

RAPID COMMUNICATION

Quadruple Immunofluorescence: A Direct Visualization Method

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SUMMARY We describe fluorescence immunostaining of four different antigens in the same section. The fluorochrome conjugates used show highest emission in the blue, green, yellow, and red regions of the visible light spectrum, respectively. Specially designed single fluorochrome filter combinations allow selective visualization of each fluorochrome label in turn, without visible crosstalk with the others. (*J Histochem Cytochem* 45:155–158, 1997)

KEY WORDS

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Multiple immunostaining, i.e. the simultaneous immunolabeling of more than one antigen in the same preparation, is instrumental to the understanding of spatial relationships and possible co-localization of different structures and molecules. Double- and triple-immunostaining techniques are well-established and almost commonplace, especially in immunofluorescence.

Recent evolution of thin-film coating technology has made it possible to precisely tailor excitation and emission filters (Reichman 1994; Johnson 1990; Marcus 1988), thus permitting selective demonstration of partly overlapping fluorochromes (Brelje et al. 1993). At the same time, the introduction of new fluorescent labels (Brelje et al. 1993; Haugland 1992; Wessendorf and Brelje 1992) has widened the array of available conjugates for immunofluorescence, which now span all regions of the visible light spectrum.

We have developed a combination of fluorochrome conjugates and filter sets that enables simultaneous immunostaining of four antigens in the same preparation. A standard fluorescence microscope is used and the four labels applied are independently visualized in blue, green, yellow, and red, in turn. The spectral characterization of filter sets required is provided to facilitate understanding and reproduction of the method.

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Materials and Methods

Tissues

Pituitaries were taken from normal rats (sacrificed in other experiments), pigs and cows (local abattoir), and human subjects (postmortem). Procedures were approved by local ethical committees and conformed to European Community regulations. Samples were fixed in 4% paraformaldehyde or in periodate-lysine-paraformaldehyde (McLean and Nakane 1974) and washed in 7–10% sucrose-PBS. Cryomicrotome sections (4–10 μm) were collected on slides pretreated with poly-L-lysine (Huang et al. 1983) or Vectabond (Vector; Burlingame, CA).

Immunostaining

Primary antisera/antibodies were: anti-human β -LH or β -FSH (from sheep: Affinity, Mamhead, UK) (Ferri et al. 1995a); anti-rat, -bovine, or -human prolactin (mouse monoclonals) (Berger et al. 1988); anti-rat GH (monkey, AFP411S); anti-human ACTH (rabbit, AFP39013082); anti-rat TSH (guinea pig, AFP3035990P); anti-neuroendocrine protein VGF (rabbit) (Ferri et al. 1995a). Four antisera/antibodies raised in different species were generally diluted together (1:400–1:4000, or 1–5 $\mu\text{g}/\text{ml}$ for monoclonals; overnight incubation) in PBS containing 3% normal donkey serum, 3% normal serum of the species being immunostained (rat, pig, bovine, or human), and 0.02% NaN_3 .

Affinity-purified IgG preparations (from donkey, or horse: Jackson ImmunoResearch, West Grove, PA; Vector; Molecular Probes, Eugene, OR) were used as secondary antibodies, conjugated with either biotin or a fluorochrome. Preference was given to preparations pre-absorbed on solid phase with serum proteins from multiple species (including donor species of other primary antisera/antibodies to be

used at the same time). Secondary antibodies were generally diluted together (medium as above: 5–10 $\mu\text{g/ml}$ or 2–5 $\mu\text{g/ml}$ for Cy3 conjugates and biotin conjugates to be followed by Cy3-avidin; 1–2-hr incubation). As appropriate, a third incubation step included an avidin-streptavidin-fluorochrome conjugate (same suppliers as above: 10–20 $\mu\text{g/ml}$ or 2–5 $\mu\text{g/ml}$ for Cy3 conjugates in PBS; 1–2 hr).

Controls included substitution of each conjugate with PBS and single immunostaining. Possible reactivity of secondary antibodies with primary antibodies from inappropriate species was checked on sections. When crossreacting, antibodies were diluted in PBS containing 3% normal serum of the inappropriate species and applied in separate incubation steps, in parallel with further controls. Slides were coverslipped with PBS-glycerol, with or without antifading agents (Vectashield, Vector; SlowFade, Molecular Probes). BX60 and BX50 microscopes (Olympus Italia; Milan, Italy) equipped with standard 100-W mercury lamps were used for observations and T-Max 400 film (Kodak; Rochester, NY) and PM-30 systems (Olympus) for photography.

Fluorochromes and Filter Combinations

Experiments were focused on conjugates of some compounds showing emission maxima in different regions of the

visible light spectrum: blue (aminomethylcoumarin acetic acid, AMCA), green (fluorescein isothiocyanate, FITC), yellow (cyanine 3.18, or Cy3) tetramethyl rhodamine isothiocyanate, TRITC), and red (allophycocyanin, APC, and cyanine 5.18, Cy5).

Filter specifications were drawn on the basis of published spectra (Brelje et al. 1993; Haugland 1992) and of previous experience with FITC/TRITC discrimination (Ferri et al. 1995a). To minimize possible crosstalk, combinations were designed as follows: for FITC, 460–495 and 510–540 nm; for Cy3/TRITC, 540–555 and 570–610 nm (excitation and emission bands, respectively). The human eye has low sensitivity to light in the 660–670-nm wavelength range (Lennie et al. 1993), corresponding to emission maxima of APC and Cy5. The relevant combination was designed to fully transmit low-wavelength emission (from about 635 nm). Hence, excitation was limited to 590–620 nm. Such specifications were passed on to Chroma Technology (Burlington, VT) for custom production, and a standard set was purchased for the blue emitter AMCA (Chroma). Filters were mounted on standard holders (Olympus) for microscopy and optical characterization.

Spectra of each excitation path and emission path were obtained, as described (Ferri et al. 1995b). Using a spectrofluorometer (Fluorolog-2; Spex Industries, Metuchen, NJ),

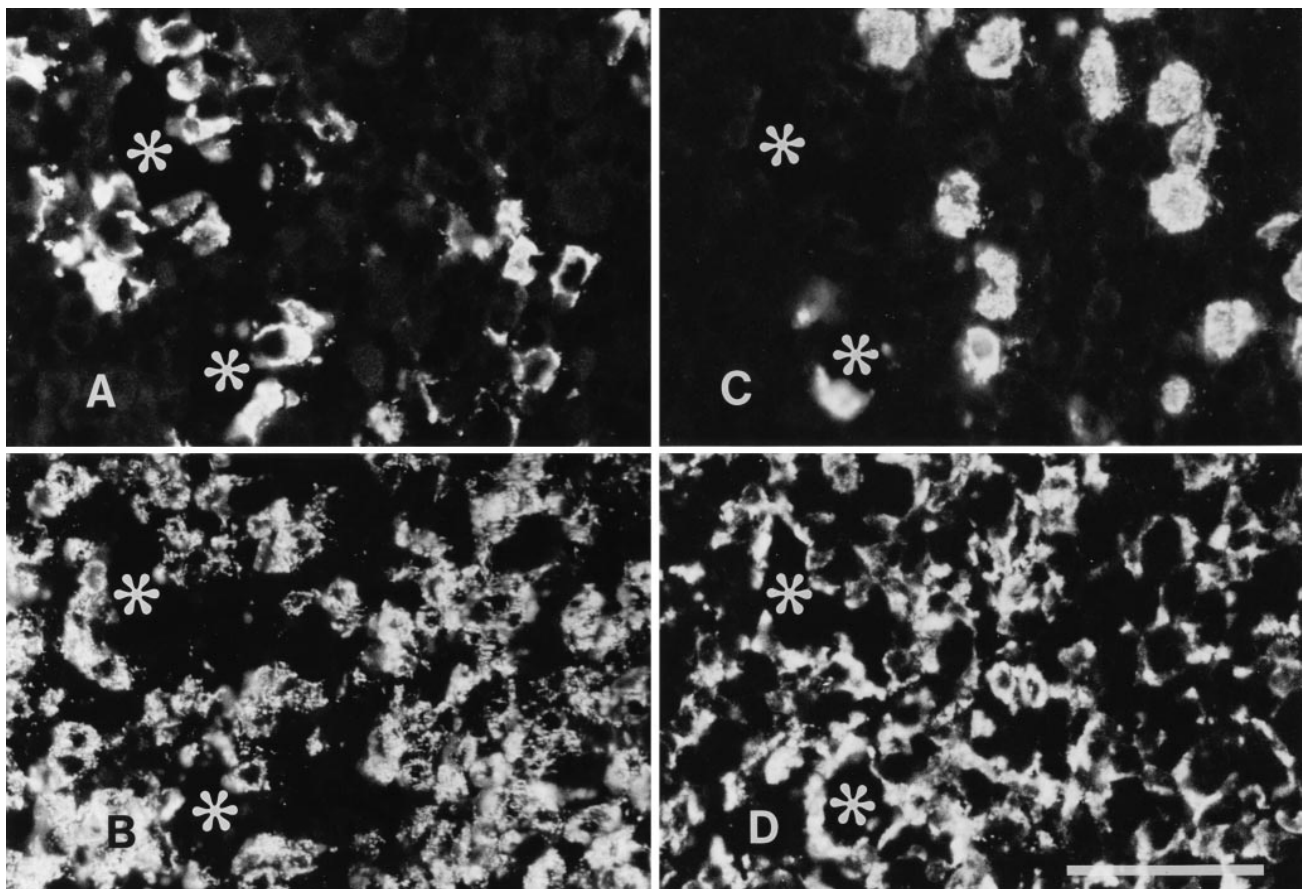


Figure 1 Quadruple immunostaining of rat pituitary: the same area was photographed sequentially through single-fluorochrome filter combinations. Separate cell populations are immunostained for the hormones: ACTH (A, AMCA-labeled); GH (B, Cy3); LH (C, FITC), and prolactin (D, Cy5). Indirect immunofluorescence; two blood vessels are indicated by asterisks. Bar = 100 μm for A–D.

monochromatic light (320–720 nm, in 1-nm steps) was projected onto the excitation filter and measured after 90° reflection by the dichroic mirror. Absorbance through a combined dichroic mirror (at 45°, as for normal use) and emission filter was measured in the same wavelength interval using a spectrophotometer (Lambda 9; Perkin-Elmer, Langen, Germany). The relative intensity of light collected from either path was plotted (output/input). Excitation and emission spectra of the fluorochrome conjugates used were obtained for comparison (PBS solution, using the same spectrofluorometer mentioned above).

Results

Features labeled with each of the fluorochrome conjugates tested showed bright staining over low background when viewed through the appropriate filter combination, and no detectable signal through either of the three inappropriate filter sets (Figures 1A–1D).

A distinct yellow signal was revealed for Cy3 and TRITC, as opposed to the red-orange color seen with most microscopes and filter combinations. Although unusual in appearance, the preparations were very bright and were associated with little eyestrain, even after prolonged observation. A deep red fluorescence was seen for APC and Cy5, resulting in an intense although somewhat fatiguing signal over a virtually invisible background. When immunostaining of the same or parallel features was subjectively compared, Cy3, TRITC, and FITC clearly appeared brighter than Cy5, APC, or AMCA (labels are listed in decreasing order of visual effectiveness).

In most cases, secondary antibodies showed no unwanted reactivity for other primary antibodies used in the procedure, and the standard two- or three-step immunostaining protocol was followed. Occasionally (about 5% of all secondary antibodies, mainly with non-pre-absorbed preparations), a minor degree of crossreactivity was revealed in control sections and

the alternative procedure (absorption with normal serum) was successfully applied.

Correctly exposed negatives were generally obtained for AMCA, FITC, Cy3, and TRITC using the standard exposure index indicated for the film (ISO 400). Under identical conditions, APC and Cy5 required considerable overexposure (ISO 12–25). Although assessment of anti-fading agents was beyond the scope of the present investigation, somewhat better signal preservation after photography was noted with FITC-conjugates.

Spectral characterization of custom-designed filter sets (Figure 2, bottom) revealed excitation and emission bands virtually superimposable to the specifications given to the supplier. The standard AMCA set and the newly designed FITC and Cy3/TRITC combinations closely matched excitation and emission maxima of the relevant fluorochrome conjugates (Figure 2, bottom vs top panels). As expected, the design strategy adopted for the APC/Cy5 set resulted in a suboptimal excitation band (about 25% of maximum: Figure 2, Cy5, top vs bottom panels). As mentioned, however, excitation proved adequate to produce a bright, directly visible signal through the microscope.

Discussion

A simple and effective method has been developed for the simultaneous but fully independent demonstration of four antigens in the same preparation. Thus, a powerful tool is provided for the investigation of many complex patterns of co-localization and of the spatial relationships between multiple cell and tissue structures.

Wide application of the method is made possible by the direct visualization approach used, all four labels being directly observed through a fluorescence microscope. The fluorochromes required are readily avail-

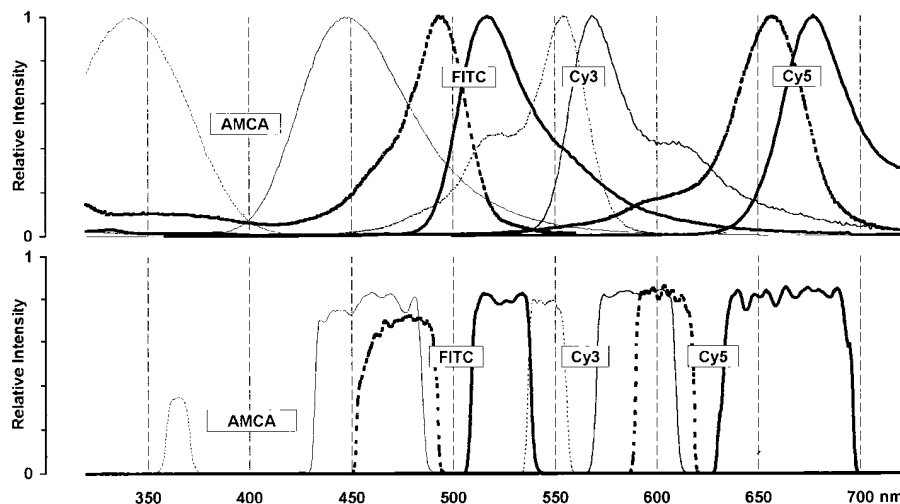


Figure 2 Excitation and emission spectra of four representative fluorochrome conjugates (upper panel, broken and solid lines, respectively) and spectra of excitation and emission paths of the corresponding filter combinations (lower panel, broken and solid lines, respectively): Relative intensity = output/input light; irrelevant transmission regions in emission filters, found at wavelengths below excitation bands, were omitted). Excitation and emission maxima of AMCA, FITC, and Cy3 (above) are closely matched by the corresponding filter combinations (below). Because the eye has low sensitivity to far-red light, the Cy5 emission band was designed to fully transmit all the “early” emission (from about 635 nm).

able on a commercial basis, conjugated to a wide range of secondary antibodies and avidins. Finally, the spectral characterization provided for each filter combination should make reproduction of the entire set-up straightforward. Because the present study was based on commonly used mercury light sources, adaptation to spectrally different sources may require some change in filter sets. Conversely, an entirely different approach would apply to laser scanning confocal microscopy because of the powerful excitation light and the highly sensitive detectors involved (Brelje et al. 1993).

A simple two- or three-step immunostaining protocol could be followed in most cases, based on one primary and one secondary incubation, each including the four relevant antisera/antibodies. As a potential source of artifacts, unwanted reactivity of secondary antibodies was monitored and proved uncommon and easy to block. Pre-absorbed secondary antibody preparations were helpful, being less prone to the latter problem.

Labeling was brightest for Cy3, followed by TRITC and FITC, in agreement with previous observations (Wessendorf and Brelje 1992). Both Cy3 and TRITC conjugates have emission maxima in the yellow region of the spectrum at about 565 and 575 nm, respectively (Brelje et al. 1993; Haugland 1992). Therefore, it is not surprising that Cy3 or TRITC, viewed through a 570–610-nm emission band, resulted in a yellow, very bright and effective signal. The human eye is very sensitive to light of such wavelength (Lennie et al. 1993), further explaining our subjective observations.

Cy5 is generally regarded as hardly suitable for direct visualization, in view of its emission maximum in the far-red region at about 670–675 nm (Brelje et al. 1993; and this study). Although sensitivity would be higher with electronic imaging, Cy5 works well in the direct visualization approach described and is being successfully applied in our laboratory to many tissues and organs. APC labeling could be easier to visualize, since its emission maximum is at a 10–15-nm lower wavelength than that of Cy5 (Haugland 1992). In our hands, however, APC resulted in a lower visual signal. The high molecular weight of APC, its chemical characteristics (Haugland 1992), and its limited availability in conjugated form appear to make it a less valuable alternative (Brelje et al. 1993). As mentioned, the selection of excitation and emission bands for the Cy5 filter set was empirical. Further work is required to reach the best possible compromise between excita-

tion efficiency and direct visibility of this fluorochrome.

As a final note, preliminary experiments with conjugates of the blue-emitter cyanine 2, or Cy2 (Rockland; Gilbertsville, PA), carried out in parallel with FITC, showed bright signal and low background when viewed with the FITC filter set and no crosstalk through the AMCA, Cy3, or Cy5 combinations.

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