC, a program that employs a pixel-by-pixel algorithm to enable users to batch-process the analyses of confocal images for pixel counts (any number of colors) as well as the assessment of all combinations of colocalization (up to three antigens) simultaneously, in a Z stack comprised of multiple slices. The simultaneous analysis of colocalized pixels was only applied to two- and three-color combinations, since any other colors are combinations of red, green and blue component pixel intensities. Hence, the presence of four or more colors can result in an inappropriate evaluation of colocalized pixels.

To demonstrate the biological relevance of accurately quantifying triple-colocalization, we studied expression of the chemokine RANTES in the mouse CNS in correlation with MV spread. RANTES is important in initiating an immune response against MV in the CNS (Manchester et al., 1999), but like all chemokines, RANTES expression needs to be tightly regulated. Accurate quantification of RANTES expression is, therefore, necessary to provide a more complete understanding of the global immune response following viral infection. Similar colocalization analyses could also be performed to address novel biological questions related to signal transduction, apoptosis and interactions between activator and repressor elements. In most of these cases, multiple proteins must interact in order to cause a cascade of events. Accurate quantification of triple-colocalized proteins would be an appropriate way to determine the extent of interaction necessary to initiate a particular biological cascade.

It is worth noting that the danger with batch-processing a gallery of Z stack slices is the alteration of pixel intensities through the image caused by bleaching of the fluorophores. Bleaching can be kept to a minimum by ensuring that: (1) the image contains optimal (bright and homogeneous) pixel intensities, (2) the image is analyzed with no more than the optimal number of overlapping Z stack slices, (3) the confocal microscope is set at optimal acquisition conditions [e.g. pinhole diameters, detector gain and laser powers must be optimized such that images have a full range of pixel intensities (0–255), with very little saturation at either end] and (4) the use of mounting media with anti-fade agent (either homemade or commercially available from numerous companies); and, additionally for the analyses of Z stack slices the use of an adjustable-threshold segmentation algorithm, which takes into account varying pixel intensities through an image, that has recently been reported (Ponomarev and Davis, 2003).

The IMAJIN_COLOC PERL script can run under the Windows and Unix/Linux operating systems. IMAJIN_COLOC will also be supported on Apple (Mac) platforms with the release of MacPerl v 5.8 in the near future. In summary, IMAJIN_COLOC has: (1) the ability to accurately count all pixels in a given image, (2) the ability to quantify all combinations of colocalization in both double- and triple-labeled images, (3) the ability to batch-process multiple confocal images in a folder, (4) the capability to visually represent true signal (i.e. above background values) in a gif image and (5) numerous applications for the analyses of novel biological processes.
ACKNOWLEDGEMENTS

This work was supported in part by an appointment to the Research Participation Program at the Center for Biologics Evaluation and Research administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the US Department of Energy and the US Food and Drug Administration.

Conflict of Interest: none declared.

REFERENCES


