Volume 47(9): 1213–1217, 1999
The Journal of Histochemistry & Cytochemistry
http://www.jhc.org

TECHNICAL NOTE

Improved Double Immunofluorescence for Confocal Laser Scanning Microscopy

Rakesh K. Kumar, Cheryl C. Chapple, and Neil Hunter

School of Pathology, University of New South Wales (RKK), and Institute of Dental Research (CCC,NH), Sydney, Australia

SUMMARY Reliable double immunofluorescence labeling for confocal laser scanning microscopy requires good separation of the signals generated by the fluorochromes. We have successfully overcome the limitation of a single argon ion laser in achieving effective excitation of dyes with well-separated emission spectra by employing the novel sulfonated rhodamine fluorochromes designated Alexa 488 and Alexa 568. The more abundant antigen was visualized using the red-emitting Alexa 568, with amplification of the signal by a biotinylated bridging antibody and labeled streptavidin. This was combined with the green-emitting Alexa 488, which yielded brighter images than fluorescein but exhibited comparable photodegradation. With appropriate controls to ensure the absence of crosstalk between fluorescence channels, these dyes permitted unequivocal demonstration of co-localization. This combination of fluorochromes may also offer advantages for users of instruments equipped with alternative laser systems. **(J Histochem Cytochem 47:1213–1217, 1999)**

KEY WORDS
immunohistochemistry
confocal laser scanning microscopy
double labeling
fluorochromes

Confocal laser scanning microscopy (CLSM) permits accurate co-localization of fluorescent markers, because the thin optical section generated by the instrument eliminates the confounding effects of out-of-focus fluorescence (Wouterlood et al. 1998). As in conventional double immunofluorescence, detection of co-localization requires appropriate controls to ensure the absence of crossreactivity and of nonspecific binding of antibodies or other fluorochrome-labeled markers. In addition, technical issues unique to CLSM must be specifically addressed.

For valid interpretation of the digital images generated by CLSM, reliable separation of the signals generated by the fluorochromes is critical. This is, in turn, dependent on the respective excitation/emission spectra and the choice of barrier filters. Overlap between the emission spectra of fluorescein and tetramethylrhodamine renders simultaneous excitation of probes labeled with these dyes unsatisfactory for CLSM (Entwistle and Noble 1992). Although the problem can be solved by sequential excitation with wavelengths selective for each of the fluorochromes if a dual laser instrument is available (Arai et al. 1996), this approach is not an option for many users. For a confocal microscope equipped with a single laser, the ability to efficiently excite fluorochromes with well-separated emission spectra depends on the spectral lines available. The combination of fluorescein and Texas Red, widely used for conventional double immunofluorescence, can be efficiently excited using a mixed gas argon–krypton laser. If desired, the dyes can be excited sequentially at 488 nm and 568 nm for even more reliable separation (Berger et al. 1995). However, Texas Red is poorly excited by the 488- or 514-nm line of the commonly used argon ion laser, necessitating so much electronic amplification of the signal that unacceptable crosstalk from the "red tail" of fluorescein emission results (Entwistle and Noble 1992; Wouterlood et al. 1998). Tandem conjugates of phycoerythrin and either Texas Red or the far red-emitting carbocyanine dye Cy5 can be excited at 488 nm. These labels have been evaluated for double immunofluorescence labeling including CLSM (Uchihara et al. 1995; Gothot et al. 1996), but exclusion of residual phycoerythrin emission necessitates the use of narrow bandpass filters, and nonspecific binding of labeled probes may make interpretation difficult (van Vugt et al. 1996).

An interesting solution to the problem of double

Correspondence to: Rakesh K. Kumar, School of Pathology, University of New South Wales, Sydney, Australia 2052.
Received for publication December 1, 1998; accepted March 16, 1999 (8T4840).

 0022-1554/99/$3.30

immunofluorescence labeling for CLSM using a single argon ion laser was proposed by Entwistle and Noble (1992). They suggested that a combination of fluorescein and lissamine–rhodamine B could be effective, provided that the rhodamine-labeled probe was used in a streptavidin–biotin amplified system for detection of the more abundant antigen. Although excitation of lissamine–rhodamine B by the 488- or 514-nm line of an argon ion laser is inefficient, it was proposed that labeling in this fashion would increase the red emission sufficiently for detection not to require high gain settings to utilize the full gray-level range, which in turn would minimize crosstalk. We describe a successful implementation of this approach using novel sulfonated rhodamine fluorochromes, which may also offer advantages for users of instruments that permit laser excitation at alternative wavelengths.

Materials and Methods

Antibodies and Labeled Probes

Mouse monoclonal anti-Type IV collagen, polyclonal rabbit antibodies to Ki-67 and to laminin, biotinylated polyclonal goat anti-mouse immunoglobulins, and swine anti-rabbit immunoglobulins labeled with fluorescein isothiocyanate (FITC) were purchased from Dako (Carpinteria, CA). Monoclonal anti-cytokeratins 4 and 13 were from Sigma (St Louis, MO). Goat anti-rabbit immunoglobulins labeled with Alexa 488 (excitation–emission spectra similar to fluorescein) and streptavidin labeled with Alexa 568 (excitation–emission spectra similar to lissamine–rhodamine B) were from Molecular Probes (Eugene, OR).

Tissues and Immunostaining

Gingival tissues from individuals with advanced adult-type periodontitis had been collected as part of a previously described study (Chapple et al. 1998) approved by the Human Ethics Committee of the Central Sydney Area Health Authority. Six-μm-thick frozen sections were cut from these biopsies and stored at −70C until use. Thawed sections were fixed in acetone at room temperature for 10 min. Nonspecific binding was blocked with 20% goat serum for 20 min, and then appropriate concentrations of antibodies or streptavidin (determined on the basis of preliminary experiments) were sequentially applied for 30 min each, with four 30-sec washes after each incubation. For rabbit primary antibodies, detection was with labeled anti-rabbit immunoglobulins; for mouse primary antibodies, detection was with biotinylated anti-mouse immunoglobulins followed by labeled streptavidin. Vectashield (Vector Laboratories; Burlingame, CA) was used as the mounting medium. Controls included sections incubated without the primary antibody or, where the reporter molecule was labeled streptavidin, without either the primary or the secondary antibody.

Immunofluorescence Microscopy

Fluorescent staining of tissues was visualized using a Leitz Orthoplan microscope equipped with a xenon arc lamp, appropriate dichroics, and a ×50, NA 1.0 water-immersion epifluorescence objective. Confocal laser scanning was performed with the Optiscan F900e personal confocal system (Optiscan; Melbourne, Australia), which uses an optical fiber both as the illumination source and the detection aperture. This system was equipped with a 50-mW argon ion laser, filters to attenuate the output of the laser to either 10% or 1% power, and filters allowing excitation with the 488-nm laser line, the 514-nm line, or a 50⁄50 split between the two wavelengths. Two channels were available for simultaneous data acquisition: Channel 1 (displayed as green) could include a 510–550-nm bandpass filter and either a 515-nm or a 530-nm longpass filter, while Channel 2 (displayed as red) could include either a 550-nm or 590-nm longpass filter. Accumulated 16-scan digital images (512 × 512 × 16 bits per channel) were converted to bitmap files for printing.

Image Analysis

Measurement of pixel intensity was undertaken to compare the relative intensity of staining with Alexa 488 and fluorescein and to assess the photostablity of fluorochromes under the conditions of CLSM. For the green fluorochromes, nuclei of cycling cells stained with Ki-67 antibody and anti-rabbit immunoglobulins conjugated to either of these labels were examined. Series of nine consecutive 16-scan monochrome images were accumulated. A 128 × 128 pixel region of each image, containing a single optimally labeled nucleus with a visible nucleolus, was defined and mean pixel intensity for the region was determined using the Image/Histogram menu option in Adobe Photoshop software (Adobe Systems; San Jose, CA). To quantify relative intensity of staining with the two fluorochromes, the first 16-scan image of each series was analyzed. To quantify photodegradation, the procedure was repeated for all images in each series and the intensity expressed as a percentage of the initial value for that series. Photodegradation of Alexa 568 was similarly assessed by examining series of images of blood vessels stained for Type IV collagen with a streptavidin–biotin-amplified detection system. The software package GraphPad Prism (GraphPad Software; San Diego, CA) was used for data analysis and preparation of graphs.

Results

Double Immunofluorescence

Initial experiments established that, using a biotinylated bridging antibody and streptavidin conjugated to Alexa 568, the red-emitting fluorochrome could be visualized by confocal microscopy when excited with the 488-nm line of an argon ion laser and detected using a 590-nm longpass filter. However, because excitation at this wavelength was inefficient, a high photomultiplier tube (PMT) gain was required to obtain a satisfactory image. As a result, when double immunofluorescence labeling was attempted, longer wavelength emissions from green fluorochromes such as Alexa 488 and FITC were amplified to unacceptably high levels, producing crosstalk from the green to the red channel.

Therefore, in subsequent experiments we used a 50⁄50 split of both 488-nm and 514-nm laser lines for excitation at 10% laser power. This yielded a satisfactory signal for both fluorochromes without inordinately high settings for PMT gain, using a 510–550-nm bandpass plus a 530-nm longpass filter for detection of green emission in Channel 1 (the emission signal from 530–550 nm was collected to exclude the 514-nm excitation line) and a 590-nm longpass filter for detection of red emission in Channel 2. As demonstrated in Figures 1A–1C, with this arrangement there was no detectable crosstalk in either direction between the non-co-localizing signals from cytoplasmic staining of epithelial cells for keratins and staining of nuclei of cycling cells for Ki-67.

Using appropriate settings for PMT gain, determined on the basis of non-co-localizing double staining for Ki-67 and cytokeratin, this combination of fluorochromes and filters also permitted demonstration of true co-localization. An example of co-localized matrix proteins in the thickened walls of blood vessels in advanced periodontitis is shown in Figures 1D–1F, stained for Type IV collagen and for laminin.

Photostability of Fluorochromes

We found that using a secondary antibody labeled with Alexa 488 consistently yielded brighter images of nuclei stained with optimal concentrations of Ki-67 antibody than were obtained with fluorescein, but substantial fading was observed with both dyes under the experimental conditions employed (Figures 2A–2F). These findings were confirmed by image analysis (Figure 3). In contrast, Alexa 568 exhibited no visible fading when used as a reporter label for blood vessels stained for Type IV collagen (Figures 2G–2I) and no fading was demonstrable by image analysis (not shown).

Discussion

Reliable double immunofluorescence labeling for confocal microscopy requires that the fluorochromes used exhibit good spectral separation. For confocal microscopes equipped with an argon ion laser with a limited output of laser lines, this is a difficult prerequisite to satisfy. A green-emitting dye such as fluorescein is efficiently excited by the 488-nm laser line, but a well-separated red-emitting fluorochrome such as Texas

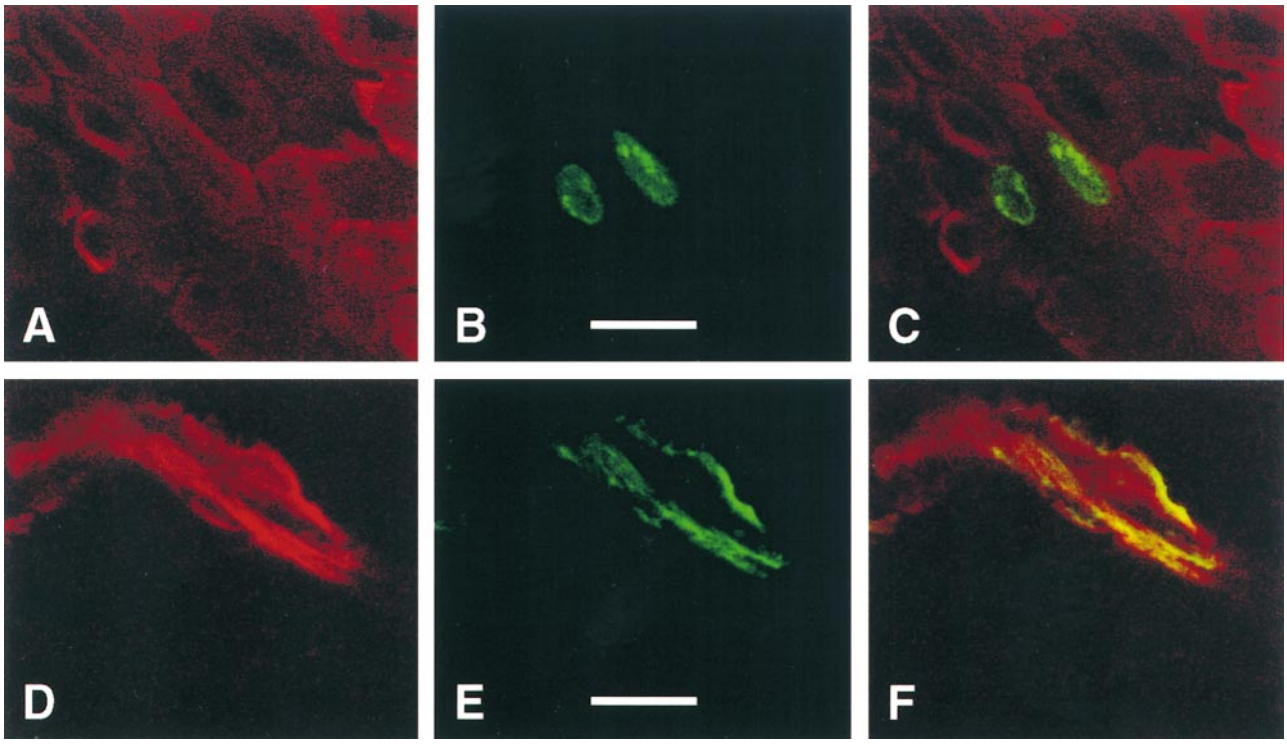

Figure 1 Double immunofluorescence visualized by CLSM. (**A–C**) Non-co-localizing staining for a mixture of cytokeratins 4 and 13, using mouse monoclonal primary antibodies detected with biotinylated anti-mouse immunoglobulins and streptavidin labeled with Alexa 568, and for Ki-67 antigen in cycling nuclei using a rabbit polyclonal antibody detected with anti-rabbit immunoglobulins labeled with Alexa 488. (**D–F**) Co-localization of staining for Type IV collagen and for laminin, using similar detection methods. Images in **A** and **D** were obtained with the PMT gain for the green channel reduced to zero and those in **B** and **E** with the red channel reduced to zero. Images in **C** and **F** were obtained by simultaneous acquisition of data in both channels. Note the absence of crosstalk in **C** and the co-localization shown as yellow in **F**. Bars = 25 μm.

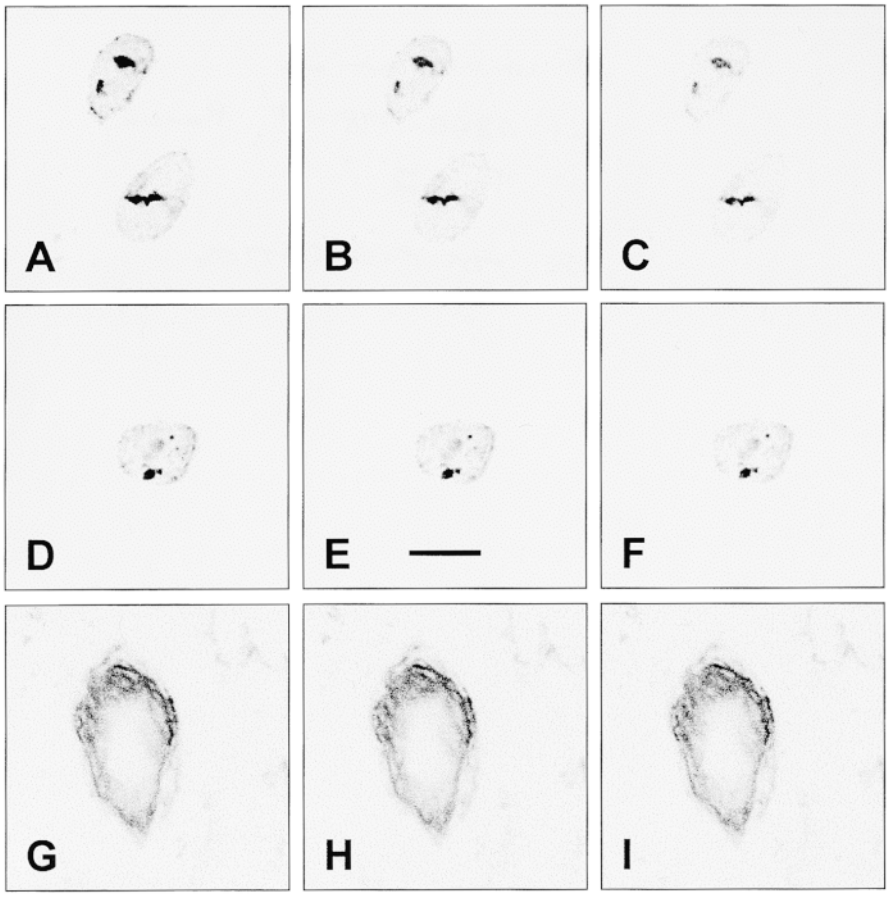

Figure 2 Inverted monochrome CLSM images demonstrating photodegradation of fluorochromes. (**A–C**) Nuclei stained for Ki-67 antigen and detected with anti-rabbit immunoglobulins labeled with Alexa 488. (**D–F**) A similarly stained nucleus using FITC as the reporter label. (**G–I**) A blood vessel stained with anti-Type IV collagen detected with biotinylated anti-mouse immunoglobulins and streptavidin labeled with Alexa 568. Each set comprises images 1, 5, and 9 from a sequence of 16-scan images of the same field. Bar = 15 μm.

Red, which is widely used for conventional double immunofluorescence microscopy, is poorly excited using the available laser lines. In this study we have successfully implemented a solution originally proposed by Entwistle and Noble (1992). This relies on the use of a red-emitting fluorochrome with excitation and emission spectra in between those of rhodamine and Texas Red, together with amplification of the red signal during the immunostaining procedure, by using a biotinylated bridging antibody and labeled streptavidin.

We used the novel fluorochromes designated Alexa 488 and Alexa 568 for these experiments. The Alexa dyes are sulfonated rhodamine derivatives, which are described by the manufacturer as brighter and more photostable than the traditional fluorochromes they replace. In our hands, these dyes proved very useful for visualizing double immunofluorescence using CLSM. Compared to fluorescein, brighter images were obtained using antibodies labeled with Alexa 488. However, whereas Entwistle and Noble (1992) found that excitation at 488 nm was adequate for lissamine–rhodamine B, we obtained better results for Alexa 568 in double immunofluorescence using concurrent excitation with the 488-nm and 514-nm laser lines.

For successful visualization of dual staining by CLSM using this approach, the more abundant antigen must be detected using the amplified reporter system and the red fluorochrome. As pointed out by Entwistle and Noble (1992), this is counterintuitive but works in practice because the output from the green-emitting fluorochrome is much greater than that from the red-emitting one. Given that the fidelity of discrimination between fluorochromes in the digital images generated by CLSM is dependent on the use of appropriate settings for PMT gain, we suggest that a non-co-localizing double-labeled section should be included as a control in each experiment to allow calibration of channels. With the inclusion of such a control, crosstalk can be eliminated, permitting unequivocal demonstration of co-localization of labels.

Photostability of fluorochromes is a major issue for CLSM, particularly for three-dimensional reconstruction of images. Green-emitting fluorochromes are a recognized problem. Alexa 488 has been demonstrated by the manufacturer to be more photostable than fluorescein for conventional immunofluorescence and was therefore investigated for CLSM. Unfortunately, in this study we found that photodegradation

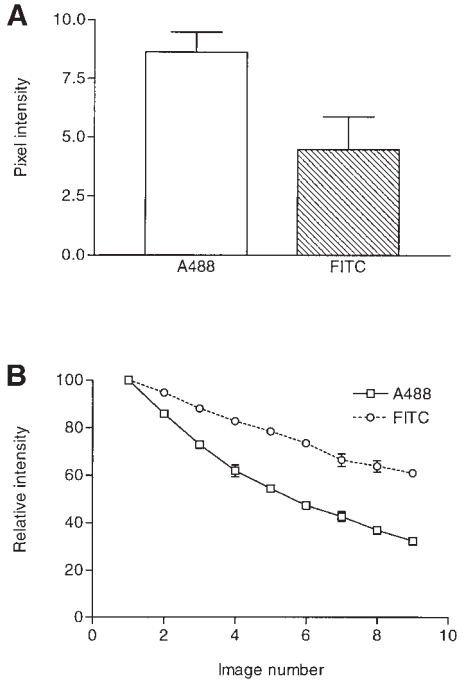

Figure 3 Comparison of labeling using the green fluorochromes Alexa 488 and fluorescein, assessed by image analysis as described in Materials and Methods. (**A**) Relative brightness of nuclear labeling for Ki-67. Data are mean pixel intensity ± SEM, $n = 7$ for both labels. Intensity of labeling with Alexa 488 was significantly greater than with fluorescein, $p<0.05$ by unpaired t-test. (**B**) Photostability of labeling in a series of consecutive 16-scan images. Data are mean percent of maximal intensity ± SEM.

of the Alexa 488 dye under the conditions of CLSM was at least as marked as that observed with fluorescein. However, there was no apparent photodegradation of Alexa 568, indicating the potential of this fluorochrome for images using a single label for three-dimensional reconstruction. Indeed, the use of red-emitting dyes as the primary labels for immunofluorescence microscopy, including CLSM, has been strongly advocated (Entwistle and Noble 1992), although the relative inefficiency of red signal data collection by photomultiplier tubes remains a problem.

The strategy for double immunofluorescence labeling that we have described for CLSM is not limited to systems equipped with an argon ion laser. Mixed gas argon–krypton lasers are able to optimally excite both of these Alexa dyes, which could be expected to yield increased green emission using Alexa 488 compared to fluorescein, because of the higher output, as well as increased red emission using Alexa 568 compared to Texas Red, because of more efficient excitation. Similar improvements could be anticipated using sequential excitation with an argon ion laser at 488 nm and a helium–neon laser at 543 nm in a dual laser system. In both cases, further amplification of the red signal during immunostaining is unlikely to be necessary.

Acknowledgments

Supported by a grant from the Dental Board of New South Wales. CCC is a recipient of a Dental Postgraduate Scholarship from the National Health and Medical Research Council of Australia.

Literature Cited

Arai R, Jacobowitz DM, Nagatsu I (1996) Calretinin is differentially localized in magnocellular oxytocin neurons of the rat hypothalamus. A double-labeling immunofluorescence study. Brain Res 735:154–158

Berger EG, Burger P, Hille A, Bachi T (1995) Comparative localization of mannose-6-phosphate receptor with 2,6 sialyltransferase in HepG2 cells: an analysis by confocal double immunofluorescence microscopy. Eur J Cell Biol 67:106–111

Chapple CC, Srivastava M, Hunter N (1998) Failure of macrophage activation in destructive periodontal disease. J Pathol 186:281–286

Entwistle A, Noble M (1992) The use of Lucifer yellow, bodipy, FITC, TRITC, RITC and Texas Red for dual immunolabelling visualized with a confocal scanning laser microscope. J Microsc 168:219–238

Gothot A, Grosdent JC, Paulus JM (1996) A strategy for multiple immunophenotyping by image cytometry: model studies using latex microbeads labeled with seven streptavidin-bound fluorochromes. Cytometry 24:214–225

Uchihara T, Kondo H, Akiyama H, Ikeda K (1995) Single-laser three-color immunolabeling of a histological section by laser scanning microscopy: application to senile plaque-related structures in post-mortem human brain tissue. J Histochem Cytochem 43:103–106

van Vugt MJ, van den Herik–Oudijk IE, van de Winkel JGJ (1996) Binding of PE-CY5 conjugates to the human high-affinity receptor for IgG (CD64). Blood 88:2358–2361

Wouterlood FG, Van Denderen JCM, Blijleven N, Van Minnen J, Hartig W (1998) Two-laser dual-immunofluorescence confocal laser scanning microscopy using Cy2- and Cy5-conjugated secondary antibodies: unequivocal detection of co-localization of neuronal markers. Brain Res Protocols 2:149–159