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Six-Colour Fluorescent Imaging of Lymphoid Tissue Based on Colour Addition Theory

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Running headline: Six-colour imaging of tissue sample

Summary

Multi-colour imaging of immunofluorescently stained tissue with the confocal microscope was accomplished by using colour addition theory. This new technique includes several improvements for immunolabelling: (1) the use of the colocalization of two or more markers on one cell for the identification of specific cell populations; (2) the use of the colocalization of two fluorescent dyes from secondary reagents for the identification of the cells; (3) a multi-step staining protocol with two primary antibodies originating from the same host species or with two or three biotin-conjugated primary antibodies. After image acquisition, colour segmentation/unmixing are applied to the single multi-colour image to generate multi-pseudochannels for individual or colocalized fluorescent dyes. With this new technique, we have been able to visualize simultaneously six cell populations in the mouse lymph node and intestine. The efficiency of this method has also been demonstrated in the three-dimensional reconstruction of thick sections from mouse ileum. Our method is simple, efficient and may be indispensable in experimental cell and tissue studies requiring multiple immunolabelling.

KEYWORDS

Six-colour imaging; Immunofluorescent staining; Confocal microscopy; Colour addition

Introduction

Laser scanning confocal microscopy represents one of the most significant advances in optical microscopy, because of its improved spatial resolution over conventional bright field and fluorescent microscopy, the elimination or reduction of background information from the focal plane, and the capability to collect serial optical sections from thick specimens.

However, for simultaneous visualisation of multiple cell types in complex tissues by immunofluorescent staining for confocal microscopy, two general problems must be solved by histochemists. The first is the development of a reliable multiple immunolabelling method with primary antibodies often available from the same host species only or with haptenated antibodies carrying the same hapten. The second is the development of a reliable and generally applicable multi-colour immunofluorescent staining protocol for the multi-colour analysis of cell and tissue samples; only a limited number of fluorescent dyes are presently available for simultaneous application to a single sample in confocal microscopy because of the restrictions in the excitation wavelength of the lasers and the overlap of emission spectra of fluorescent dyes.

The application of multiple primary antibodies originating from the same host species is problematic because of the cross-reaction of secondary species-specific antibodies with the primary antibodies. To solve this problem, several approaches have recently been proposed (Buchwalow et al., 2005). These methods include: (1) the use of directly labelled primary antibodies (Tsurui et al., 2000); (2) a two-step protocol using two or three primary monoclonal or polyclonal antibodies (Lewis Carl et al., 1993; Negoescu et al., 1994; Shindler and Roth, 1996; Brouns et al., 2002); (3) the use of different haptenated primary antibodies (Tsurui et al., 2000; Brouns et al., 2002); (4) the use of specific anti-Ig isotype/subclass

secondary antibodies (Tidman et al., 1981; Suzuki et al., 2000; Buchwalow et al., 2005); (5) the use of immune complexes of primary antibody and the Fab fragment of the secondary antibody for staining (Ino, 2004); (6) the use of two or more biotin-conjugated primary antibodies (Ferri et al., 1999). The immune labelling of cell or tissue samples with either of these methods is however not without its limitations.

Multiple immunofluorescent staining is a powerful strategy for visualizing the spatial and temporal relationship between different antigens, cell populations and tissue components in histological sections. This approach has found widespread applications, including the tracing of dynamic cellular processes. However, because of the limitations of the dyes employed in confocal microscopy, only a few studies with four or more colour immune stains have been reported (Buchwalow et al., 2005). A seven-colour imaging technique using mainly directly labelled primary antibodies has been reported for the simultaneous detection of up to seven antigens on thymus tissue (Tsurui et al., 2000). With respect to multi-colour staining at the single-cell level, a four-colour labelling method has been described for the identification of mesenchymal stem cells (Schieker et al., 2004).

Colour addition is the process of mixing three primary colours, viz. green, red and blue, at variable intensities to generate a wide range of defined secondary colours, such as yellow, cyan and magenta. Based on this concept, we describe a new multi-colour imaging technique that is intended to solve the two above-mentioned problems in immunohistochemistry.

Materials and methods

Mice

The Balb/c mice (8-12 weeks old) used in all the experiments were obtained from Harlan Winkelmann (Borchen, Germany) and maintained under specific pathogen-free conditions.

Antibodies and reagents

The antibodies and reagents are shown in Table 1.

Immunofluorescent staining

Mice were sacrificed by CO₂ narcosis and organs were quickly taken out, embedded in Tissue-Tek® OCT Compound (Sakura Finetek, Torrance, CA, USA) and snap-frozen in liquid nitrogen. Cryosections (10 or 30µm) were prepared with a Reichert-Jung Frigocut 2800 Cryostat.

Sections were fixed at 4°C in methanol for 3 minutes and in acetone for 3 minutes, respectively. All the following steps were performed at room temperature. The sections were air-dried for 1 hour and then rehydrated for 10 minutes in phosphate-buffered saline (PBS). Unspecific streptavidin-binding sites and naturally biotinylated protein sites were saturated by using the Streptavidin/biotin blocking kit (Vector, Burlingame, CA, USA) according to the manufacturer's instructions. Non-specific antibody binding sites were blocked for 20 minutes with 2% goat serum (Sigma Chemical Company, St. Louis, MO, USA) in PBS. The sections were incubated for 1 hour with primary antibodies. The secondary antibodies were diluted and incubated with mouse serum (1% in antibody solution) for 10 minutes to avoid any cross-reaction of secondary antibodies with mouse proteins, including mouse immunoglobulin, on the sections. The sections were incubated for 30 minutes with secondary antibodies or reagents, washed with PBS, drained, air-dried and mounted with Kaiser's glycerol gelatine

(Merck, Darmstadt, Germany) using 24mmx60mm coverslips (Menzler-Gläser, Braunschweig, Germany)

Confocal microscopy

Confocal imaging was performed with a LSM META510 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-Apochromat 40x/1.0 oil immersion objective lens. After image acquisition, the images were adjusted and analysed by using LSM software.

Image processing and three-dimensional reconstructions

The images were processed with Adobe Photoshop CS. LSM software and Adobe Photoshop CS were used to segment the secondary colours. Three-dimensional reconstructions were performed with Imaging J from National Institutes of Health (NIH). The images from optical sections were exported to Adobe Photoshop CS as serial images (Format JPEG), processed and saved as JPEG documents. Three-dimensional projections were performed on this image sequence and the movie documents generated from the image stack were saved as uncompressed AVI files. The settings for the three-dimensional projection were: slice spacing (pixels): 1; rotation angle increment: 10; opacity: 0; surface depth-cueing: 50%; interior depth-cueing: 50%; projection methods: brightest. The files were then opened in Virtual Dub (Version 1.5.10, www.virtualdub.org), compressed, further processed and saved as normal AVI documents. The DivX Pro™ 5.1.1 Codec was used for the compression of these AVI files.

Results

General principles of the proposed multi-colour imaging technique

To perform the multi-colour imaging, several new approaches have been applied in our new technique. They include: (1) the use of multi-cell markers for the identification of the cells; (2) the use of two fluorophore-conjugated secondary antibodies against one primary antibody; (3) a multi-step protocol that permits the application of two antibodies of the same isotype; (4) colour segmentation /unmixing of multi-colour images. Compared with other methods, which are based on the linear or spectral unmixing of fluorophores, our method is built on the combination of fluorophores for the characterization of the antigen or cells. This has been carried out by the combination of immunohistochemistry and image processing, which involves the use of colour addition theory for image acquisition and colour segmentation/unmixing for the generation of pseudochannels for each antigen.

Colour addition means that secondary colours can be generated by mixing up to three primary colours together (Figure 1A). To apply this well-known principle in the development of our new technique, we demonstrated it in the immunolabelling of sections for confocal microscopy. This was performed by the immunolabelling of B cells in the mouse spleen. The sections were incubated with anti-B220, followed by a cocktail of secondary antibodies that included Alexa Fluor 488-, Alexa Fluor 546- and Alexa Fluor 633-conjugated goat anti-rat IgG. Images for these three fluorescent dyes are shown in Figure 1B (B1-B3). The results for colour addition of green, red and blue are shown in Figure 1C (C1-C3). The white colour, which is the product of the addition of all three primary colours, is shown in Figure 1D. This principle was also demonstrated for the streptavidin/biotin immunolabelling system (anti-

B220-biotin plus Alexa Fluor 488-, Alexa Fluor 546- and Alexa Fluor 635-conjugated streptavidin) and similar results were obtained (data are not shown). These results thus demonstrate that three primary colours for visualizing three dyes can be combined to generate defined secondary colours that can be used for the identification of a specific cell population.

Colocalization analysis in digital images is a typical “dual-images pixel point” process, between a pair of input images to generate a single output image (Shotton, 1993). In the present study, colour segmentation/unmixing have been performed in a similar way to the colocalization analysis. The basic idea is that all the voxels in both channels have the common events defined as signal intensity above a chosen threshold or within a certain range. This principle is shown in Fig 1E (for Fig 1C3). Analysis of the distribution pattern of intensity pairs allows for the identification of colocalization and for the discrimination of background, cross talk between channels, fluorescent attenuation and channel misregistration (Demandox and Davoust, 1997). All the voxels, which are in Region 3 in Fig 1E, are displayed in Figure 1F. This image can be regarded as an image generated from Figure 1C3 after background elimination.

Colocalization of two cell markers for the identification of cells

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. In our study, the colocalization of two cell-surface markers and colour addition theory were used for the identification of a specific cell population. For example, CD3 and CD4 molecules are expressed on a variety of cells. However, the study of coexpression of these two molecules could provide a reliable means of identifying T helper or regulatory cells in relationship to other CD3- or CD4-positive cells in a particular organ. To demonstrate this, spleen sections were used for staining the surface markers CD3, CD4 and B220. This was carried out by incubating sections with Alexa Fluor

488-conjugated anti-B220, anti-CD3e and anti-CD4-biotin followed by Alexa Fluor 633-conjugated goat anti-hamster IgG and Alexa Fluor 546-conjugated streptavidin. As shown in Figure 2A, T helper or regulatory cells that are positive for both CD3 and CD4 can be visualized in magenta, which is the product of the addition of blue and red. By using a similar approach, the relative expression of MHCII on B cells and macrophages was studied and the results are shown in Figure 2B. Cells positive for B220 and MHC II can be visualized in cyan. Moreover, with this approach, F4/80+MHCII+ macrophages can be identified with the second colour magenta in spleen red pulp (Figure 2C).

Use of two secondary antibodies against one primary antibody

To perform multi-colour staining, a combination of two fluorophores was used as new pseudo-fluorescent dye for the characterization of defined antigen or cell population. To demonstrate this, spleen sections were incubated with rabbit anti-mouse laminin, rat anti-B220 antibodies followed by an antibody cocktail of Alexa Fluor 633-conjugated goat anti-rat IgG, Alexa Fluor 488-conjugated goat anti-rabbit IgG and Cy3-conjugated goat anti-rabbit IgG. The results are shown in Figure 3A. Laminin-positive tissue components (the most abundant structural and biologically active component in basement membranes) are in yellow, which is the product of the addition of green and red. The colour segmentation method (Colocalization analysis) in the LSM software was used to extract the yellow components from the images to generate an additional “yellow channel”, which is similar to the image generated by combination of green and red channels (Figure 3B).

Use of two primary antibodies from the same host species

Application of two primary antibodies from the same host species is possible by using a new sequentially multi-step protocol. To demonstrate this, spleen sections were incubated with rat anti-B220 and hamster anti-CD3 antibodies followed by Alexa Fluor 488-conjugated goat

anti-rat IgG and Alexa Fluor 633-conjugated goat anti-hamster IgG. Subsequently, sections were incubated with rat anti-CD31 followed by Alexa Fluor 546-conjugated goat anti-rat IgG. As shown in Figure 4A, B cells could be visualized in yellow, CD3⁺ T cells in blue and blood vessels in red. Inside the T cell zone, a central artery can be observed. Splenic sinusoids can be visualized by the red CD31 stain in the red pulp. The staining mechanisms are shown schematically in online Supplementary Materials SF1. By a combination of Alexa 488 and Alexa 546, B cells and blood vessel were identified in yellow (green from Alexa 488 and red from Alexa 546) and red (red from Alexa 546), respectively.

With another similar approach, rat anti-B220, hamster anti-CD3 and rat anti-Ki67 were used to determine the proliferating cells in the B- and T-cell compartments in the spleen. Spleen sections were incubated with rat anti-B220 and hamster anti-CD3 followed by Alexa Fluor 488-conjugated goat anti-rat IgG and Alexa Fluor 633-conjugated goat anti-hamster IgG. Subsequently, sections were incubated with rat anti-Ki67 followed by Alexa Fluor 546-conjugated goat anti-rat IgG. The results are shown in Figure 4B. B cells receive a “green stain” from Alexa Fluor 488 and a “red stain” from Alexa Fluor 546-conjugated goat anti-Rat IgG. Therefore, they exhibit a yellow colour on their cell membranes. Since Ki67 is a nuclear protein marker used for detection of cells that are associated with cell proliferation, some replicating B cells have a yellow colour on their cell membrane and a red colour in the nucleus. The presence of CD3 and Ki67 in some T cells can also be observed. Although both anti-B220 and anti-Ki67 originate from rat, they can be visualized in different colours on one single section to study the proliferation of B cells and other cells under physiological and pathological conditions.

A similar approach was used for the staining of tissue samples with two or three biotin-conjugated antibodies to demonstrate the efficiency of this multi-step protocol in haptenated antibody systems. Anti-ERTR-9-biotin and anti-MOMA-1-biotin were applied to a single section to identify the macrophage subpopulations in marginal zones of the spleen (Figure

4C). This was carried out with a multi-step protocol. The sections were incubated with anti-B220-Alexa 488 and anti-MOMA-1-biotin, followed by Alexa 546-conjugated streptavidin. After washing with PBS, the sections then were incubated with anti-ERTR9-biotin, followed by Alexa 635-conjugated streptavidin. The staining mechanisms for this staining are presented in online Supplementary Materials SF2. ERTR-9 positive macrophages are located in a different region from MOMA-1-positive macrophages. Furthermore, three biotin-conjugated primary antibodies can also be applied on one section for the immunolabelling of three different populations of macrophages. For example, biotin-conjugated anti-MOMA-1, anti-macrophage marker F4/80 and anti-Mac-1 can be applied simultaneously to one section to visualize three macrophage subpopulations in the spleen (Figure 4D). Mac-1+ and F4/80+ macrophages, which are located mainly in the spleen red pulp, are revealed by their magenta and red colours, respectively. The MOMA-1-positive cells can be visualized in white, which is the product of the addition of red, green and blue.

Six-colour imaging of mouse lymph node and small intestine

Based on the improvements and modifications described above, a six-colour immunofluorescent staining method was developed for the simultaneous detection of six antigens in tissue samples. In the first example, two unconjugated rat anti-mouse primary antibodies (anti-B220, anti-CD31), one Syrian hamster anti-mouse antibody (anti-CD3e), two biotin-conjugated anti-mouse antibodies (Armenian hamster anti-CD11c and rat anti-ERTR-9) and one rabbit antibody (anti-LYVE1) were applied for six-colour imaging of the mouse inguinal lymph node in a four-step protocol. Sections were incubated with anti-CD31, anti-CD11c-biotin, anti-CD3 and anti-LYVE1, followed by Alexa Fluor 633-conjugated goat anti-rat IgG, Alexa Fluor 635-conjugated streptavidin, Alexa Fluor 633-conjugated goat anti-Hamster IgG, Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 546-conjugated goat anti-rabbit IgG. After washing with PBS, the sections were incubated with

anti-ERTR-9-biotin and anti-B220 followed by Alexa Fluor 546-conjugated streptavidin, Alexa Fluor 488-conjugated goat anti-rat IgG. The results are shown in Figure 5A. The antibodies against B220, CD3, ERTR-9 and CD11c stain B cells, T cells, medullary macrophages and dendritic cells, respectively. Blood vessels and lymphatic vessels are revealed by the antibodies against CD31 and LYVE1. The staining process and mechanisms for this staining are presented in online Supplementary Materials ST1, ST2. Since lymphatic endothelial cells are also weakly positive for CD31 (data not shown), a faint blue component is seen associated with the lymphatic vessels; this must be taken into account in the imaging analysis. By using the colour segmentation methods, all six components can be extracted to form six pseudo-channels for the representation of six cell populations or components. The yellow and cyan pseudo-channels generated for the lymphatic vessels and blood vessels are shown in Figure 5C. The segmentation of these two structures was performed by using the **Calculations** functions of RGB channels (In Menu Image) in the Adobe Photoshop CS.

A similar protocol was used for the immune staining of murine ileum sections. Two unconjugated rat anti-mouse primary antibodies (anti-B220, anti-CD31), two biotin-conjugated hamster anti-mouse antibodies (anti-CD3-biotin and anti-CD11c-biotin) and two rabbit antibodies (anti-LYVE1 and anti-PGP9.5) were used for the simultaneous detection of nerve fibres, blood and lymphatic vessels and three other immune cell populations in the section of mouse ileum (Figure 5B). The section was incubated with rat anti-B220, anti-CD3-biotin and rabbit anti-LYVE1 followed by Alexa 488-conjugated goat anti-rat IgG, Alexa 635-conjugated streptavidin and Cy3-conjugated goat anti-rabbit IgG. After washing with PBS, the sections were incubated with anti-CD11c-biotin, rat anti-CD31 and rabbit anti-PGP9.5 followed by Alexa Fluor 546-conjugated streptavidin, Alexa Fluor 633-conjugated goat anti-rat IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG. B cells are in cyan, T cells are in magenta, lymphatic vessels are in yellow, blood vessels are in blue and the PGP9.5+ nerve fibres are in green. Anti-PGP9.5 was used to study the distribution of nerve

cells/fibres in the lamina propria of villi of the ileum, because they may also play an important role in fine tuning of peripalsis, in activity regulation of intestinal endocrine cells and in modulation of mucosal immunity. By using the Imaging J software, a three-dimensional reconstruction of this thick ileum section was performed on the images from the optical sections. The three-dimensional architecture of a villus of murine ileum is presented in online Supplementary Materials SM1, SM2.

Discussion

In the present study, we have described a new multi-colour immunofluorescent staining technique based on colour addition theory. The basic idea of this method is to use colocalization of two or more fluorescent dyes for the generation of additional colour channels to allow the identification of cells or of tissue components. This new technique, which is a combination of immunohistochemistry and image processing, can be summarized as follows: multi-dyes (three series of dyes), three channels and three colours in versus one multi-colour image and multi-pseudochannels out. It enables us to perform up to six-colour immunolabelling in an easy and reliable way. To our knowledge, we are the first to accomplish this kind of six-colour indirect immunofluorescent staining for confocal microscopy.

Colocalization of fluorescent dyes for the identification of cells

Colocalization analysis is a powerful tool for the demonstration of spatial and temporal overlap in the distribution patterns of fluorophores. In the present study, the application of colocalization has been “stretched” to the multi-colour imaging of cells or tissue samples. The first improvement is the use of multiple cell markers for the characterization of cells. Since few cells have an exclusive cell marker, two or more cell markers can be combined together for the accurate identification of cells in histological sections. The colocalization of two markers means that this cell will have a secondary colour that is the product of the addition of two primary colours originating from two fluorescent dyes. For example, detection for the CD4 and CD25 can be combined and the secondary colour generated can be used for the identification of T regulatory cells. The second is the combination of two fluorophore-

conjugated secondary antibodies against one primary antibody to generate a pseudo secondary colour marker. Because of the limitations of the dyes that can be applied in confocal microscopy, the generation of the secondary colour is very helpful. Furthermore, the use of spectral overlapping fluorescent dyes, such as Alexa 546 and Texas Red on the same section or same cell can be avoided by using our multi-colour labelling method. The colour separation method can make two colours distinguishable for the identification of cells. For example, let us consider two primary colours A and B. Colour C is the product of colour addition of A and B. A and B are proposed to identify two types of cell, respectively. With the normal protocol and because of cross-reaction, both cells have a mixed colour of A and B, which therefore cannot be used for cell identification. With our methods, Colour A and C are used to identify these two cells. Although C has colour components from A, it can be “unmixed” from A. Since the two colours A and C are distinguishable, they can be used to identify these two kinds of cells or tissue components. Therefore, in our method, colocalization and colour segmentation based on it provide a more refined and convenient approach for the identification of cells.

Immunolabelling with two primary antibodies from the same host species

The cross-reaction of secondary specific antibodies or reagents with primary antibodies makes it difficult to apply two or more primary antibodies or haptenated antibodies to one single section.

The first solution is the use of directly labelled primary antibodies or antibody complex to avoid this kind of cross-reaction. Since directly labelled primary antibodies with special dyes are not always commercial available and large amounts of antibodies are needed for labelling, this method cannot be generally applied. Furthermore, the sensitivity of antibody detection can be reduced because the signal amplifications in the indirect methods are not available in direct immunolabelling. Another alternative is the use of immune complexes of the primary

and secondary antibodies for the direct labelling of antigens (Ino, 2004). However, soluble immune complexes often show reduced or no immunoreactivity to antigens on sections. Some methods, e.g. the antigen retrieval of tissues by heating, may improve immunoreactivity for soluble immune complexes. This method is based on similar principles with the new Zenon Technology (Molecular Probes, Invitrogen, Eugene, OR, USA), viz. of complexing primary antibodies with dye-labelled or enzyme-labelled Fab fragments of secondary antibodies directed against their distinct Fc regions (Bradford et al., 2004; van Duijnhoven et al., 2005). The primary antibody types that can be recognized and detected by Zenon labelling reagents are determined by the immunospecificity of the Fab fragments from which they are prepared. Simple mixing of the Zenon reagent with an intact primary antibody forms a labelling complex rapidly and quantitatively. This has some advantages, e.g. it is economical and the complex is easy to prepare and can be readily employed in quantitative labelling. It also permits the application of multiple primary antibodies from the same host species in the same immunolabelling protocol, as in our methods. However, the labelling efficiency with this technique may be reduced when Zenon Labelling Kits are used selectively to label antibodies in hybridoma supernatants and samples containing serum. Furthermore, in order to generate the immune complex for direct labelling, the primary antibodies are mixed with Fab fragment of secondary antibodies and then a non-specific IgG is used to complex the unbound Fab fragments of the secondary antibodies. The efficiency of this neutralization can affect the staining of cells or tissue samples. Another limitation of this technology is that, because of the lower immunoreactivity of the immune complex and antigens, the signals are sometimes weaker than that of the stain when used in indirect methods. We have confirmed this in one of our studies by staining human cells with two mouse antibodies and the Zenon labelling Kit (data are not shown). In general, labelled antibodies with Zenon labelling reagents are similar in brightness to directly labelled (i.e. chemically labelled) antibody. The brightness of the Zenon complex can be adjusted somewhat by changing the amount of Zenon labelling reagent

used during the labelling process. Therefore, due to the above-mentioned limitations, these methods must be confirmed before their application to the multi-colour immunolabelling in future studies.

The second approach is to use different detection systems for the primary antibodies. For example, biotin- and digoxigenin-conjugated antibodies can be used simultaneously for immune labelling (Tsurui et al., 2000). In addition, tyramide signal amplification, a highly sensitive method allowing the dilution of the first antibodies far beyond the detection limit of fluorescently labelled secondary antibodies, has also been used for this purpose (Brouns et al., 2002). Nevertheless, these methods can only be applied in certain situations.

The third approach involves indirect staining with specific anti-Ig subclass antibodies when they are available. A general applicable protocol has been proposed and four-colour staining has been accomplished on sections of papillary lesion of human mammary gland immunostained with three primary mouse antibodies (IgG 3, IgG1, IgG 2a) and a rabbit antibody (Buchwalow et al., 2005). This method can only be applied when the primary antibodies originating from the same species have different isotypes and when high specific anti-Ig subclass antibodies are available. However, it cannot be used with monoclonal antibodies from the same species and isotype (e.g. anti-B220 and anti-CD31 in this study) or with polyclonal antibodies (e.g. anti-LYVE1 and anti-PGP9.5).

The fourth approach is by indirect staining in a two-step protocol. This can be performed in several ways. (1) The monovalent Fab fragment of the antibodies or the antibodies themselves are used to saturate the remaining binding sites of the primary secondary antibodies. This method relies on the efficiency of the monovalent antibodies and on the saturation of the remaining binding sites. Indeed, both polyclonal and monoclonal antibodies from the same host species have so far been applied in the immunolabelling of single sections (Moore and Fay, 1992; Lewis et al., 1993). We have also tested this method in our study of both primary antibodies and biotin-conjugated antibodies. Our results indicated that cross-reactions

couldnot be completely avoided (data are not shown). For example, anti-CD31 and anti-B220 were used to study spleen sections in a similar two-step protocol but the blood vessel and B cells were not clearly distinguishable. The situation is similar when applying two or more biotin-conjugated antibodies (data are not shown). On the contrary, by our method, these kinds of cross-reactions are used for the immunolabelling of the cells. This technique can be described as a multi-step staining protocol: application of the first stain (primary antibody + secondary antibody), then the second stain, followed by the third stain. The secondary antibody in the second stain will also bind to the primary antibody in the first stain (SF1A, ST1). The primary and secondary antibody in the second stain can probably also associate with secondary antibody in the first stain (online Supplementary Materials SF1B, ST2). The same applies when using two or more haptenated antibodies (online Supplementary Materials SF2A, SF2B, ST1 and ST2).

Multi-colour imaging of cells or tissue samples

To perform multi-colour imaging, one of the methods that can be employed is the direct labelling of some or all primary antibodies. With this method, seven-colour imaging of thymic tissues with an epifluorescence microscope was carried out and seven channels for seven antigens CD8, TCR β , CD4, I-Ad/I-Ed, CD44, CD18 and CD11c could be clearly separated (Tsurui et al., 2000). However, this method requires the availability of individual primary antibodies with properly selected dyes, suitably designed filter sets and complicated image processing. A similar approach is the combination of Zenon technology and linear or spectral unmixing methods. If fluorophores are not only visible in one channel but ‘bleed through’ into the next channel, they can be linearly unmixed (Zimmermann, 2005), i.e. spectrally overlapping fluorescent dyes (e.g. Alexa 546 and Texas Red), which cannot be separated by using standard filter sets, can be linearly unmixed in the staining of one single cell (Schieker et al., 2004).

Our colour addition-based method has several advantages compared with the methods described in literature. It provides a solution for selective immunolabelling with two or more primary antibodies from the same host species (even the same isotype) or antibodies carrying the same hapten. All the reagents are commercially available and no labelling of antibodies is necessary prior to staining. There is no need for a UV laser or additional filter sets and instruments. Only standard image processing software is required.

For image or data acquisition, two things must be taken into account for efficient colour addition. (1) Colour addition also means the addition of two colours in every single voxel. Both colours should be imaged in the same dynamic range (full 8 or 16 bit) in order to apply our new techniques; otherwise the information will be difficult to interpret. (2) During data acquisition, a defined correlation of both colours is important for colour addition. This can be achieved in the several ways. The first is optimization of the antibody concentration. For example, to detect listeria and LYVE-1, the rabbit anti-listeria antibodies can be used at a lower concentration than in normal single staining, since the immunostaining of listeria in histological sections is usually very strong. The second is the optimization of instrument settings during image acquisition. For example, detector gain and amplifier offset in confocal microscopy must be optimally adjusted for this purpose. In some brightfield fluorescent microscopes, similar settings, e.g. black level and sensitivity, have to be adjusted for better colour addition. The black level here is similar to the amplifier offset in confocal microscopy. The third is the adjustment of contrast, brightness or other parameters by using LSM software or other programs.

Background, which is present to variable degrees in any image, is a major factor restraining the power of colour addition, colour segmentation and colocalization analysis. It can be reduced through the optimization of the staining protocol or instrument settings in order to obtain images that have a lower background and a high signal/noise ratio. For example, by using the Alexa Fluor dye conjugates that are more fluorescent and more

photostable than their commonly used spectral analogues, such as fluorescein, rhodamine 6G, Cy3 (Panchuk-Voloshina et al., 1999), background can be reduced to a low level and signal/noise ratio can be improved. Another method to reduce the background is to employ quantum dots instead of normal fluorescent dyes (Giepmans et al., 2005). Quantum dots have several advantages over conventional fluorophores for light microscopic imaging including their higher brightness and photostability. Another advantage is that they can be excited by one laser line and they have much narrower emission spectra than the normal dyes.

However, high intensities of background will occasionally make our colocalization analysis and colour addition less efficient. Various methods can be proposed to solve this problem. High densities background can be successfully suppressed by low pass, median and Gaussian filters (Demandolx and Davoust, 1997). A great advantage is their ease of handling and their speed. The disadvantage is that some information may be lost after filtering. False-negative or false positive results can be generated. We have confirmed this in our study of three-dimensional reconstructions of thick sections of mouse ileum. When median filters were applied to the image sequences from optical sections from confocal microscopy, some T cells in a magenta colour, which was the product of the addition of blue and red, were not observed compared with the original images without filter processing (data are not shown). Another possible method is image restoration with deconvolution (Landmann and Marbert, 2004). Deconvolution (Shaw and Rawlins, 1991; Van der Voort and Strasters, 1995) makes use of the image properties of the optical systems in the form of point spread function for “putting the light back where it is coming from”. The general principle of this method for our application is shown in Fig1E. A threshold is chosen to define the background level. The voxels in Region 3 are voxels in which red and blue fluorescent dyes colocalize. By using this method, background can be removed almost entirely without substantial loss of useful information in the images. This is indeed a good method and images in our study have been optimized by using principles of this method. This restoration of images can be performed by

the colocalization analysis in some softwares. Similar processing can also be performed by using the normal Adobe Photoshop. For example, the adjustment in Levels or Curves (In Menu Image) in this software can be used to suppress the background. However, since this method depends on the threshold set for the background level, the discrimination of signal from background is crucial for this method. This can be achieved by adjustment of all the photomultipliers to their respective channel intensities followed by the recording of control specimens probed with one fluorochrome only as a multichannel data set (Landmann and Marbet, 2004). When the problems caused by the background are solved, our methods can also be efficiently applied to fluorescent microscopy. The images from fluorescent microscope should be transferred to greyscale images and assigned with different colours in order to perform the colour addition.

Since our colour segmentation methods have some principles in common with the linear or spectral unmixing methods, we have named it “Colour Unmixing”, which means that, instead of separating the mixture of spectra directly detected by the detectors in confocal microscopy, we used secondary Colour (A+B) and primary Colour A to distinguish two antigens. Since six colours in one single image are not easy to visualize and will make the characterization of multi-antigens less convincing, multi-pseudo channels can be generated by using colour segmentation/unmixing methods. Not only the normal three secondary colours, but also other colours, e.g. bright green, which according to the RGB presentation is (102, 255, 0), can be also used for the identification of cells. Colour addition can also therefore be applied to different intensities of colour. The principles of segmentation for our method are presented in Fig.6.

Applications of multi-colour immunofluorescent staining

Multi-colour imaging is a powerful tool for revealing different cells in relation to their tissue environment. In the present study, four cell populations in addition to blood and

lymphatic vessels in the medullary region of mouse lymph node have been simultaneously visualized on one single section. Some ERTR-9 positive medullary macrophages are located in the medullary sinus of the lymph node. In this staining, the two primary antibodies are derived from hamster but are from Armenian hamster (for anti-CD11c-biotin) and Syrian hamster (for anti-CD3e), respectively; no cross-reaction between goat anti-Syrian hamster IgG and Armenian hamster anti-mouse CD11c antibodies has been observed in another study (data are not shown). In the staining of ileum, two polyclonal antibodies originating from rabbit were used to study the relative distribution of nerves and lymphatic vessels in the villus of mouse ileum. By combining other markers, we were able to visualize six cell populations in a small area of the functional unit of mouse intestine and to determine their relative distribution. We expect that the study of the location of a variety of functionally distinct cell types and tissue components by our method will facilitate the estimation of cell-cell or cell-matrix interactions in vivo.

In this study, a three-dimensional reconstruction of a thick section of ileum was performed to demonstrate the efficiency of our method in this kind of application. In the near future, we will apply this method in three dimensional reconstructions of serial thin sections from mouse small intestine and other organs. This will make it possible to visualize six cell populations or tissue components in one single organ in three-dimensional models. This will allow the evaluation of statistically significant numbers of individual structures while retaining their localization within the organ and with respect to each other.

Although our method has been developed mainly using lymphoid and intestinal tissues, it can also be used in other tissues or at the single cell level. For cell culture studies our method also has some advantages, when identification of specific cell types and states relies on the study of coexpression of multiple antigens. For example, for the identification of mesenchymal stem cells, when four antibodies rat anti-collagen-I, rabbit anti-collagen-IV, goat anti-fibronectin and mouse anti-CD44 are applied and visualized by FITC, Alexa 546,

Texas Red and AMCA conjugated to secondary antibodies, linear unmixing of Alexa 546 and Texas Red had to be performed (Shieker et al., 2004). However, using our method linear unmixing would not be required and the four primary antibodies did not have to originate from four different host species.

In summary, we describe a simple and efficient technique to perform multi-colour immunofluorescent staining. This technique can be applied not only in confocal microscopy, but also in normal fluorescence microscopy. We expect that this technique can be widely used in immunohistochemical studies of a variety of cells and tissue samples.

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Legends to figures

Figure 1. Colour addition principle in RGB colour model and its application in confocal microscopy. (A) The three primary colours, red, green and blue can be mixed together to generate secondary colours, yellow, magenta, cyan and white. (B)-(D) Immunolabelling of B cells in splenic follicle with anti-B220 and three Alexa dye-conjugated secondary antibodies. Scale bar: 20 μ m. (B) Three channels for Alexa 488, Alexa 546 and Alexa 633 are shown in the three primary colours green (B1), red (B2) and blue (B3), respectively. (C) Different combinations of two of three channels can yield secondary colour pseudo-channels yellow (C1, green and red), cyan (C2, green and blue) and magenta (C3, red and blue). (D) The merged picture is in white because of the addition of the three primary colours. (E) Principle of colocalization analysis. Colocalization analysis of topographically corresponding voxels from two channels in Image C3 by using the LSM software. Every single voxel is characterized by two (e.g., red and blue) intensities that are used for arranging it in a two-dimensional histogram along a red (x-axis, intensities from 0-255) and a blue (y-axis, intensities from 0-255). The absolute frequencies of voxels are displayed by using different colours (colour bar, bottom). The intensities of red and blue of voxels in the two channels in

Region 3 are above a threshold level set for discriminating the background from signals. (F) This image from Region 3 can be regarded as the image of C3 after background suppression.

Figure 2. Colocalization of two cell markers for the identification of specific cell population in mouse spleen. (A) B220 (green), CD3 (blue) and CD3CD4 (magenta) stained B cells, T cells and CD3+CD4+ T helper or regulatory cells, respectively. Scale bar: 20 μ m. (B) B220 (green), Mac-1 (red) and MHCII reveal B cells and Mac-1+ positive macrophages and MHCII expression on all cells. B cells in merged picture are in cyan, because of the colocalization of MHCII (blue) and B220 (green). No colocalization of MHCII and Mac-1 can be observed. Scale bar: 20 μ m. (C) Colocalization of MHCII and F4/80 in spleen red pulp. MHCII+ cells are blue, F4/80+ cells are in red, while F4/80+MHCII+ cells are in magenta. The cells in blue in the merged picture are B cells or other cells in spleen red pulp. Scale bar: 10 μ m

Figure 3. Colocalization of two fluorescent dyes for the identification of tissue components in mouse spleen. (A) Alexa 488- and Cy3-conjugated goat anti-rabbit IgG are used to reveal the laminin positive “skeleton” of mouse spleen (yellow in the merged micrograph). This includes fibres, vessels, extracellular matrices and other tissue components. (B) Two similar additional yellow channels are generated with two different methods. The first (top) is a simple combination of the green and red channels. The second (bottom) is an extraction of the yellow components in the merged picture by using the method described in the colocalization analysis (Figure 1E). Scale bar: 20 μ m.

Figure 4. Application of two primary antibodies originating from the same host species, or two or three biotin-conjugated primary antibodies for immune staining on one single section. (A) CD3 (blue), B220 (yellow) and CD31 (red) staining of T cells, B cells and blood vessels

(including splenic sinusoids) in mouse spleen, respectively. A central arteriole (**CA**) is surrounded by T cells (**PALS**, periarteriolar lymphoid sheath). Scale bar: 50µm. (B) CD3 (blue), B220 (yellow) and Ki67 (red) staining of T cells, B cells and cells associated with proliferation, respectively. A B220+Ki67+ cell is indicated by the arrow. Some T cells that are associated with cell proliferation can also be observed. Scale bar: 10µm. (C) Identification of spleen marginal macrophages by using biotin-conjugated anti-ERTR-9 and anti-MOMA-1 antibodies. ERTR-9+ macrophages (blue) are located outside whereas the MOMA-1+ macrophages (Metallophilic macrophages, magenta) lie inside the marginal zone. Scale bar: 20µm. (D) Three biotin-conjugated anti-mouse macrophages markers identify three macrophage populations in the mouse spleen. MOMA-1+ macrophages are in white, Mac-1+ cells are in magenta, whereas F4/80+ macrophages are in red. Anti-B220 (green) stains B cells in spleen white and red pulp. Scale bar: 20µm.

Figure 5. Six-colour imaging of mouse inguinal lymph node and ileum. (A) B220 (green), CD3 (blue), ERTR-9 (red), CD11c (magenta), CD31 (cyan) and LYVE1 (yellow) staining of B cells, T cells, medullary macrophages, dendritic cell, blood vessel and lymphatic vessel in the medullary region of mouse lymph node, respectively. Some ERTR-9-positive macrophages lie inside the medullary sinus. Scale bar: 20 µm. (B) B220 (cyan), CD3 (magenta), CD11c (red), CD31 (blue), LYVE1 (yellow) and PGP9.5 (green) staining of B cells, T cells, dendritic cells, blood vessels, lymphatic vessels and nerve fibres in one villus of mouse ileum, respectively. Two B cells and two dendritic cells are indicated by arrows and stars, respectively. Scale bar: 10µm. (C) Segmentation of lymphatic vessel (yellow) and blood vessel (cyan) from the image of Figure 5A using the Adobe Photoshop CS.

Figure 6. Principles of colour segmentation. (A) The values of three colours span a 3D colour space (i.e. colour cube), thus every pixel can be regarded as a 3D vector in this space. At each corner of the colour cube is a representative of one colour class (red, green, blue,

yellow, magenta, cyan, white). (B) Each pixel is classified in up to six (or seven) classes by determine the class representative with minimal angular deviation. After colour classification/segmentation, an intensity value can be obtained by projecting the colour vector of each pixel onto its class representative (using the scalar product).

Online Supplementary Materials

Figure SF1. Application of two rat anti-mouse primary antibodies to a single section in a four-step protocol. (A) Mechanism I for colour addition in this staining protocol. The secondary antibody, Alexa 546-conjugated goat anti-rat, in the fourth step binds with both primary antibodies anti-B220 and anti-CD31 antibodies. (B) Mechanism II for colour addition in this staining protocol. The secondary antibody, Alexa 488-conjugated goat anti-rat, has two binding sites for rat antibody. One is occupied by anti-B220; the other can also bind anti-CD31 in the third step. In the last step, Alexa 546-conjugated goat anti-rat binds with anti-CD31 to the blood vessel and anti-CD31 associated with the anti-B220/goat anti-rat IgG complex. The final staining results are probably generated through a combination of Mechanism I and Mechanism II. Finally, T cells, B cells and blood vessel are visualized in blue, yellow and red, respectively.

Figure SF2. Application of two biotin-conjugated primary antibodies on one single section in a four-step protocol. (A) Mechanism I for colour addition in this staining protocol. The secondary reagent, Alexa 635-conjugated streptavidin in fourth step, binds with both anti-ERTR-9-biotin and anti-MOMA-1-biotin. (B) Mechanism II for colour addition in this

staining protocol. The secondary reagent, Alexa 546-conjugated streptavidin has four biotin-binding sites. One is occupied by anti-MOMA-1-biotin; the others can also bind with anti-ERTR-9-biotin in the third step. In the last step, Alexa 635-conjugated streptavidin binds to anti-ERTR-9-biotin on the ERTR-9-positive macrophages and also anti-ERTR-9-biotin associated with the complex of anti-MOMA-1-biotin/Alexa 546-conjugated streptavidin on the MOMA-1-positive macrophages. The final staining results are probably generated through a combination of Mechanism I and Mechanism II. Only two molecules of biotin on the biotin-conjugated primary antibodies are shown in this Figure.

Table ST1. Staining mechanisms I of colour addition for six-colour imaging of mouse lymph node.

Table ST2. Staining mechanisms II of colour addition for six -colour imaging of mouse lymph node.

Movie SM1. Animation of 18 optical slices (Z stack) taken by confocal microscopy.

Original images from a 30µm thick cryosection of mouse ileum were transferred to Imaging J and processed by the methods described in Material and methods. B220 (Cyan), CD3 (Magenta), CD11c (Red), CD31 (Blue), PGP9.5 (Green). Scale bar: 10µm.

Movie SM2. Three-dimensional view (rotation along Y axis) of a villus from the same section as that in Movie SM1.