

Six-Color Segmentation of Multicolor Images in the Infection Studies of *Listeria monocytogenes*

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ABSTRACT Multiple immunofluorescent staining is a powerful strategy for visualizing the spatial and temporal relationship between antigens, cell populations, and tissue components in histological sections. To segment different cell populations from the multicolor image generated by immunostaining based on color addition theory, a systems approach is proposed for automatic segmentation of six colors. After image acquisition and processing, images are automatically segmented with the proposed approach and six-pseudo channels for individual or colocalized fluorescent dye are generated to distinguish different cell types. The principle of this approach is the classification of each pixel into one of six colors (red, green, blue, yellow, magenta, and cyan) by choosing the minimal angular deviation between the RGB vector of the given pixel and six classically defined edge vectors. In the present infection studies of *Listeria monocytogenes*, the new multicolor staining methods based on the color addition were applied and the proposed color segmentation was performed for multicolor analysis. Multicolor analysis was accomplished to study the migration and interaction of *Listeria* and different cell subpopulations such as CD4CD25 double positive T regulatory cells; we also visualized simultaneously the B cells, T cells, dendritic cells, macrophages, and *Listeria* in another experiment. After *Listeria* infection, ERTR9 macrophages and dendritic cells formed cluster with *Listeria* in the infection loci. The principle of color addition and the systems approach for segmentation may be widely applicable in infection and immunity studies requiring multicolor imaging and analysis. This approach can also be applied for image analysis in the multicolor in vivo imaging, multicolor FISH or karyotyping or other studies requiring multicolor analysis. *Microsc. Res. Tech.* 70:171–178, 2007. © 2006 Wiley-Liss, Inc.

INTRODUCTION

Multiple immunofluorescent staining is a powerful strategy for visualizing the spatial and temporal relationship between antigens, cell populations, and tissue components in histological and pathological studies. However, multicolor imaging with confocal microscopy is difficult because of the restriction of the excitation wavelength of the lasers and the fluorescent dyes. Only a few multicolor immunostaining methods for more than four colors have been reported. The use of directly labeled primary antibodies and spectral imaging and unmixing has allowed seven-color imaging of thymic tissue (Tsurui et al., 2000). Recently, a new method based on color addition theory has been developed for the simultaneous visualization of six cell population in secondary lymphoid tissue (Ma et al., 2006). This provides an easy and efficient way for the multicolor imaging of cell and tissue samples. However, for a better visualization and interpretation of the image data, an automatic color segmentation method is needed to generate multipseudo channels, which represent different antigens or cell populations in complex tissue samples.

Image segmentation is the process of separating or grouping an image into different parts or of isolating the objects and finding the relation between them (Jain, 1998). Earlier research work in the segmentation field has mainly dealt with monochrome images. Color is a useful and important property that adds much more information to the images. Therefore, color image segmentation algorithms are frequently derived from monochrome image segmentation methods. The color perceived by the human eye is a combination of three color stimuli, viz. red (R), green (G), and blue (B), which form the normal, widely used, RGB color space.

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TABLE 1. Antibodies and reagents used for the immunolabeling

Target	Conjugate	Species	Dilution	Isotype	Clone	Company	Location
Biotin	Alexa 546		1:300	Streptavidin		Molecular Probes	Eugene, OR
Biotin	Alexa 635		1:300	Streptavidin		Molecular Probes	Eugene, OR
CD45/B220	Alexa 488	Rat	1:300	IgG2a, κ	RA3-6B2	BD Pharmingen	San Diego, CA
CD3e		Syrian hamster	1:200	IgG, group 2, κ	500A2	BD Pharmingen	San Diego, CA
CD4	PE	Rat	1:300	IgG2a, κ	GK1.5	BD Pharmingen	San Diego, CA
CD11c	Biotin	Armenian hamster	1:500	IgG1, λ	HL3	BD Pharmingen	San Diego, CA
CD25	Biotin	Rat	1:300	IgM	7D4	BD Pharmingen	San Diego, CA
ERTR-9	Biotin	Rat	1:300	IgM		BMA Biomedicals	Augst, Switzerland
Hmastert IgG(H+L)	Cy5	Goat	1:300	(Fab') ₂		Jakson Immuno Research Laboratories	West Grove, PA
Rabbit IgG (H+L)	Alexa 488	Goat	1:300	(Fab') ₂		Molecular Probes	Eugene, OR
Rabbit IgG(H+L)	Alexa 546	Goat	1:300			Molecular Probes	Eugene, OR
Rabbit IgG(H+L)	AMCA	Goat	1:300	(Fab') ₂ , Fc specific		Jakson Immuno Research Laboratories	West Grove, PA
Rat IgG(H+L)	Alexa 488	Goat	1:300			BD Pharmingen	San Diego, CA
Listeria		Rabbit	1:300			Biodesign International	Saco, ME

Many color models are used to represent the colors in various representations: RGB (red, green, and blue), HSV (hue, saturation, and intensity), and CMYK (cyan, magenta, yellow, and key). Compared with monochrome images, color images contain the information of brightness, hue, and saturation for each pixel. The existing color image segmentation techniques can be classified into a few approaches based on clustering, edge detection, region growing, neural network, fuzzy and histogram thresholding (Deshmukh and Shinde, 2005). Since our new multicolor staining method relies on color addition theory, a pixel-based three-dimensional (3D) clustering type of color segmentation has been developed to generate multiple pseudochannels for each color. The principle of this approach is the classification of each pixel of the image into one of six defined color classes (red, green, blue, yellow, magenta, and cyan). The efficiency of this approach has also been demonstrated in the important research field of immunology-infection studies of *Listeria monocytogenes*.

L. monocytogenes has been widely used as a model to investigate the pathogenesis of an intracellular bacterial pathogen, cellular immunity, and host defense (Czuprynski and Haak-Frendscho, 1997). Murine listeriosis is one of the most important experimental models for these studies. The investigations of the migration and locations of various cell populations, including B cells, T cells, dendritic cells and macrophages, are of great interest for cellular immunity studies. To visualize these multiple cell populations in one single section, we have applied our new multicolor immunostaining method (Ma et al., 2006). To generate multipseudo channels for the identification of six cell populations or subpopulations in the infected mouse spleen, the proposed color segmentation approach has been used to facilitate the powerful multicolor imaging method.

MATERIALS AND METHODS

Bacteria

The *L. monocytogenes* virulent wild-type strain EGDe was grown in brain heart infusion broth overnight at 37°C, with shaking. The next day, suspensions were diluted and grown for 2 h until log-phase growth was attained. Bacteria were then centrifuged, washed

twice, and resuspended in sterile phosphate-buffered solution (PBS). The optical density of the bacterial suspensions was adjusted by spectrophotometer at 600 nm. The intended number of colony-forming units (CFU) was extrapolated from a standard growth curve, and appropriate dilutions with sterile PBS were made to prepare the inocula for the mice. The exact bacterial concentration was verified by plating dilutions on brain heart infusion agar plates (Difco, Detroit, MI) and by counting colonies after overnight growth.

Animal Experiment

BALB/C female mice (8–12 weeks old) were obtained from Harlan Winkelmann (Borchen, Germany). All animal procedures were approved by the local district governments and were carried out according to the guidelines of the German Law for the protection of animal life.

For *L. monocytogenes* infection, 5×10^5 CFU in 100 μ L PBS were used for i.v. infection and 1.0×10^9 CFU in 100 μ L PBS were inoculated for oral infection. Mice were supplied with autoclaved, acidulated water and fed ad libitum with autoclaved standard diet. At 24 and 48 h after infection, mice spleens were harvested for further immunostaining.

The antibodies and reagents used are shown in Table 1.

Immunofluorescent Staining

Mice were sacrificed by CO₂ narcosis and organs were quickly removed, embedded in Tissue-Tek[®] optimal cutting temperature compound (Sakura Finetek, Torrance, CA) and snap-frozen in liquid nitrogen. Cryosections (10 μ m) were prepared with a Reichert-Jung Frigocut 2800e Cryostat.

The general staining protocol was described in Ma et al. (2006). For the staining in the i.v. infection, spleen sections were incubated with rabbit anti-*Listeria*, Alexa Fluor 488-conjugated rat anti-B220, PE-conjugated rat anti-CD4, and biotin-conjugated anti-CD25 antibodies, followed by AMCA-conjugated goat anti-rabbit IgG and Alexa Fluor 635-conjugated streptavidin.

For the staining of oral infection experiments, sections were incubated with anti-B220, anti-CD11c-biotin, anti-CD3, and anti-*Listeria*, followed by Alexa Fluor 488-conjugated goat anti-rat IgG, Alexa Fluor

TABLE 2. Parameters and settings for confocal microscopy

Parameters				
Fluorescent dye	Lasers	Excitation wavelength (nm)	Emission filter (nm)	Detector
AMCA	Enterprise (max. 12%)	364	BP380-430	Normal
Alexa 488	Argon (max. 7%)	488	BP505-530	Normal
Alexa 546, PE	HeNe1 (max. 20%)	543	BP560-615	Normal
Alexa 635, Cy5	HeNe2 (max. 42%)	633	654-718	META

635-conjugated streptavidin, Cy5-conjugated goat anti-Hamster IgG, Alexa Fluor 488-conjugated goat anti-rabbit IgG, and Alexa Fluor 546-conjugated goat anti-rabbit IgG. After being washed in PBS, the sections were incubated with anti-ERTR-9-biotin followed by Alexa Fluor 546-conjugated streptavidin.

Confocal Microscopy

Confocal imaging was performed with an LSM META510UV confocal scanning laser system on an Axiovert 200 M microscope (Zeiss, Jena, Germany). The instrument settings are shown in Table 2. Images were obtained with a Plan-Neofluar 40×1.3 oil immersion objective lens. After image acquisition, contrast and brightness of the images were adjusted by using LSM software.

Segmentation Program Development

Image J (Version 1.36b) was downloaded from the website (<http://rsb.info.nih.gov/ij/>) of National Institutes of Health, USA. The segmentation program was written in a Java-like macro language for Image J (<http://rsbweb.nih.gov/ij/developer/macro/macros.html>). Our segmentation programs and details of algorithms are available by request.

Image Segmentation and Processing

1. For image processing and the running of the program, a Dell Optiplex GX620 PC (Pentium IV processor) was used (CPU, 3.20 GHz; RAM, 3.5 G). The operation system was Microsoft Windows XP (Professional edition).
2. The images were first processed with Adobe Photoshop CS (levels adjustment) and saved as JPEG documents. The adjustment included the following: reducing the background, improving the contrast, etc.
3. Segmentation was performed with our segmentation program integrated in Image J.
4. After segmentation, the images in six colors were transferred to 8-bit gray-scale images and mapped with six corresponding colors by using the color mapping macro (for example, use macro (run "Magenta") to add magenta to the gray-scale image). According to the definition, magenta is (255, 0, 255) for RGB model, which implies the mixing of red and blue at the same intensity. Further processing in the Image J program included "smooth" and "adjustment of brightness/contrast."

RESULTS

General Principles of the Proposed Multicolor Segmentation Technique

A pixel-based 3D clustering algorithm has been used to develop the segmentation program. In our study,

segmentation is carried out in the RGB color space. The definition of six color classes (red, green, blue, yellow, magenta, and cyan), which are represented by six-edge vectors, is shown in Figure 1A. The principle of this approach is the classification of each pixel into one of these six classically defined color classes by choosing the minimal angular deviation between the RGB vector of the given pixel and six-edge vectors (Fig. 1B and Eq. (1)).

$$C_i, \text{ where } \max_{i=r,g,b,y,m,c} \left(\frac{\vec{P} \cdot \vec{C}_i}{|\vec{P}| \cdot |\vec{C}_i|} \right) \quad (1)$$

To test the validity of the methods, segmentation of a 2D computer spectrum, which had a continuous band of primary and secondary colors, was performed and the results are shown in Figures 2A and 2B. After segmentation, the images were mapped with the six defined colors for better visualization and interpretation (Fig. 2C).

Color Segmentation Based on Colocalization Analysis of Two Antigens

The colocalization of two cell-surface markers has been widely used for the study of the expression of two antigens on one cell population (Agnati et al., 2005). Since few types of cells have an exclusive cell-surface identification marker, more than one marker is required for the accurate identification of a particular cell type, e.g., CD4CD25 double-positive T regulatory cells.

To demonstrate this feature, mice were inoculated i.v. with *L. monocytogenes* and spleens were studied for the migration and localization of various cells populations, such as CD4CD25, B220CD3, B220 CD25, and other cells. By using our segmentation program, six pseudochannels in six colors, which represented six subpopulations, were generated by color addition theory (Fig. 3). With the addition of a further fluorescent image for *Listeria* (UV excited channel), the distribution of *L. monocytogenes* after infection can be investigated (Fig. 3B2). The six-color segmentation results of multicolor image for noninfected and infected spleen are shown in Figures 3C and 3D.

Multicolor Imaging in *Listeria* Infection Studies

Multicolor staining methods based on color addition were applied in our infection studies. The mice were inoculated with *L. monocytogenes* by oral delivery. Using antibodies against B220, CD3, CD11c, ERTR9, and *Listeria*, B cells, T cells, dendritic cells, and *Listeria* were visualized simultaneously on one single section of spleen from the noninfected and infected mouse. The staining results for normal mouse spleen are

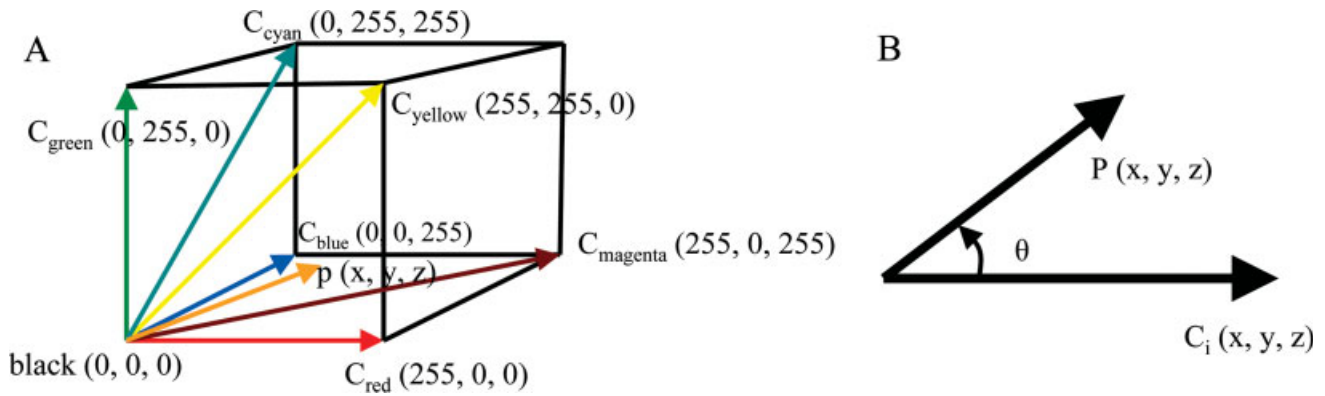


Fig. 1. Principles of the six-color segmentation algorithm. (A) Six classes of color are represented by six-edge vectors in the RGB color space. (B) $C_i(x,y,z)$ is the vector assigned for one standard color, e.g., red or yellow. θ is the angular deviation between the RGB vector of one given color and that of one defined standard color class.

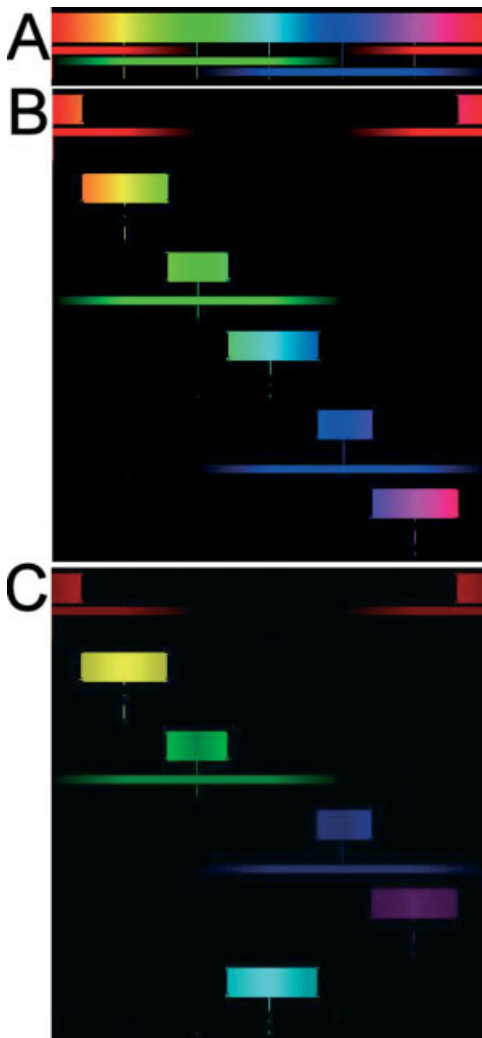


Fig. 2. Segmentation of a continuous 2D computer spectrum (A). A computer spectrum containing various colors (<http://en.wikipedia.org/wiki/Image:Computerspectrum.png>). This spectrum can be regarded as the combination of three colors: green, red, and blue (at the bottom). (B) Six color classes are separated. (C) The pixels in the six color classes are mapped with the six colors as defined. The brightness and contrast were adjusted after color mapping.

shown in Figure 4A. The infection loci in the white pulp and red pulp of spleen from mouse at 24 h after inoculation are shown in Figures 4B1 and 4B2, respectively. The segmentation results of Figure 4B1 are shown in Figure 4C. With the same approach, the segmentation of other two images (Figs. 4A and 4B2) were also performed (data are not shown). By 48 h after infection, huge infection loci, which included many ERTR9 macrophages and dendritic cells, were observed in the mouse spleen (data are not shown).

DISCUSSION

Six-Color Segmentation of Multicolor Images

In the present study, we have accomplished an automatic segmentation algorithm to facilitate the six-color analysis of multicolor images. This approach provides an easy and efficient way for color segmentation in multicolor imaging studies. The program is applied with the multicolor staining protocols, in which color addition is used for the generation of up to six colors for different antigens, cell populations, or structures. Since color is a constant property in the multicolor images, the pixels of images are mapped into a certain color class and the cells or other structures, which are the objects of interest, will manifest themselves as clusters. Therefore, our method is a pixel-based segmentation method, in which a 3D clustering algorithm is used.

Another popular pixel-based segmentation method, which is normally used for the study of co-expression of two antigens, is colocalization analysis. Colocalization analysis in digital images is a typical “dual-image pixel point” process (Shotton, 1993). Since it selects and segments all voxels, where both channels have common events defined as signal intensity above a chosen threshold or within a certain range, this approach is a 2D histogram thresholding algorithm, which is among the most popular techniques for segmenting the gray-scale images. However, color images are more complicated than the gray-scale images so that the color image segmentation is a more difficult task. Celenk and Uijt de Haag (1998) have independently undertaken the thresholding of three histograms based on

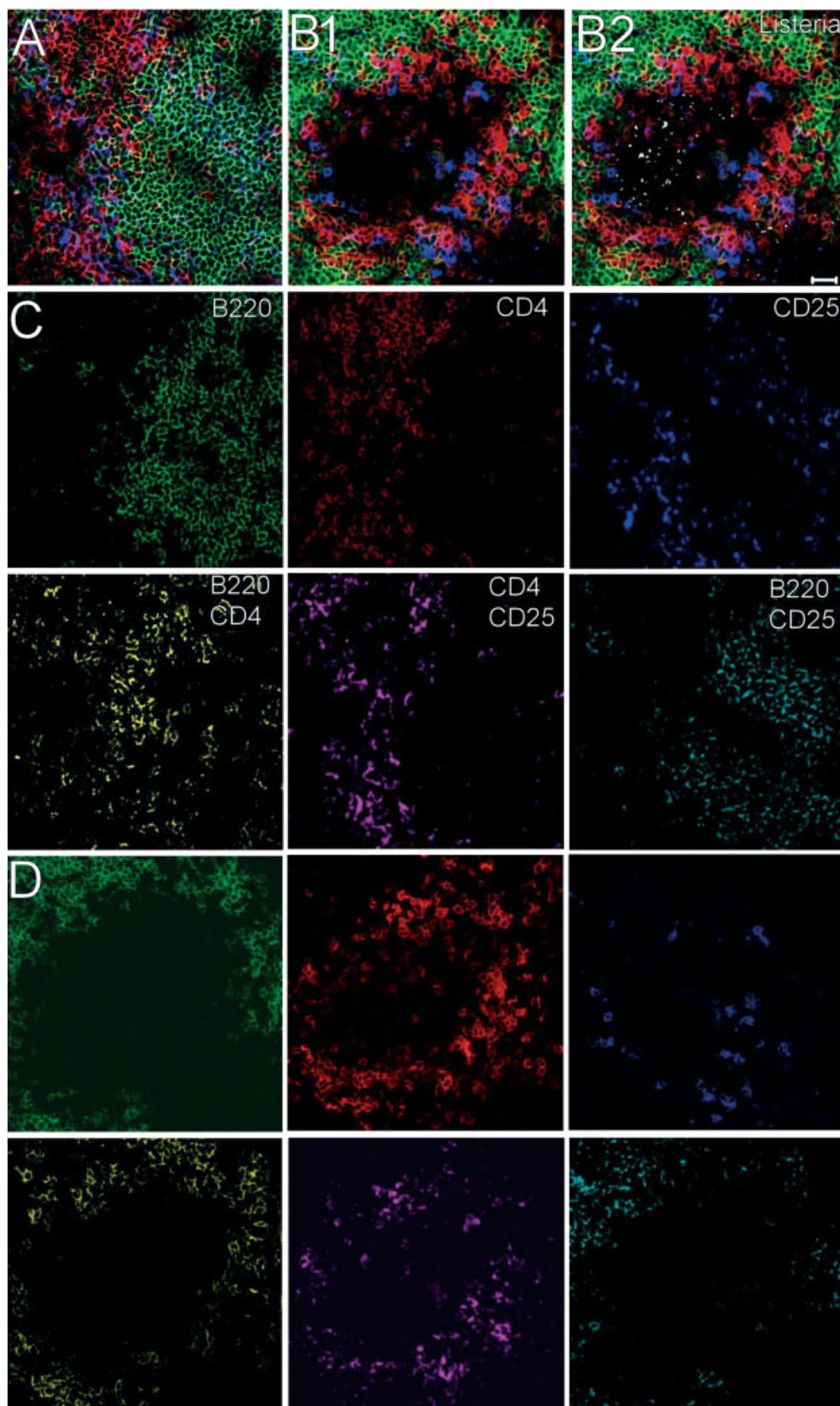


Fig. 3. Multicolor segmentation of six cell populations in the white pulp of noninfected and infected spleen. B220, CD4, and CD25 are stained with green, red, and blue, respectively. Scale bar: 20 μm . (A) Distribution of various cell populations in the spleen white pulp from noninfected BALB/c mouse. (B) BALB/c mice were inoculated i.v. with 5.0×10^5 CFU of *L. monocytogenes* EGDe and their spleens were prepared for histology 24 h after inoculation. Typical micrographs for the infection loci in the white pulp are shown. The additional channel (UV laser excited channel, mapped with white color, for goat anti-rat AMCA) for *Listeria* is shown in (B2) with AMCA staining. Six-color segmentation of (A) and (B1) are shown in (C) and (D), respectively. Double-positive cells for B220/CD4, CD4/CD25 and B220/CD25 are shown with yellow, magenta and cyan, respectively. After infection, CD4⁺ cells and CD4/CD25 cells (including regulatory T cells and other cells) migrate and are located around the *Listeria*.

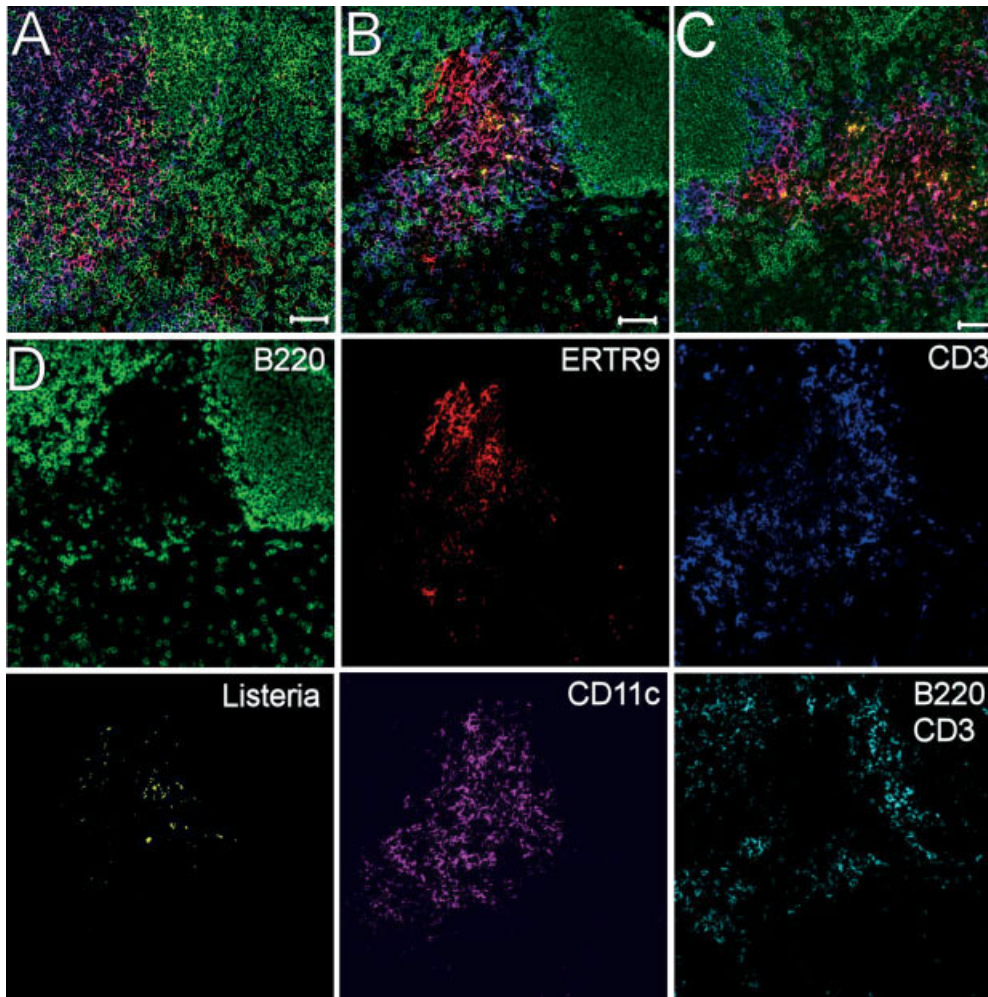


Fig. 4. Five-color imaging of mouse spleen infected with *L. monocytogenes*. BALB/c mice were inoculated p.o. with 5.0×10^8 CFU of *L. monocytogenes* EGDe and their spleens were prepared for histology 24 h after inoculation. The images are 2×2 tile scans. Scale bar: 50 μ m. Typical images of spleen from noninfected mouse (A) and mouse 24 h after infection (B and C). (A) ERTR-9 macrophages are located mainly in the marginal zone of the white pulp. CD11c-positive dendritic cells are present in the T cell region and marginal zone. (B and C) Images of the infected mouse spleen white pulp and red pulp, respectively. By 24 h after infection, dendritic cells and ERTR9 macrophages migrate and form clusters around *L. monocytogenes*. (D) Six-color analysis of B. Four cell populations, identified by B220 (B cell), ERTR9 (macrophages), CD3 (T cell), and CD11c (dendritic cell), are shown in green, red, blue, yellow, and magenta, respectively. *L. monocytogenes* is shown in yellow. Double-positive cells for B220/CD3 are shown in cyan.

RGB coordinates by maximizing within-group variance and have combined the three results with a predicative logical function. One advantage of this method is that the background information can be eliminated, since the intensities value lower than the threshold are set to zero. This kind of background elimination is especially useful when high background, which may be maximally up to 30% of signal intensity, is present in the confocal images. However, the relationship between the three color components (RGB) is not taken into account. Furthermore, for one colocalization analysis, only one secondary color can be generated and the primary colors are difficult to segment. Therefore, the method is not suitable for fast and efficient automatic image analysis also. To perform the six-color segmentation, a 3D thresholding algorithm, which performs the thresholding of three primary color channels at the same time, should be applied. One advantage of this method is that good segmentation results can be still obtained when the expression of antigens on one cell varies widely. For example, for the analysis of Ki-67 in the cell nuclei, a cell differentiation associated antigen, 3D thresholding may be a good choice. If our methods are used, the color classes should be defined again so as to perform the proper color segmentation.

Other kinds of segmentation algorithms can also be used for color image segmentation. For example, a method based on the calculations of three color channels/layers has been described in our previous studies (Ma et al., 2006). Since the principle of this method is that the three color channels used for color addition are calculated to generate the pseudo channels for each antigen or cell population, this approach is an image-based segmentation technique. One advantage of this method is that the segmented structures are normally continuous, with one object not being separated from others within a few regions. However, it is a manual operation, which is time consuming and tedious, and it cannot be used on large numbers of images or image stacks.

To perform our proposed color segmentation of secondary colors, comparable color intensities from both primary colors are needed. If two fluorescent-dye conjugated secondary antibodies are used against one primary antibody, comparable signals from two primary color channels are easy to obtain. If co-expression of two antigens is used for the identification of certain cell population or subpopulation, in most cases, comparable signals can still be obtained through the following ways: (1) the co-expression of two antigens is in

comparable level; (2) the co-expression of two antigens is not in comparable level, but after staining signals from two channels are comparable because of the staining step; (3) the instrument settings in the confocal microscope, such as detection gain, amplified set will make two color intensities comparable; (4) some image processing steps can adjust the contrast and brightness to make two color signals comparable.

Preprocessing of multicolor images is necessary for easy and efficient image segmentation. First, since the manipulation is carried out on single pixel, optimized images with high resolution should be used for the segmentation analysis so as to avoid loss of information and generate better segmentation result. Second, some image filtering and restoration methods such as deconvolution can be used to improve the efficiency of the color segmentation analysis (Landmann, 2002; Landmann and Marbet, 2004). Third, background information can be reduced or eliminated before grouping of pixels to each color class. This can be performed by comparing the pixel intensities with the defined threshold set for each color, similar to the thresholding in colocalization analysis. This step can be integrated in the segmentation program and can also be performed with the Photoshop or other processing software. In the present study, the levels adjustment in the Photoshop was used to reduce the background and improve the image quality. In addition, noises can be reduced or eliminated by Photoshop and other softwares before segmentation.

Images with background without preprocessing can also be used for the segmentation analysis. However, in this case, there may be a problem of over- or under-segmentation of background information. For example, let us consider the background pixels (30, 34, 45) according to the RGB model. These background pixels will be grouped to cyan, which should be taken into account for the further analysis. This kind of mis-segmentation can also be observed when strong unspecific staining is present. The segmented results in one channel cannot be simply regarded as a single component containing only the signals for certain object of interest, which is similar to the case in the single staining. That is to say, in the single staining, we have to take into account all information, including background, signal, noise, and unspecific staining. For segmentation analysis, we also have to do the similar work for better interpretation of the image data.

Postsegmentation processing is also important to optimize the segmentation results. In our study, the images generated were first mapped with the defined color, e.g., (255, 255, 0) for the yellow color according to the RGB color model. After that they were further processed through smooth and brightness/contrast adjustment, which improved their visual quality. However, by applying this method, some information in the images, for example, the intensity ratio between the CD4 and CD25 antigen, may be lost after mapping the color on the segmented results.

Multicolor Imaging of Infected Mouse Spleen

Multicolor immunofluorescent staining is especially useful for studying the dynamics of a wide range of cell populations with respect to cellular immunity to patho-

gens. Since *L. monocytogenes* is a widely used bacterium for this kind of study, we have employed it for the demonstration of the efficiency of our multicolor staining and segmentation methods. The use of four sets of antibodies allows seven color analysis (six colors from segmentation plus one additional channel for bacteria) for the study of the various subpopulations of cells or cells needed for identification with two antigens.

Prior to the identification of FoxP3, the expression of CD4 and CD25 was used to define the cell population referred to as CD4+CD25+ regulatory T cells. In this case, the double-positive cells can be identified, with our method, by their magenta color, which represents the color addition of red from the staining of CD4 and blue from the staining of CD25. Therefore, our method provides a means of identifying cells by segmentation of cells in secondary color.

In the present studies, the CD4CD25 cells together with the CD4 cells formed cluster at the periphery of infection loci. The interaction of CD4 and CD4CD25 can be visualized if two pseudochannels are combined together. However, the use of CD25 as marker of T regulatory cell is a little problematic, since CD25 is also expressed on nonregulatory cells in the normal mouse spleen and in the immune response to pathogens, which has also been shown in our studies (Figs. 3C and 3D). In the future study, we will attempt to identify different subset of Foxp3-expressing cells by the combination of these three markers. For example, cells positive for CD4CD25Foxp3 can be identified through the addition color of these three antigens, which makes it possible to distinguish naturally occurring regulatory cells, induced regulatory cells, and other subsets of regulatory cells.

Macrophages are the cells that are mainly responsible for early events during *L. monocytogenes* infection since they are infected first and serve as a vehicle for bacteria multiplication and dissemination throughout the host. In the early stage following oral infection, the ERTR9 macrophages in the marginal zone of the white pulp of mouse spleen are involved in the uptake of *L. monocytogenes*. By 24 h after infection, ERTR9 macrophages form clusters in the infection loci both in the white pulp and red pulp. Many dendritic cells, which are also responsible for the uptake of bacteria, are observed in these infection loci. After segmentation of the images, five pseudochannels for five cell populations can be generated for the better visualization of the dynamics of the immune cell populations. Therefore, our method permits the investigation of various subsets of macrophages, dendritic cells, or other cells simultaneously in tissue samples for infection studies.

Potential Applications of the Multicolor Segmentation Approach

The multicolor images from confocal microscopy were used for the demonstration of the validity of our multicolor segmentation. Certainly, fluorescence microscopy can also be used for this kind of imaging and segmentation analysis. Confocal microscope has a higher spatial resolution power, but wild-field microscopy coupled with appropriate deconvolution procedures is also suitable for fluorescent imaging, and even has some advantages. One advantage is much less bleaching of the dyes, which is especially important for the imaging of living cells or tissues.

The principles of our labeling and segmentation analysis may find many applications in biomedical studies. Firstly, multicolor imaging/staining is useful not only in immunostaining, but also in many other fields of histological and pathological studies. Various labeling methods, such as fluorescent labeling, immunolabeling, or the use of fluorescent proteins (such as enhanced green fluorescent protein, yellow fluorescent protein) can be combined for the multicolor analysis of cell populations or structures. Secondly, this method can be used for studies of infection by various pathogens in host tissue, such as multiple bacterial infections in the same organ. Thirdly, it is an extremely useful procedure for *in vivo* or *in situ* live imaging in combination with cell transfer techniques. Different kinds of immune cells or labeled bacteria, which can later be identified by up to six colors, can be transferred to animals for the study of the immunodynamics of the infection process. Fourth, the method can be used in multicolor fluorescence *in situ* hybridization and other similar studies (Speicher et al., 1996). For example, up to seven different color channels for probe detection in the *in situ* hybridization can be used, which allows the simultaneous high resolution localization of multiple-point-like sources in biological specimens at a thickness of 30 μm (Maierhofer et al., 2003). However, appropriate selected fluorescent conjugated dyes and complicated filter sets are needed for this kind of analysis. With the color addition theory, multicolor FISH can be performed in a similar way with the color addition theory, in which only three set of dyes are necessary and no spectral linear unmixing is required.

In the near future, the proposed pixel-based clustering segmentation method will be improved for better and intelligent color segmentation. For example, in our method, no spatial relationship between the neighbor pixels was considered. Therefore, other image-based segmentation algorithms can be introduced to the segmentation program. For example, the region growing algorithm may be helpful for the improvement of the segmentation results. Let us consider the case in which a dendritic cell (red) lies under the extracellular matrix or fibers (green) in the spleen; the overlapping area will be yellow, which will not be distinguishable from the yellow bacteria by means of the program. The use of thinner sections will be helpful in reducing this false-positive effect, although it will make the visual-

ization of some larger cells or structures difficult. However, combination of the region growing algorithm with the clustering method may make the separation of dendritic cells and fibers more easily.

In conclusion, when combined with a variety of staining or labeling methods, the proposed segmentation approach described here should find many further applications in the multicolor analysis of cells and tissues in biomedical research fields.

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