Immunology Letters 161 (2014) 1–5

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Easy performance of 6-color confocal immunofluorescence with 4-laser line microscopes

Nathalie Eissing[a,1], Lukas Heger[a,1], Anna Baranska[a], Robert Cesnjevar[b], Maike Büttner-Herold[c], Stephan Söder[d], Arndt Hartmann[d], Gordon F. Heidkamp[a,*,2], Diana Dudziak[a,*,2]

[a] Friedrich-Alexander Universität Erlangen-Nürnberg, Universitätsklinikum Erlangen, Department of Dermatology, Laboratory of Dendritic Cell Biology, Erlangen, Germany
[b] Friedrich-Alexander Universität Erlangen-Nürnberg, Universitätsklinikum Erlangen, Department of Paediatric Cardiac Surgery, Erlangen, Germany
[c] Friedrich-Alexander Universität Erlangen-Nürnberg, Universitätsklinikum Erlangen, Department of Nephropathology, Institute of Pathology, Erlangen, Germany
[d] Friedrich-Alexander Universität Erlangen-Nürnberg, Universitätsklinikum Erlangen, Department of Pathology, Erlangen, Germany

ARTICLE INFO

Article history:
Received 5 February 2014
Received in revised form 27 March 2014
Accepted 1 April 2014
Available online 12 April 2014

Keywords:
6-Color
Confocal immunofluorescence
4-Laser
Stokes shift
Human
Murine
Tissue
Pathology
Dendritic cell

ABSTRACT

Confocal laser scanning microscopy is an advanced technique for imaging tissue samples *in vitro* and *in vivo* at high optical resolution. The development of new fluorochrome variants do not only make it possible to perform multicolor flow cytometry of single cells, but in combination with high resolution laser scanning systems also to investigate the distribution of cells in lymphoid tissues by confocal immunofluorescence analyses, thus allowing the distinction of various cell populations directly in the tissue. Here, we provide a protocol for the visualization of at least six differently fluorochrome-labeled antibodies at the same time using a conventional confocal laser scanning microscope with four laser lines (405 nm, 488 nm, 555 nm, and 639 nm laser wavelength) in both murine and human tissue samples. We further demonstrate that compensation correction algorithms are not necessary to reduce spillover of fluorochromes into other channels when the used fluorochromes are combined according to their specific emission bands and the varying Stokes shift for co-excited fluorochromes with the same laser line.

1. Introduction

Typical microscopic analyses allow the simultaneous visualization of three to four different cellular markers using fluorochrome-labeled antibodies, which contrasts with multiparameter flow cytometry – or newly developed mass spectrometry-based analyses [1–3]. One recently invented technology is the imaging flow cytometry, which combines the advantages of traditional FACS analysis with sensitive fluorescence imaging of cells in flow [4]. Another resourceful method, the Multi-Epitope-Ligand-Cartography technology (MELC) allows collecting information of up to 100 molecules in tissues or single cells [5,6]. Here, signals could be collected with select FITC- or PE-labeled antibodies in serial application/bleaching steps. Unfortunately, this technique is limited to non-confocal detection and the application of antibodies with high binding affinity for signal amplification is not feasible.

Due to the invention of new fluorochromes used for labeling of antibodies and the generation of new fluorescent proteins, recent advances have been achieved in the detection of cellular proteins by multicolor immunofluorescence microscopy [5,7–10]. Remarkably, Kongure et al. were able to excite six transiently transfected fluorescent proteins (CFP, mMiCy, EGFP, YFP, dKeima570, and mKeima) with comparable excitation maxima and sufficiently large Stokes shift [10]. Thus, six fluorescent proteins were excited by one laser

Abbreviations: BDCA, blood dendritic cell antigen; DCs, dendritic cells; FITC, fluorescein isothiocyanate; MARCO, macrophage receptor with collagenous structure; mDC, myeloid dendritic cell; PE, phycoerythrin; SA, streptavidin.

* Corresponding authors at: Friedrich-Alexander Universität Erlangen-Nürnberg, Universitätsklinikum Erlangen, Department of Dermatology, Laboratory of Dendritic Cell Biology, Research Campus Module II, Hartmannstrasse 14, 91052 Erlangen, Germany. Tel.: +49 91318539346.
E-mail addresses: gordon.heidkamp@uk-erlangen.de (G.F. Heidkamp), diana.dudziak@uk-erlangen.de (D. Dudziak).
[1] These authors contributed equally to this work.
[2] These authors are co-senior authors.

http://dx.doi.org/10.1016/j.imlet.2014.04.003
0165-2478/

line (458 nm) and emitted fluorescence was detected in subcellular structure localizations as separate signals. However, due to the high spillover between the emission bands and the restriction to biochemical applications, some tissue types, in particular human tissue cells in health and disease, are less prone for such analyses.

Recently, a sophisticated study of murine lymph node dendritic cell (DC) subpopulations demonstrated five laser line based co-staining of six different fluorochrome-labeled antibodies in single tissue sections [8]. Although commercially available microscopes were used to discriminate numerous fluorochromes on tissue sections at high resolution and cellular positioning was excellently examined by histo-cytometry, further application of compensation algorithms for correction of spillover and deconvolution might limit the advantages of this method for the daily laboratory use.

Here, we provide detailed protocols for the application of up to six differently fluorochrome-labeled antibodies in murine and human tissue sections for the simultaneous detection of lymphoid cell populations using a four-laser-line microscope. We further demonstrate that select combinations of different fluorochrome-labeled antibodies reduce cross-talk of the different laser lines and allow for fast and direct visualization of several cell subsets in one tissue section without application of advanced mathematical algorithms.

2. Materials and methods

2.1. Murine and human samples

Six to eight weeks old female C57BL/6 mice were purchased from Janvier (France) and maintained under specific pathogen-free conditions according to institutional and national guidelines. Human spleen samples from patients requiring therapeutic splenectomy were collected following national ethical guidelines regulating the use of human tissues, with informed consent obtained in accordance with the Declaration of Helsinki (Ethical approval Ethical committee Erlangen: no. 3761).

2.2. Staining of human and murine tissues

The different tissues blocks (8 mm × 8 mm × 5 mm) were washed in PBS, dried with kimwipe (KIMWPIE, Kimberly-Clark corporation, USA), embedded in O.C.T. Tissue Tec (Sakura Finetechnical Co., Tokyo, Japan) frozen on dry ice and kept at −80 °C till further usage. Murine and human spleen blocks were cut into tissue slices of 10 μm using a cryotome (JUNGCM 3000, Leica, Bensheim, Germany), fixed in acetone for 2 min at room temperature and dried for at least 12 h. Before staining, the tissue slices were rehydrated in PBS (Invitrogen) for 10 min, and endogenous biotin was blocked using streptavidin/biotin solution (AbD Serotec, Düsseldorf, Germany). Murine spleen samples were stained with the following antibodies: anti-B220 (CD45R) Alexa Fluor® 647 (1:50, clone: RA3-6B2, BioLegend); anti-CD3e eFluor® 450 (1:50, clone: 145-2C11, eBioscience); anti-CD11c PE (1:50, clone: HL3, BD Biosciences) amplified with goat IgG anti-PE (1:400, polyclonal, Novus Biologicals) and anti-goat Cy3 (1:200, Dianova/Jackson Immuno Research), anti-CD4 Biotin (1:50, clone: GK1.5, eBioscience) secondarily stained with Streptavidin-PacificOrange™ (1:100, life technologies); anti-MARCO Alexa Fluor® 488 (1:50, clone: ED31, Serotec) and anti-CD8 PerCP-eFluor® 710 (1:25, clone: 53–6.7, eBioscience). Human tissue sections were stained with the following antibodies: anti-CD11c Alexa Fluor® 647 (1:25, clone: 3.9, BioLegend); anti-CD11c Horizon™ V450 (1:50, clone: ICRF44, BD Bioscience), anti-BDCA1 (CD1c) PE (1:50, clone: AD5-8E7, Miltenyi Biotech) amplified with goat IgG anti-PE (1:400, polyclonal, Novus Biologicals) and rabbit anti-goat Cy3 (1:200, Dianova/Jackson Immuno Research); anti-CD19 Brilliant Violet™ 650 (1:25, clone: HIB19, BioLegend), anti-CD3 Alexa Fluor® 488 (1:50, clone: UCHT1, BioLegend), anti-HLA-DR Biotin (1:50, clone: G46-6, BD Bioscience) secondarily stained with Streptavidin-PerCP-eFluor® 710 (1:100, eBioscience). After staining, the slides were washed with PBS, mounted with MOWIOL 4-88 (Roth, Germany), covered with a coverslip, and dried in the dark for at least 12 h at room temperature before they were analyzed with the confocal microscope LSM700 (ZEISS). Isotype control stainings were performed to rule out unspecific binding of the used antibodies (data not shown).

2.3. Confocal microscopy

A ZEISS LSM700 was used for confocal microscopy of human and murine spleen samples and data were recorded using 63×/1.4 NA oil immersion objective and ZEN Software 2009 (ZEISS, Germany). For the 405 nm laser the fluorescences were detected with a short-pass (<490 nm) filter for classic emission detection (BD Horizon™ V450, PacificBlue™, eFluor® 450). The Stokes shift of PacificOrange™ allowed an orange-to-yellow discrimination using a bandwidth filter of 505–600 nm. Similarly, Brilliant Violet 605™ as well as Brilliant Violet 650™ could be separated in the red range using a long-pass filter of >590 nm. Fluorochromes that were excited with a 488 nm laser line were detected with a band-pass (490–555 nm) filter (Alexa Fluor® 488, FITC) in combination with a long-pass (640 nm) filter due to a far red-shifted emission (PerCP-eFluor® 710, PerCP-Cy5.5). PE/Cy3 antibodies were excited with 561 nm and detected with a band-pass (490–635 nm) filter. Alexa Fluor® 647 labeled antibodies were excited with 633 nm and detected with a long-pass (>640 nm) filter. To cover a representative area of the tissue 8 × 8 tiles (812 μm) were recorded and for detailed analysis single cells were optically zoomed in separately. The acquisition time was approximately 45 min with a pixel dwell time of 10.2 μs and an optimal resolution of 512 × 512 pixels according to the acquisition criteria. For experimental setup fluorescence spectral analyzer from BioLegend was used (http://www.biolegend.com/spectraanalyzer).

3. Results

3.1. Stokes shift differences allow for 6 color confocal immunofluorescence microscopy

In order to apply more than four differently fluorochrome-labeled antibodies on tissue samples without the application of compensation correction algorithms [8,9] on a standard four laser confocal immunofluorescence microscope, we analyzed the absorption and emission spectra of commercially available fluorochromes. We noticed that a few fluorochromes with similar absorption maxima significantly differed in their Stokes shift, which describes the differences in energy levels of the exciting and the emitted wavelengths. Based on these observations we concluded that in parallel it should be possible to easily excite more than one fluorochrome by the same laser line (Fig. 1). Indeed, we found that Horizon™ V450 or PacificBlue™ labeled antibodies could be visualized by combining them with PacificOrange™, Brilliant Violet605™, or Brilliant Violet650™ conjugated antibodies and excited at 405 nm (Fig. 1A and B). Further, we also suggested that the fluorochromes Alexa Fluor® 488 and PerCP-eFluor® 710 or PerCP-Cy5.5 might be excited at 488 nm (Fig. 1C), and detected separately with different filters (Fig. 1D). Unfortunately, up to now we found no fluorochromes available for the simultaneous detection of antibodies within the 561 nm or the 633 nm laser lines, thereby it is possible to detect only a single fluorochrome of either Cy3/PE (Fig. 1E and F) or Alexa Fluor® 647, respectively (Fig. 1G and H).