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Microwaving for double indirect immunofluorescence with primary antibodies from the same species and for staining of mouse tissues with mouse monoclonal antibodies

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Abstract Many methods have been devised for double immunocytochemical staining. We now describe that moderate microwaving does not elute antibodies, but prevents their reactions with subsequently applied reagents. Thus, microwaving performed in between the first and second staining cycles permits double indirect immunofluorescence staining with antibodies raised in the same species. Moreover, microwaving also inhibits reactions with endogenous immunoglobulins present in extracellular compartments. This substantially reduces background in indirect immunostaining of mouse tissues with mouse monoclonal antibodies.

Introduction

Several different methods for detection of multiple tissue-bound antigens have been devised. In their simplest form these methods make use of primary antibodies that are directly labelled by, for example, different fluorophores, gold particles or enzymes (Boorsma 1984; Nairn 1976; Pryzwansky 1982). Although useful in certain applications, direct methods suffer from a lower sensitivity than indirect techniques. Moreover, the direct immunocytochemical approach requires access to antibodies in sizeable quantities and to labelling methods that do not adversely affect antibody activity.

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For these reasons many investigators use indirect (two or more layer) methods. Provided primary antibodies from different species are available these can be directly used for double or multiple stainings in combination with differently labelled species-specific second antibodies (Campbell and Bhatnagar 1976; Ferri et al. 1997; Nairn 1976; Pryzwansky 1982). Similarly, different subclasses of monoclonal antibodies can be used in conjunction with differently labelled subclass-specific antibodies.

However, quite often the appropriate combination of antibodies is not available. Consequently, many methods have been constructed to achieve double stainings with primary antibodies raised in the same species. Apart from stainings of adjacent sections or mirror sections, true double stainings were first carried out in conjunction with antibody elution techniques (for review see Larsson 1988). Since the elution methods were not always reliable and occasionally damaged tissue antigens, alternative methods were sought. A generation of such methods were based on what may be called the "blocking principle". In these approaches, precipitation of peroxidase reaction products or of metallic silver around tissue-bound antigen-antibody complexes physically shielded these from reaction with subsequently applied immunoreagents (Butterworth et al. 1985; Hunyady et al. 1996; Jackerott and Larsson 1997; van den Pol 1985; Sternberger and Joseph 1979). However, the reaction products often also shielded against detection of nearby antigens. Shindler and Roth (1996) employed detection systems of different sensitivities so that the least sensitive detection system only detected antibodies applied at low dilutions. Although such mixed sensitivity approaches can be useful (cf. Larsson 1981) they are restricted to situations where at least one antigen occurs in sufficient quantities to be detected by less sensitive methods. An alternative approach was described by Wang and Larsson (1985) who found that formaldehyde vapour treatment inactivated the antigen-combining sites on the secondary antibodies present in tissue-bound antigen-antibody complexes. This method completely eliminated the shielding effect on nearby antigens, but was associated with the risk of rendering formaldehyde-sensitive antigens

undetectable. Further improvements were introduced by Negoescu et al. (1994) who employed labelled monovalent Fab fragments as secondary reagents instead of the bivalent secondary antibodies. Although this approach is theoretically very elegant we have not succeeded in getting it to work, presumably because the labelled Fab fragments available have been of too low avidity. This can lead to exchanges during incubation resulting in contaminating mixed staining. In an alternative approach, Carl et al. (1993) employed monovalent Fab fragments for blocking residual sites on tissue-bound antibodies. Recently, Lan et al. (1995) reported on the use of microwaving for double immunoenzymatic staining. Since immunoenzymatic detection was used it could not be examined whether the microwaving procedure actually eluted antibodies and left the enzyme reaction product in situ or if the microwaving had a denaturing effect on the secondary antibodies similar to the formaldehyde method of Wang and Larsson (1985). Moreover, with immunoenzymatic detection it can often be difficult to reveal antigens that coexist in the same compartment. This is due both to problems with shielding effects (vide supra) and with discerning mixed colours. The latter problem is particularly severe if the coexisting antigens occur in variable proportions. In contrast, immunofluorescence is very useful for revealing coexisting antigens. Thus, a simple change of selective filters allows the selective visualisation of differently fluorochromed antibodies. Moreover, the sizes of most fluorescent reporter molecules are small enough to keep shielding effects at a minimum. Double immunofluorescence, however, requires that the fluorochromed antibodies are not eluted. We decided to study if the above-mentioned microwave method could be extended to double immunofluorescence. If microwaving worked by antibody elution, the treatment would remove the immunofluorescent antibodies. Conversely, if antibody denaturation was involved, the fluorescent antibodies would remain, but become unreactive to subsequently applied reagents. We found that both mechanisms occurred, but that elution was only detectable after repeated microwaving. Thus, the microwaving technique is also useful for double immunofluorescence staining. Furthermore, the method was also helpful for staining mouse tissues with mouse monoclonal antibodies in that it eliminated detection of endogenous mouse immunoglobulins present in the interstitial fluid.

Materials and methods

Tissue material

Wistar rats and Balb/c mice were anaesthesised with carbon dioxide and intracardially perfused with 1–2 ml saline, followed by 20 ml 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PFA). Specimens from different organs were postfixed in PFA overnight at 4°C, dehydrated in graded ethanols and embedded in paraffin at 60°C under vacuum. In addition, specimens were cryoprotected in 30% sucrose in 0.1 M sodium phosphate buffer pH 7.4, embedded in Tissue Tek OCT compound (Miles, Elkhart, Ind., USA) and frozen in n-heptane cooled by liquid nitrogen for cryostat sectioning.

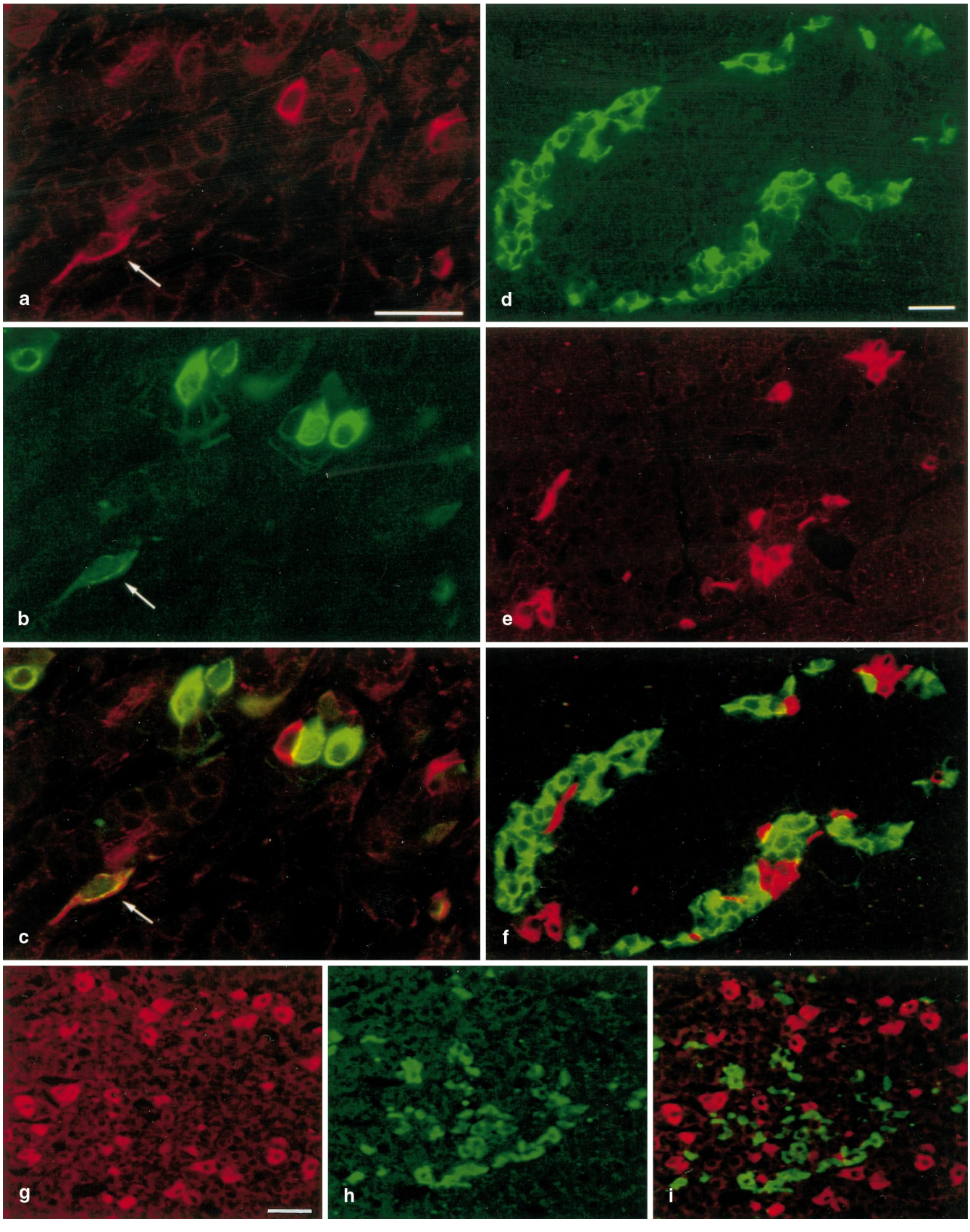
Immunocytochemistry

Sections (5 µm) were hydrated and pretreated with 1% bovine serum albumin (Sigma, St. Louis, Mo., USA) or 10% serum from the species donating the secondary antibodies (diluted in 0.05 M TRIS, 0.15 M NaCl, pH 7.4) for 30 min at room temperature and then immunocytochemically stained with rabbit antisera to synthetic human gastrin I (number 2717; kindly donated by Dr. J.F. Rehfeld, Rigshospitalet, Copenhagen, Denmark; Larsson and Rehfeld 1979), human glucagon (Dako, Glostrup, Denmark), human pancreatic polypeptide (HPP; kindly donated by Dr. Ron E. Chance, Indianapolis, Ind., USA) and synthetic somatostatin-14 (Dako). In addition, mouse monoclonal antibodies to glucagon (Novoclone, Novo-Nordisk, Bagsværd, Denmark), somatostatin (Novoclone), thyrotropin-stimulating hormone (TSH), follicle-stimulating hormone (FSH; Biodesign International, Kennebunk, Me., USA) and fetal antigen-1 (FA1; kindly donated by Dr. B. Teisner, Odense University, Denmark) were used. The site of the antigen-antibody reaction was revealed by fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (Dako), Texas red-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratory, Westgrove, Pa., USA), aminomethylcoumarin (AMCA)-conjugated goat anti-rabbit IgG (Dako) or FITC- or Texas red-conjugated goat anti-mouse IgG (Jackson). A triple layer method employing a second layer of biotin-labelled goat anti-mouse IgG (Dako) followed by AMCA-labelled streptavidin (Vector Laboratories, Burlingame, Calif., USA) for 30 min at room temperature was also used. In addition, the site of the antigen-antibody reaction was revealed by horseradish peroxidase-labelled secondary antibodies (Dako) followed by development in FITC-labelled tyramide (Dupont, NEN Research Products, Boston, Mass., USA) as described by the manufacturer. Specimens were examined in a Leica epillumination microscope using FITC, AMCA and Texas red selective filter blocks. The AMCA filter block was supplemented with a 490 long pass filter.

Microwave treatment

A Polar Patent PP780 microwave oven (Miele, Axlab, Copenhagen, Denmark) was used. After staining for the first antigen, five slides each were submerged in 50 ml 10 mM citrate buffer pH 6.0 in capped plastic jars. The jars were placed in a tray containing 1 l tap water and microwaved at 780 W for 5 min. This treatment was repeated 1–6 times with replacement of citrate buffer and tap water between cycles. After microwaving, slides were left in the citrate buffer at room temperature for 20 min.

Fig. 1 a–c Rat antropyloric mucosa first stained with rabbit anti-somatostatin serum and Texas red-labelled anti-rabbit IgG and subsequently microwaved 3×5 min and then stained with rabbit anti-gastrin serum and fluorescein isothiocyanate (FITC)-labelled anti-rabbit IgG. **d–f** Rat pancreas first stained with monoclonal mouse anti-glucagon antibodies and FITC-labelled anti-mouse IgG and subsequently microwaved 3×5 min and then stained with monoclonal mouse anti-somatostatin antibodies and Texas red-labelled anti-mouse IgG. **g–i** Rat anterior lobe of pituitary first stained with mouse monoclonal anti-follicle-stimulating hormone (FSH) and Texas red-labelled anti-mouse IgG and subsequently microwaved 3×5 min and then stained with mouse monoclonal anti-thyrotropin-stimulating hormone (TSH) and FITC-labelled anti-mouse IgG. The sections were photographed with a selective Texas red filter (**a, e, g**), selective FITC filter (**b, d, h**) and with a double filter (**c, f, i**). Note in **a–c** that the methods allow the detection of gastrin cells as well as somatostatin cells without contaminating staining while simultaneously detecting a G/D cell (cf. Larsson et al. 1995, 1996) storing both hormones (*arrow*). Note in **d–f** that the method separately detects islet glucagon and somatostatin cells with no evidence of contaminating staining and in **g–i** that FSH and TSH cells are separately detected. *Scale bar* 40 µm (**a–f**); 30 µm (**g–i**) ▶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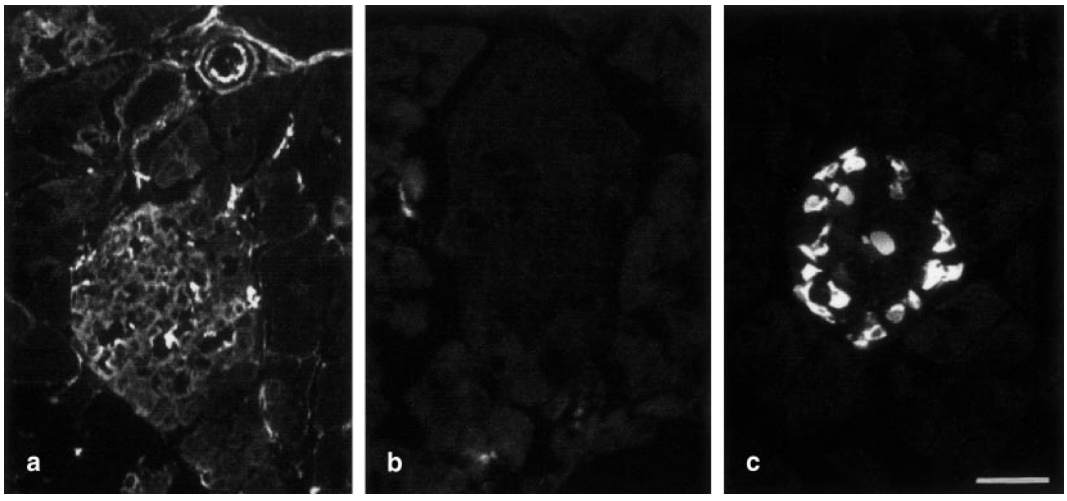

Fig. 2 Mouse pancreas stained with Texas red-labelled anti-mouse Ig without (**a**) or after (**b**) 3×5 min microwaving. Note that without microwaving much staining is present in the interstitial tissue and, in particular, in the blood vessels of a well-vascularized islet of Langerhans in the centre of the field. Note that this staining is considerably reduced in the adjacent section shown in **b**. In **c** is shown another adjacent section immunostained with a mouse monoclonal anti-glucagon antibody and Texas red-labelled anti-mouse Ig after a preceding round of microwaving. Note that only specific staining of the islet A cells is seen. *Scale bar* 45 µm

Results

Initial experiments using microwaving of immunofluorescence specimens revealed that treatments for up to 2×5 min did not lead to detectable losses of fluorescence. With more cycles of microwaving, fluorescence was gradually weakened and showed marked reductions after five or six 5-min cycles. With the FITC-tyramide detection system, no reduction in fluorescence intensity was observed upon repeated microwaving. This was expected since, in this case, the fluorescence was emitted by the peroxidase-catalysed deposits of fluorescent tyramide precipitates and not upon remaining fluorescent antibodies. Nevertheless, this experiment revealed that the microwaving had no direct untoward effect on the FITC fluorescence per se.

Next, the influence of graded microwave treatments on the cross-talk between the first and second antibody staining cycles was examined. The optimal time to avoid contaminating staining was 3×5 min. With all antibody combinations tested, this time produced an optimal inactivation of cross-talk while not leading to overt elution of antibodies. Using different combinations of polyclonal (rat pancreas: rabbit anti-glucagon/rabbit anti-HPP; rat stomach: rabbit anti-somatostatin/rabbit anti-gastrin; Fig. 1) or of monoclonal antibodies (rat pancreas: mouse anti-glucagon/mouse anti-FA1; mouse anti-glucagon/mouse anti-somatostatin; rat pituitary: mouse anti-FSH/mouse anti-TSH; Fig. 1) successful double-stainings were obtained using an intermediate 3×5-min microwave treatment.

As this result applied also to mouse monoclonal antibodies we asked ourselves whether microwaving could also inhibit the often troublesome background observed when mouse tissues were stained with mouse monoclonal antibodies. Our studies revealed that treatment of mouse tissue section with microwaves for 3×5 min before indirect immunofluorescence staining with mouse monoclonal antibodies virtually eliminated background staining in blood vessels and interstitial tissues (Fig. 2). In contrast, staining of intracellular immunoglobulins in plasma cells and lymphocytes was not appreciably changed (data not shown).

Discussion

Our results demonstrate that microwaving is a useful method for immunofluorescence double staining. However, it should be noted that excessive microwaving also elutes antibodies. Thus, for individual applications different durations of microwaving should be tested. In the present study, successful double staining of a number of antigens was achieved by a standard 3×5-min microwaving at 780 W. This time may not necessarily apply to other ovens, antibody combinations or buffers used.

In addition to permit double stainings with primary antibodies raised in the same species, microwaving can also be used to significantly reduce the background observed when mouse tissues were stained with mouse monoclonal antibodies. This is highly useful since the increased availability of mouse monoclonals paired with the use of different mouse transgenic models make such stainings highly desirable. It is worthy of note, however, that microwaving only blocked the staining of endogenous mouse immunoglobulins in interstitial tissues and blood, but not in plasma cells and/or lymphocytes. This presumably relates to the higher concentrations of immunoglobulins stored by these cells. Nevertheless, the elimination of intercellular staining was highly useful for studies of non-lymphoid cells and organs. Studies of the latter would probably require more elaborate methods for complete elimination of reactions with cellular stores

of immunoglobulins (cf. Lu and Partridge 1998). Microwaving is a highly successful method for detection of a number of tissue-bound antigens which otherwise may be masked by adverse tissue-processing methods (Shi et al. 1998 and references therein). However, in our experiments, certain antigens, such as insulin, show a diminished immunoreactivity after microwaving in citrate buffer pH 6.0 (D. Tornehave and L.-I. Larsson unpublished data). Undoubtedly, use of alternative buffers and formulas for antigen retrieval (AR) may amend this type of problem (cf. Shi et al. 1998), but as the present double-staining method depends on compromised reactivity of endogenous and exogenous immunoglobulins, the scope for future improvements may be more limited than in ordinary AR methods. Accordingly, the potential masking and demasking effects of microwaving on individual antigen-antibody combinations need to be examined before double-staining studies are commenced.

The large number of methods devised for double stainings with primary antibodies raised in the same species indicates that no single method is universally ideal. Although the microwaving technique also has its drawbacks, we find it very simple and useful in many practical situations.

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