INTRODUCTION

To study the three-dimensional organization of the nucleus it is necessary to visualize specific DNA sequences or proteins while preserving the native nuclear structure. Yeast provides specific problems, since unlike higher eukaryotes, in which the nuclear lamina provides a rigid support for the shape of the nucleus, yeast nuclei collapse in nonionic detergents 1-3. Morphological work in yeast therefore requires that cells or nuclei are well-fixed by chemical crosslinking prior to permeabilization of the nuclear envelope. An alternative approach is to localize endogenous proteins in yeast by fusion with Green Fluorescent Protein (GFP) 4, or derivatives thereof, or to localize specific chromosome domains by targeting a DNA-binding factor fused to GFP (e.g. laci- or the tet-repressor) to clusters of its recognition consensus inserted at the chromosomal site of interest 5-7. However, these methods are not particularly well adapted to labeling multiple targets, nor do they allow localization of a specific protein relative to a given DNA sequence or a given structure, such as the nuclear envelope. The combined immunofluorescence (IF)/Fluorescent in situ hybridization (FISH) protocol described here has been optimized to analyze the subnuclear localization of several proteins at once and to localize proteins relative to DNA sequences 8-9.

Earlier FISH protocols for budding yeast included treatments with protease after fixation or extraction with SDS and TritonX-100, to enhance hybridization efficiency 10-11. The protocols we present here omit protease and harsh detergent treatments. Cells are fixed after spheroplasting in osmotically buffered growth medium, to efficiently preserve nuclear structure. In this way, the cell wall does not itself become a target of the crosslinking reagents, a phenomenon that can prevent efficient diffusion of the fixative to the nucleus. At the same time we obtain high efficiency labeling with both antibodies and DNA probes. The described protocol can also be performed with cells that are fixed prior to spheroplasting, if preservation of the cell shape is desired 9.

This double in situ/ immunofluorescence staining provides a powerful tool for the characterization of nuclear protein localization and the positioning of specific chromosomal
regions. Confocal microscopy of the fixed cells demonstrates that the nuclei maintain their three-dimensional organization throughout the procedure\(^8\). We have found that immunolabeling with antibodies specific for the nuclear pore complex is a sensitive means to monitor both the integrity of the nuclear envelope and the size of the nucleus. The diameter of an intact diploid nucleus is about 2 µm, while that of a flattened chromatin mass is \(~ 6-8 \) µm. By aiming to maximize the maintenance of nuclear integrity, we limit the sensitivity of the FISH assay, and unique sequence probes need to contain roughly 6 kb for reproducible detection. Moreover, the combination of immunofluorescence and FISH can result in a reduced sensitivity for immunolocalization, since not all antibodies resist the hybridization conditions. For specific applications, such as immunostaining alone, immunostaining on cells fixed prior to spheroplasting, or double GFP-immunolocalization, this protocol can be modified as indicated at the end of the chapter.

**Yeast Strains and Media**

Diploid yeast cells are preferred for these studies because nuclei are nearly twice the size of haploid nuclei. For cell synchronization experiments a/a diploids can be used after release from alpha factor arrest\(^{12}\). There is a significant variation in the efficiency with which different strains are converted to spheroplasts, probably reflecting differences in the cell wall composition. Whenever mutant and wild type strains are compared, it is preferable that they share the same genetic background. Moreover, it is important to note that the efficiency of spheroplasting can be affected by growth conditions, i.e. carbon source, rate of growth and stage of growth at the time of harvest. Best results are obtained with cells grown on rich medium (YPD)\(^{12}\) to mid-logarithmic phase (1-2 x \(10^7\) cells/ml).

**Antibody Purification and Specificity**

Polyclonal antibodies are a good tool for immunofluorescence because they recognize a variety of different epitopes, and are likely to recognize at least one present on the denatured protein in fixed cells. However, most rabbit sera react with a variety of yeast proteins in addition to the specific antigen, and therefore we strongly recommend that all antibodies used for yeast immunofluorescence be affinity purified against recombinant antigen as follows:

Perform a Western transfer of the recombinant antigen onto nitrocellulose filter. After staining with Ponceau red (0.05% in 3%TCA), cut out the strip containing the protein band. Wash the nitrocellulose strip 3 x 10 minutes in 1xTEN (20 mM Tris-Cl, pH 7.5, 1 mM
EDTA, 140 mM NaCl), 0.05% Tween20. Block excess protein binding sites by incubating in 1xTEN + 0.05% Tween20 + 1% dry milk powder, at room temperature for 20 minutes. Incubate the strip with 10-50 µl of serum (depending on the titer of the antibody) in 1 ml of 1xTEN, 0.05% Tween20, 1% dry milk powder, overnight at 4°C with constant agitation ( rocker or wheel).

Remove the supernatant, wash the strip 3 x 10 minutes in 1xTEN, 0.05% Tween20 at room temperature. Elute the bound Ig with 300 µl of 100 mM glycine, pH 3.0, for 2 minutes. Immediately raise the pH to 7.0 by adding 1M Tris base (the volume needed should be determined before starting), and place on ice.

Repeat the elution once or twice and pool the elutions. The number of elutions required depends on the antibody and the first time elutions should be checked separately. It may be necessary to use glycine at a lower pH (pH 1.9, for example) to elute the antibody.

Once purified, the antibodies can be stored as aliquots at -80°C. Stabilization is enhanced by addition of 1-2% ovalbumin and 20% glycerol. The antibody is used at a dilution of 1:2 or more for immunofluorescence. The specificity of the purified antibodies should be demonstrated by Western blot and immunofluorescence on strains lacking the protein in question.

Monoclonal antibodies have the advantage of recognizing a single epitope thereby decreasing the probability of nonspecific background staining. Therefore they do not have to be affinity purified before use. However, since this same epitope may be shared by other proteins, it is essential to test the specificity of the antibody on Western blots. The obvious disadvantage of monoclonal antibodies is that the unique epitope may be masked or denatured under the conditions used to fix and permeabilize cells.

Secondary antibodies coupled to fluorophore are used to detect primary antibodies. They should be tested for a lack of cross-reactivity with yeast proteins, by performing immunofluorescence in the absence of the primary antibody. To eliminate non-specific background, it is advisable to preabsorb the secondary antibody on fixed yeast cells as described in part I (below).

**Choice of the fluorophore**

The choice of the fluorophore depends on the number of different probes that need to be visualized on each sample, and on the filter sets available for the microscope. It is important to choose those that can be excited and visualized independently. If there is overlap between the emission spectra, some signal must be attenuated in order to avoid “bleed through”. Some of the more commonly used fluorophores are listed below:
Immunofluorescence

1. Grow the cells overnight to about 1-2 x 10^7 cells/ml in 50ml YPD or selective media.12
2. When maintenance of the cell shape and cytosolic structures is required, you should first fix the cells in growth medium for 20 minutes at 30°C in 4% paraformaldehyde, prior to spheroplasting. The paraformaldehyde should be freshly prepared before the experiment begins by mixing 5 g of paraformaldehyde, 15 ml H_2O and 25 µl 10 N NaOH. Dissolve at 70°C in a closed bottle for about 30 minutes with occasional shaking (do not let fumes get into eyes!).
3. Harvest the cells at 1200xg for 5 minutes at room temperature in preweighed 50ml polypropylene tubes (keep the Erlenmeyer flasks).
4. Decant the supernatant and weigh the cell pellet.
5. Resuspend the cells in 1 ml/0.1 gram of cells 0.1 M EDTA-KOH (pH 8.0), 10 mM DTT.
6. Incubate at 30°C for 10 minutes with gentle agitation.
7. Collect the cells by centrifugation at 800xg for 5 minutes at room temperature.
8. Carefully resuspend the cell pellet in 1 ml/0.1 gram cells YPD + 1.2 M sorbitol (mix 22g sorbitol with 100 ml YPD). To resuspend evenly, suspend the cell pellet first in 500 µl.
9. Add lyticase (β-glucanase) to 3000 U/ml and Zymolyase (20T, Seikagaku) to 600-1000 µg/ml.
10. Incubate at 30°C in the original Erlenmeyer flask with gentle agitation and monitor spheroplast formation in the microscope at 5, 10, 15 and 20 minutes. The cells should be checked microscopically every 5 minutes and spheroplasting is completed when they appear transparent. Buds should remain attached and cell shape is usually maintained.
11. You can then “re-fix” or not (or if you have not fixed in the culture do it now), depending on the sensitivity of your antigen. To “fix” at this point, transfer to the polypropylene tube and add paraformaldehyde to a final concentration of 4% from a stock of 20%. Fix 10 minutes at room temperature (keep the tubes horizontal with occasional gentle shaking).
12. Dilute with YPD + 1.2 M sorbitol to 40 ml. Centrifuge 5 minutes at 800xg.

13. Wash twice in 40 ml YPD + 1.2 M sorbitol, resuspending gently. Centrifuge 5 minutes at 800xg.

14. Resuspend 0.5 g spheroplasts in 0.8 ml YPD with or without sorbitol. The concentration of the cells in this suspension should be such that only one layer of nonconfluent cells will adhere to the slide. Leave a drop on each spot of the slide (Polylabo, super-teflon slides) for 1-2 minutes to allow the spheroplasts to adhere on the surface of the slide, and take away as much liquid as possible using a pipet. Superficially air dry 2 minutes. All the following washes are performed immersing the slide in a Coplin jar containing the appropriate buffer.

15. Put the slides in methanol at -20°C for 6 minutes (prechill the methanol).

16. Transfer the slides to acetone at -20°C for 30 seconds (prechill the acetone).

17. Air dry 3 minutes

18. Incubate slides in 1x PBS$^{13}$ + 1% Ovalbumin + 0.1% Triton X-100 for 20 minutes or more. Shake gently 2 or 3 times at room temperature. After this step the cell appear transparent and the nucleus can be seen as a dark spot. This is an indication of good spheroplasting. If this is not the case, it may help to leave the slides for a longer time in PBS + 1% Ovalbumin + 0.1% Triton X-100.

19. Dry the black surface and bottom surface of the slides with a paper towel.

20. Cover each spot on the slide with 10 µl of the appropriate primary antibody diluted as required in PBS containing 0.1% Triton X-100. If affinity purified antibodies are used, they should be diluted two or three fold in 0.5 x PBS + 0.1% Triton X-100 to avoid high salt concentrations.

21. Incubate for 1 hour at 37°C in a humid chamber or overnight at 4°C. In the latter case the slides should be covered with a coverslip to avoid drying of the antibody solution.

22. Preabsorb the secondary antibody on yeast cells. For this purpose, use the remaining fixed spheroplasts by washing them 3 x in PBS and resuspending them in 1 ml of PBS. Dilute the secondary antibody (stock is usually 1mg/ml) 1:50 in this spheroplast suspension and incubate for 1 hr on a rotating wheel at 4°C in the dark. Centrifuge at top speed, collect the supernatant and add Triton X-100 to a final concentration of 0.1%. Store on ice until use.

23. After the primary antibody incubation, wash the slides 3 x 5 minutes in PBS + 0.1% Triton X-100 at room temperature.
24. Dry the black surface and bottom surface of the slides. Cover each slide with 10 µl/spot of the fluorescent secondary antibody prepared as described in step 22 and incubate for 1 hr at 37°C in the humid chamber in the dark.

25. After the secondary antibody, wash the slides 3 x 5 minutes in PBS + 0.1% Triton at room temperature.

26. Add 15 µl per spot of antifading (PBS, 50% glycerol, 24 µg/ml diazabicyclo-2,2,2, octane or DABCO, pH 7.5). Cover with a coverslip avoiding air bubbles and seal with finger nail polish. Keep the slides at 4°C in the dark.

To visualize the DNA, various DNA staining agents as Ethidium Bromide (diluted to 1 µg/ml in antifading), DAPI (1 µg/ml), or POPO-3 (Molecular Probes) can be used, depending on the filters of the microscope and on the secondary antibodies which have been used. It is important to check that the wavelength of Absorbance and Emission do not interfere with the fluorescent probes already used.

VARIATIONS

An alternative way to localize proteins both in living and in fixed cells is to fuse the gene with that encoding GFP⁴. However, proteins fused to GFP must be assayed to ensure that they retain as many of their physiological attributes as can be measured. For very abundant or overexpressed protein, the GFP fluorescence will survive the immunofluorescence protocol. When this is not the case, antibodies raised against GFP can be successfully used in combination with antibodies raised against the second antigen of interest.

There are specific applications in which it is not necessary to preserve nuclear integrity to draw some conclusions about the localization of DNA sequences, e.g. assays for the pairing of mitotic or meiotic chromosomes¹⁰-¹¹. However, one must be careful not to draw conclusions about nuclear architecture from results obtained with flattened or spread preparations.

Evaluation of the method and troubleshooting

As an assay for nuclear integrity, dimensions of the nucleus can be calculated from EtBr or DAPI fluorescence. When nuclei are well preserved we measure a diameter of 2.0 +/- 0.2 µm in the XY plane, and 2.4 +/- 0.2 µm in the Z axis. This 20% distortion along the Z axis is also observed when cells are fixed prior to spheroplasting and reflects an integral Z-stretch function of the confocal microscope and software program. On the other hand, if cells are treated with detergents
prior to fixation, or if spheroplasts are protease-treated prior to FISH, cells become flattened, no
longer maintaining this spherical shape and the number of Z-sections possible is reduced to
one or two, indicating that the flattened nucleus is less than 0.5 µm in height.

As an independent assay for nuclear integrity, a mouse monoclonal antibody raised against the
human nuclear pore protein p62, a homologue of yeast Nsp1p can be used. Immunofluorescence of Nsp1p shows a ring-like staining at the nuclear periphery typical of nuclear pore staining. This staining is lost if cells are spheroplasted to completion prior to formaldehyde fixation, or if the nuclear envelope is disrupted by detergents. This confirms that the presence of a punctate/ ring-like staining with anti-p62 correlates with a degree of nuclear integrity. In addition, this antibody allows to monitor the relationship of the immunofluorescence signals to the nuclear periphery as defined by the nuclear pore signal.

The resolution of the light microscope depends on the fluorochrome used. Under optimal
conditions with DTAF or FITC, a resolution of 0.2 µm can be achieved in the XY axis with optimal optics and alignment. In the Z-axis resolution is much less efficient (0.6 µm). Quantitation of colocalization of two signals can be carried out on computer graphic representations of the fluorescent signals from one focal section taken as near as possible to the equator of the field of cells. A threshold for contour tracing can be set using Adobe Photoshop v4.0 software, after a normalization of each filter channel independently to give the same maximum signal. To control whether a given localization is statistically significant, a simulation by computer randomization for a given size and number of signals has been developed (contact Philippe Bucher, ISREC, e-mail pbucher@eliot.unil.ch). It is essential to check that under standard imaging conditions no signal from one fluorochrome can be detected on the other filter set, and that image capture and background subtraction is done uniformly on all images to allow direct comparison.

REFERENCES


