In "Cell Cycle - Materials and Methods"
M. Pagano, Editor
Springer-Verlag, Heidelberg
1995, pp. 15-28

2 Immunofluorescence Techniques in Cell Cycle Studies

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1 Background

A major goal in the study of cell cycle division, as in any area of cell biology, is to define the function of each cellular component and its interplay with other factors. Immunofluorescence microscopy allows the study of the abundance and subcellular localization of any protein throughout the cell cycle, and thus provides important information towards an understanding of their role. This technique combines the sensitivity of fluorescent probes with the spatial resolution of the light microscope and the specificity of antibodies. Several different fluorescent probes can be selectively detected in a mixture of molecules due to their distinct and characteristic excitation and emission wavelengths. By choosing different color fluorophores, simultaneous detection of multiple antigens within a cell is possible providing a direct means of comparing their relative spatial and temporal distribution.

2 Principle and Applications

Multiple labeling in fluorescence microscopy is an extraordinary powerful means of sorting out the factors involved in a particular cellular process in vivo. This possibility is particularly advantageous when dealing with families of proteins that show high level redundancy in in vitro assays, as in the case of the cyclin-dependent kinase family and their cyclin counterparts. Immunofluorescence makes it possible to follow the expression and subcellular localization of different cyclins, cyclin-dependent kinases or other proteins throughout the cell cycle and to compare their distribution with other family members or potential partners. In the same samples, specific phases of the cell cycle can be identified by monitoring incorporation of nucleotide analogues into the DNA (S phase) and by counterstaining DNA with Hoechst 33258 (mitosis) (Heald and McKeon 1990; Pines and Hunter 1991; Ookata et al. 1992, 1993). The combination of microinjection or transfection with multiple antigen detection by immunofluorescence was successfully used to demonstrate a requirement of Cyclin D1 during progression through G1 phase (Baldin et al. 1993), of Cyclin A and Cdk2 for entry into S phase (Girard et al. 1991; Pagano et al. 1992; Zindy et al. 1992; Pagano et al.

1993; Tsai et al. 1993), of Cyclin B and Cdc2 for progression through mitosis (Riabowol et al. 1989; Gallant and Nigg 1992), and of Wee 1 to prevent premature mitosis without completion of DNA replication (Heald et al. 1993). Furthermore, the possibility of simultaneous detection of multiple antigens provided direct evidence that distinct cellular processes like DNA methylation, DNA replication, and cell cycle regulation, which are traditionally viewed as separate and independent processes, occur at the same subnuclear compartments and are thus temporally and spatially coordinated (Leonhardt et al. 1992; Cardoso et al. 1993). Immunofluorescence thus complements biochemical and genetic assays in that it takes into account the limited spatial and temporal availability of the factors under study and allows analysis at a single cell level, avoiding problems resulting from mixed cell populations. In addition, important in vivo interactions can escape detection by biochemical means if they are part of insoluble structures.

Using a combination of in situ detergent and salt extraction followed by immunofluorescence staining, Bravo and MacDonald-Bravo (1987) showed that, during the cell cycle, there are two populations of PCNA, one that is nucleoplasmic and extracted by nonionic detergent treatment (non-S phase cells), and another that is resistant to high salt and detergent extraction and associates with sites of ongoing DNA replication during S phase. Also, the retinoblastoma protein (pRb) was shown to be tightly bound in the G1 phase nuclei and extractable under low salt conditions at S phase, and this change in extractability correlates with its phosphorylation and inactivation as a growth suppressor protein (Mittnacht et al. 1991). Moreover, Cyclin A and Cdk2 specific association with nuclear DNA replication foci is resistant to Triton X-100 extraction (Cardoso et al. 1993 and M.C.C. and H.L., unpubl.).

Fluorescence microscopy can not only yield qualitative and semi-quantitative data, but it is also a very sensitive method to precisely quantify cellular components within the cell. Fluorescence images acquired from either conventional or confocal microscopes can be digitalized and subsequently computer processed using image analysis software. This field of electronic image acquisition and analysis is rapidly evolving, but exceeds the scope of this chapter. Fluorescence microscopy, due to its sensitivity and specificity, is a unique biophysical tool to directly study specific components inside living cells. In the cell cycle field, however, the use of this technique to probe living cells is still at an early stage and we will focus in this chapter on protocols for fluorescent staining of fixed cells. A good source of information on this exciting area of fluorescence microscopy is gathered in *Fluorescence Microscopy of Living Cells in Culture, Parts A and B* (Wang and Taylor 1989). We will also not address here technical questions pertaining to light microscopy in general, which can be found in *Light Microscopy in Biology: a Practical Approach* (Lacey 1989). The goal of this chapter is to describe basic immunofluorescence protocols typically used in cell cycle studies of

mammalian cells in culture, to discuss possible variations and, most importantly, to point out common problems and necessary controls.

3 Immunofluorescence Protocols

Antibodies

Immunofluorescence requires that an antibody specifically recognizes a particular antigen in the presence of very high concentrations of other molecules. Polyclonal antiserum, although usually very good for staining cells because it contains many different antibodies that can bind to multiple epitopes on the antigen, often include high concentrations of nonspecific antibodies that give rise to high background. In most cases, simple titration of the antiserum will eliminate this problem by reducing the spurious signals below detection level. The signal strength of an antiserum is directly dependent upon the concentration of the antibody multiplied by its affinity constant: the higher the affinity, the greater the working dilution. When titration is not sufficient, the antiserum can be preadsorbed with the fixed cells prior to use. Indeed, one often obtains cleaner signals by reusing the same antibody dilution multiple times. Another method to decrease unspecific staining, which is frequently used with polyclonal antisera, is immunoaffinity purification of the antibodies (see chapter by M. Pagano, Part Four).

Monoclonal antibodies can be used either as tissue culture supernatants or as ascites fluid, and can also be pooled, which generally improves signal strength, granted that the individual antibodies bind noncompetitively to the antigen. Although monoclonal antibodies usually give clean signals, some monoclonals will not react in cell staining. This can be due to the fact that their specific epitope is either masked within the cell or destroyed during fixation.

Sample preparation

Cells for staining are usually first attached to a solid support (glass slide or coverslip, No.1 thickness) to facilitate the subsequent manipulations. In the case of adherent cultures, cells should be grown directly on previously sterilized coverslips (or, alternatively, in chamber slides).

Suspension cells can be centrifuged (approximately 1000 *g* for 10 min, at room temperature) onto glass slides using a cytospin apparatus, or can be attached to the slide with poly-L-lysine or, simply, by drying (cell smears). Slides are coated by dipping into a 1 mg/ml solution of poly-L-lysine in water for 10 min and washed with water. Slides can be dried and stored or used immediately. Cells in suspension are then spotted onto the coated slide and allowed to attach for about 10 min. Binding of cells to the positively charged poly-L-lysine covered surface, although not covalent, is usually strong enough to resist most steps of the staining procedure. Hereafter cells are processed as adherent cultures.

When dealing with cell-surface antigens, cultures can be handled in suspension either as live or as formaldehyde-fixed cells and solutions are changed by carefully spinning (at 200 g for 5 min) and resuspending the cells. The steps in the staining procedure are otherwise similar to the protocol for adherent cultures.

Fixation/permeabilization

This step converts soluble native cellular constituents into insoluble derivatives, thereby preserving cellular architecture. The most commonly used fixatives are organic solvents and cross-linking agents. The former agents (mostly alcohols, such as methanol or ethanol, and acetone, or a mixture of both) extract lipids and dehydrate the cells, precipitating the proteins. The latter agents (generally aldehydes, such as formaldehyde and glutaraldehyde) form intermolecular bridges via free amino groups creating an intracellular network of linked constituents. Stabilizing the content of biological specimens during fixation often causes denaturation of protein antigens, which is why antibodies raised against denatured proteins are often better for cell staining.

The choice of fixative can only be established empirically, so we routinely try two agents, methanol and formaldehyde, process the samples, and then evaluate the results. Often, one of the methods destroys the antigen and thus cannot be used. These tests should be run with each new antibody and prior to attempting multiple labeling procedures. With pooled monoclonals, each should first be checked individually.

Cells are first washed in phosphate-buffered saline (optionally, it can be supplemented with 1.5 mM $MgCl_2$ and 1 mM $CaCl_2$) and fixed either by incubating with –20 °C methanol for 2–5 min or with 3.7% formaldehyde (Fisher, certified ACS) in PBS for 10–15 min at room temperature. Unless otherwise stated, all manipulations are at room temperature. Methanol-fixed specimens can be air-dried and stored dehydrated at –70 °C for several months. When needed, the methanol fixed samples are brought to room temperature and rehydrated by incubating for some minutes in PBS. Formalin-fixed samples are rinsed with PBS to remove fixative and can be stored at 4 °C in PBS with 0.02% NaN_3 (to prevent microbial contamination) for several weeks.

Reactive aldehyde groups, which can increase background fluorescence, can be quenched by incubating the fixed specimen for 10 min in a solution of 50 mM glycine in water. The amino group of the glycine reacts with free aldehyde groups, thereby blocking spurious crosslinks in the rest of the procedure.

After aldehyde fixation, cultures must be still permeabilised to allow access of the antibodies inside the cells. When dealing with extracellular epitopes of membrane components, this step is generally omitted. Cells can be permeabilized either by incubation for 30 s in acetone or, more commonly,

by the action of detergents. In our hands, a 10-min-incubation with 0.25% Triton X-100 in PBS works well with most cell types.

An optional procedure includes permeabilization prior to fixation. Multiple conditions can be used and are reviewed in Pachter (1992). These protocols allow one to perform in situ fractionation of the cells, which can be followed either by biochemical or cytological analyses. For the purpose of this chapter, extraction and subsequent immunofluorescent detection can provide valuable information on the association of a particular antigen to insoluble cellular components. Often, specific localization patterns are difficult to visualize by conventional microscopy due to masking by either background fluorescence or by the soluble fraction of the antigen, or both. This is specially critical when dealing with low abundance nuclear proteins and/or low affinity antibodies. Incubating the unfixed cultures for about 30 s in a physiological buffer (100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM PIPES pH 6.8) containing 0.5% Triton X-100, releases approximately 70% of the cellular protein content, 75% of the tRNA, and almost all the phospholipids in the "soluble" fraction, retaining the nuclei, polyribosomes, and cytoskeleton. These Triton-extracted cells (Triton cytoskeletons) can be fixed and stained or further treated for 2 min in PBS containing different salt concentrations (NaCl from 10 mM up to 1 M). The salt-extracted cytoskeletons are then fixed and processed.

Using procedures similar to the one just described, it has been possible to distinguish during the cell cycle, populations of PCNA and pRb with different nuclear affinities (see Sect. 2, Principle and Applications).

Antibody binding and detection

Once the cells are fixed (after or before the permeabilization step), the coverslips or slides (cell-side-up) are transferred to a humidified dark chamber where all subsequent antibody incubations and washes are performed. A diagram of this setup is presented in Fig. 1. It is simply a petri dish covered with aluminum foil to prevent long exposure of the fluorochrome-conjugated

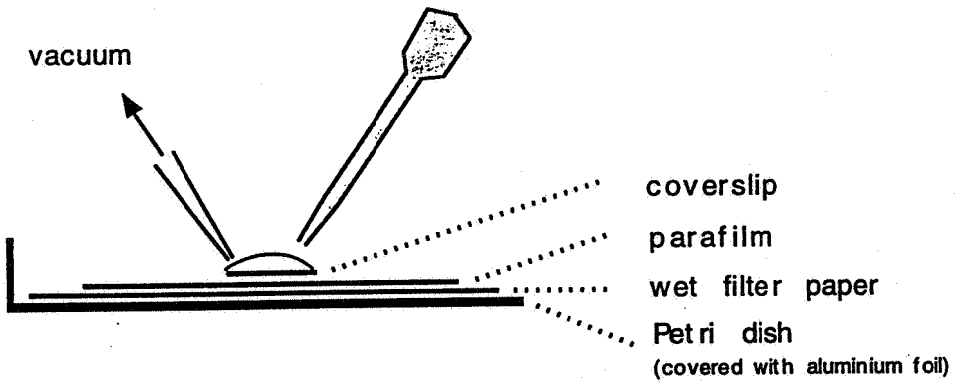

Fig. 1. Diagram of the setup for immunostaining

reagents to light with consequent fading of the signal, and with a wet filter paper covered with parafilm in the bottom of the dish to avoid drying out of the sample.

Before adding the antibody solutions, nonspecific binding should be inhibited by preincubation of the sample for about 30 min with blocking agents, a commonly used one being bovine serum albumin (BSA) at 3% in PBS (which also blocks reactive groups from aldehyde fixation). In addition, one should add the same blocking agent to all antibody dilutions. Even though BSA can be an efficient blocker and an inexpensive one, the most appropriate agent to saturate the specimen is 5% normal serum (diluted in PBS) from the same host as the labeled antibody. Normal sera, which have been lipid extracted and dialyzed, are available from a variety of commercial suppliers. For example, an anti-rabbit IgG conjugated antibody raised in goat is best blocked by the use of 5% normal goat serum in PBS. In all instances, never block a specimen with normal serum from the same host species of any of the primary antibodies, because the labeled secondary antibody would react with it.

After blocking, the antibody is added to the sample. Depending on the size of the glass coverslip, volumes as small as 10 µl can be enough to cover the cells. The hydrophobic parafilm layer will help to prevent the liquid from running off the edge of the coverslip, and it also facilitates recovering the coverslip from the dish with fine forceps. As in other immunoassays, the primary antibody can be itself labeled (in that case, the staining is referred to as direct immunofluorescence), or it can be detected with a labeled secondary antibody, which binds specifically the primary antibody (it is then called indirect immunofluorescence). Both methods are commonly used, even in combination when performing multiple stainings. The direct detection, though, requires labeling of purified primary antibody and gives less signal intensity, since the number of fluorochromes is lower. The indirect method is more sensitive because more than one second antibody molecule can react with each first antibody. The major disadvantage of the indirect method is that the background can be higher and one has to control for possible interspecies cross reaction when doing multiple antibody combinations.

Primary antibodies should be tested at concentrations between 0.1–50 µg/ml, depending on the binding affinity. As a general guideline, monoclonal antibodies are used as straight hybridoma tissue culture supernatant, ascites fluid and polyclonal antiserum should be assayed at various dilutions between 1/100–1/10 000. Incubation is carried out for 1–2 h. Since the antigen is immobilized, the time needed for antibody binding is longer than when both antigen and antibody are free in solution. With some antibodies, raising the incubation temperature to 37 °C may help improving signal strength.

Even though antibodies bind to their particular antigen with high specificity, they can also give rise to nonspecific binding. This can be particularly dangerous in cell staining since nonspecific reactions are not as easy to rec-

ognize as in other immunoassays where, for example, proteins are separated in a gel. The major causes for nonspecific binding are:

- antibodies present in the polyclonal antiserum reacting with other cellular antigens;
- cross reaction of the specific antibody with other molecules in the cell that are different but have similar or identical epitopes;
- or, when more than one molecule share the same epitope.

The first type of artifact can be easily detected by appropriate negative controls. The antibody staining should always be compared with a similar dilution of a preimmune serum of the same animal used to prepare the specific antiserum (or an antiserum preadsorbed with the original antigen), in the case of polyclonal antibodies, or to an ascites fluid or tissue culture supernatant from the same source as the specific antibodies, in the case of monoclonal antibodies. When possible, antibodies from the same class and subclass should be used as controls. In addition, if the antigen is known to be absent from some cell types, these can also be used as negative staining controls. These controls do not need to be run every time but must be performed when trying a new antibody. The second cause of nonspecific binding, i.e., cross reaction with other molecules, can generally be eliminated by further dilution of the antibody, since the affinity of the antibody for these other molecules is usually lower than to the specific antigen. The third cause is more difficult to trace and to eliminate. Testing the antibodies also in western blots of cell extracts should help addressing these types of problems.

After incubation with the first antibody, the sample is washed with several changes of 0.1% NP-40 in PBS over approximately 5 min. If background is a problem, the detergent concentration can be increased to 0.5%. When the first antibody is unlabeled, a second conjugated antibody recognizing the first one is added. This should also be previously titrated and, as a general rule, used between 0.1–10 μg/ml. Incubation should be carried out for 30–60 min, followed by extensive washes again in NP-40/PBS as above. The secondary antibody can be either fluorescently labeled or, for instance, biotinylated. In the latter case, a third incubation with fluorescently labeled streptavidin for 15–30 min has to be performed, followed by washing. Once more the reagent should be first titrated with a positive first antibody control. The first time the secondary reagent(s) is used, a negative control should be included in which the first antibody is omitted, to check for nonspecific staining by the reagent(s) used to detect the primary reagent. An additional important control that should always be performed is a positive control (i.e., an antibody previously known to react with the sample in use). If no staining is observed, this generally means that a reagent was either inadvertently omitted or the wrong reagent was used instead. Care must also be taken not to allow the specimen to dry out at any time during the procedure and to pre-

vent evaporation of the antibody solution during incubation, since it would result in intense background and overstaining, respectively.

Mounting After the last antibody incubation, the nuclei can be counterstained with the DNA-intercalating agent Hoechst 33258 (bisbenzimide). Hoechst 33258 staining of condensed chromosomes allows identification of mitotic cells and, in more detail, of the specific stage of the mitotic process the cell is undergoing. The staining is accomplished by incubating the sample for 5 min in wash buffer (NP-40/PBS) with 1 µg/ml Hoechst 33258 (stock solution 1 mg/ml in H_2O), followed by washing. The samples are now ready to be mounted in an aqueous-based mountant. We use a polyvinyl alcohol-based mounting media that hardens within a few hours, forming a permanent preparation. The pH of the mountant is very important, particularly when using fluorescein conjugates, since their maximal fluorescence occurs only at pH 8.5 or higher. In addition, an anti-fading agent such as DABCO (1,4-diazobicyclo-[2.2.2]-octane) should be added to diminish bleaching, which is specially critical with fluorescein. Other anti-fading agents that can be used instead are the antioxidants *n*-propyl gallate (added to 5%) or *p*-phenylenediamine (added to 0.1%). To prepare the mounting media, add 8 g of Mowiol 4-88 (or Gelvatol, or Elvanol) to 40 ml of 0.2 M Tris-HCl pH 8.5 and dissolve by heating at 50–60 °C with occasional stirring. After cooling down, add 20 ml of glycerol and 1–2.5% DABCO. Centrifuge at 5000 rpm for 15 min and store in aliquots at –20 °C. After thawing, mount the coverslip in one drop of mounting media and let it harden at room temperature for some hours, in the dark. Usually, in 30 min the specimen can already be viewed. If this mountant is not available, the samples can be mounted in 90% glycerol in PBS (if possible with an anti-fading agent added) and the coverslips sealed to the slide with nail polish. These are not permanent mounts though. The mounted samples should be stored in the dark, at 4 °C or frozen.

Multiple labeling Fluorescence microscopy is suited for the detection of multiple antigens in the same specimen, due to the availability of different color fluorochromes with well resolved excitation and emission spectra. In the field of mammalian cell cycle, this technique has been successfully exploited to sort out the spatio-temporal localization of the several cell cycle regulators isolated thus far. Furthermore, the ability to relate subcellular localization of a specific protein to the distribution of its possible partner(s) and to cellular processes such as DNA replication and mitosis, has provided invaluable information on cellular functions in vivo. In a broader perspective, it can explain how the organization of the cytoplasm and nucleoplasm into metabolically distinct compartments may have general regulatory functions including cell cycle control. This functional organization can be best studied by multiple staining techniques.

The choice of fluorophores to use depends, obviously, on the instrument setup and the degree of color separation between the fluorophores (in order to minimize overlap between excitation and emission spectra). A good combination of fluorochromes for a triple labeling is AMCA/FITC/Texas Red. (AMCA and Texas Red are trademarks of BioCarb Chemicals and Molecular Probes, Inc., respectively.) Filter sets (i.e., an excitation filter, transmitting a portion of the emission from the light source; a dichroic mirror, reflecting the selected light to the sample; and an emission filter, blocking any residual excitation light and transmitting the longer wavelengths) that match the spectral properties of a vast array of fluorescent dyes and have good transfer efficiency, are available from microscope manufacturers and also from other companies, including Omega Optical and Chroma Burlington. The latter also provide filter sets for multiple-dye combinations, which can be very useful for colocalization studies using multiple labeling immunostaining. These filters avoid problems such as optical shift, often encountered when recording dual fluorescence images by doubly exposing photomicrographs.

To use more than one fluorochrome in the same specimen, care must be taken to ascertain oneself that each antigen is recognized by only one labeled reagent. The safest way is to use primary antibodies that are labeled with different fluorochromes. The staining can then be performed simultaneously, but control samples individually stained should be included to monitor for cross reaction. Most commonly, though, the primary antibodies are not labeled and only indirect detection is possible. In this case, the antibodies should be from different species, class, or subclass. Then, one can use labeled secondary antibodies that are specific for the individual primary antibodies. Always the specificity of the detection reagent must be checked since antibodies against one species, class or subclass may cross react with a number of other species, class, or subclass unless they have been specifically adsorbed (secondary antibodies preadsorbed with immobilized serum proteins from other species are available commercially from several manufacturers). Hence, one should try to use secondary antibodies that are derived from the same host species. For example, when using a rabbit polyclonal antibody and a mouse monoclonal antibody, one should use anti-rabbit IgG and anti-mouse IgG antibodies both produced in, for instance, goat and the blocking agent should be 5% normal goat serum. In addition, an important control to monitor species cross reactivity of the detection reagents, is to perform the immunostaining using each of the primary antibodies with the secondary antibody specific for the other primary antibody.

Multiple stainings can also be done sequentially with fixation steps in between to help minimizing cross reaction. When dealing with two antibodies, such as two mouse monoclonal antibodies that are from the same type, which happens quite often, it is better to have one directly labeled. It is also advisable to carry out the staining sequentially, with the labeled primary an-

tibody incubated last, to avoid possible binding of the labeled secondary to this antibody.

In summary, in order to perform multiple labeling immunostaining one must take into account two important criteria:

- the fluorochromes chosen must have well resolved spectral characteristics
- the secondary antibodies available are derived from the same host so that they do not recognize one another. They must also not recognize the other primary reagents used or any other immunoglobulins present in the assay, and they should not react with the sample directly.

Monitoring S phase by BrdU incorporation

For the study of cell cycle progression it is essential to have a procedure to identify cells in S phase and in M phase. The latter is easily assayed with any DNA-intercalating dye such as Hoechst 33258, as mentioned earlier. The former is feasible by labeling the cultures with biotinylated dUTP, followed by detection with fluorescently labeled streptavidin, or, more commonly, with the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU), followed by immunofluorescence staining with an antibody to BrdU (see also chapter by M. Giunta and C. Pucillo, Part One). Labeling can be carried out for several hours to allow detection of any cell that was replicating its DNA throughout the period of time prior to fixation. The continuous labeling protocol has been very useful in experiments where antibodies or antisense constructs were microinjected into the cells and the function of the inactivated products in the G1/S phase transition was being assayed. Pulse-labeling the cultures for periods of 2–10 min allows visualization of sites within the nucleus where replication occurs. These studies have shown that replication takes place in discrete patterns of nuclear foci, which undergo characteristic changes throughout S phase (Nakamura et al. 1986; Mills et al. 1989; Nakayasu and Berezney 1989; Fox et al. 1991). Furthermore, DNA synthesis proteins (Bravo and Macdonald-Bravo 1987; Hozak et al. 1993), DNA methyltransferase (Leonhardt et al. 1992) and cell cycle regulators (Cardoso et al. 1993) have been shown to specifically localize to DNA replication foci within the nucleus, providing a direct link between these different cellular processes.

Immunodetection of BrdU incorporation requires partial denaturation of the DNA to allow access of the monoclonal antibodies to the antigenic thymidine analogue. Various procedures can be used to produce single-stranded DNA exposing the halogenated pyrimidines. These include treatment with HCl, thermal denaturation in the presence of up to 50% formamide (Beisker et al. 1987) and enzymatic digestion using either restriction endonucleases alone or endonucleases followed by exonuclease III (Dolbeare and Gray 1988). We will describe two relatively mild procedures, which fall in the first and third categories, and thereby can be used in conjunction with multiple labelings.

BrdU is added to the culture media at a final concentration between 20–100 μM for the desired period of time (from a short 2–10 min pulse to several hours). Cells can be simultaneously fed with 5-fluoro-2'-deoxyuridine (0.4 μM), an inhibitor of thymidilate synthetase, which increases the amount of incorporated BrdU into replicating DNA by lowering the competition with endogenous thymidine. After fixing and permeabilizing (alternatively, the cells can be extracted prior to fixation), in the enzyme digestion-based method, the samples are treated for 30 min with EcoRI (15 units in 100 μl of the respective enzyme buffer), followed by 30 min exonuclease III digestion (30 units in 100 μl of the recommended buffer). Following this treatment, they are incubated with anti-BrdU monoclonal antibody and detected as usual (Dolbeare and Gray 1988; Fox et al. 1991). The enzyme digestion can also be done at the same time as the antibody incubation and together with other antibody binding in dual labeling experiments (Cardoso et al. 1993). A cocktail of diluted anti-BrdU monoclonal antibody with nucleases is commercially available from Amersham and Boehringer Mannheim.

In the acid denaturation method, fixed and permeabilized cells are incubated 10 min in 1.5–4 M hydrochloric acid (inclusion of Triton X-100 to

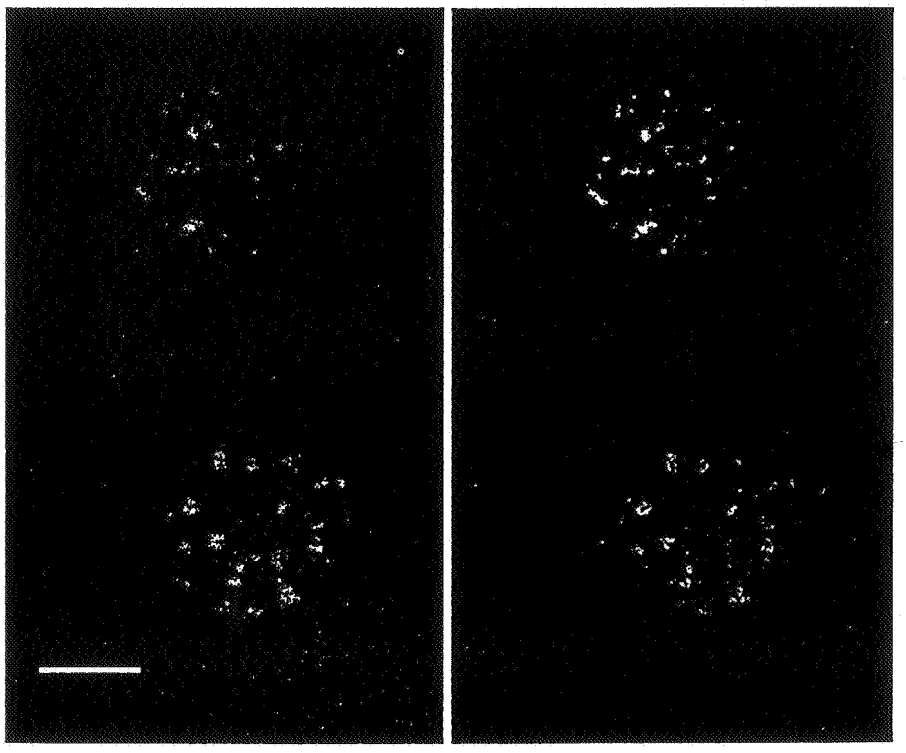

Fig. 2. Specific localization of Cyclin A (*right*) at subnuclear sites of ongoing DNA replication (*left*) as visualized by double staining with BrdU-specific monoclonal antibodies. *Bar* 10 μm

0.7% helps to extract chromatin proteins), neutralized 10 min with PBS and finally stained with anti-BrdU monoclonal antibody as described above. When performing dual stainings, the samples should first be stained for the other antibody as explained earlier, and sequentially fixed again, denatured, neutralized, and finally stained with the BrdU-specific antibodies. An example of the latter staining procedure, where Cyclin A was immunolocalized in an S phase nucleus of a mouse myoblast cell and analyzed by optical dissection with a confocal laser microscope, is presented in Fig. 2 (for specific details see Cardoso et al. 1993).

Fluorescently labeled dUTP analogues are now commercially available (Boehringer Mannheim) and make it possible to directly label and visualize sites of DNA replication in living cells.

The previous sections should have made it clear that there is no such thing as a universal and perfect protocol for cell staining. The procedure has to be optimized for each antibody and application. Possible variations and controls to be performed are described in the respective sections. The following simple and basic protocol may serve as a starting point and is only one of numerous, possible protocols.

Basic protocol for immunofluorescent staining

1. Wash cells in PBS and fix for 10 min in 3.7% formaldehyde (all solutions are made in PBS and incubations at room temperature).
2. Wash in PBS and permeabilise for 10 min in 0.25% Triton X-100.
3. Block cells for 10 to 30 min in 5% normal serum derived from the same species as the labeled antibody (blocking buffer).
4. Incubate for 60 min with primary antibody diluted in blocking buffer in a humidified chamber (see Fig. 1).
5. Wash with several changes of 0.1% NP-40 over 5 min.
6. Incubate for 30–60 min with a labeled secondary antibody diluted in blocking buffer.
7. Wash with several changes of 0.1% NP-40 over 5 min.
8. Counterstain DNA by adding Hoechst 33258 at 1 μg/ml to the second but last washing step.
9. Mount coverslip in mowiol (pH 8.5) with 2.5% DABCO.

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