

Choosing a cell fixation method

Cell fixation is often the harshest treatment in an immunofluorescent detection procedure. The fixation techniques listed in the procedures of this section provide a good starting point, but will need to be optimized experimentally for each type of cell and specific tagged protein. To make sure that your fixation treatment will not destroy the antigen you are trying to detect, do the following:

- ▶ Consult the literature or colleagues with experience in immunofluorescent detection to be sure that the cell fixation method you use will not destroy the antigenicity of the epitope tag.
- ▶ In initial experiments, try several fixation methods to determine which gives the optimal fluorescent signal.

Fix cells differently depending upon whether the cell antigen you are detecting is located at the cell surface or within the cell. For example, some fixation methods (such as methanol or acetone fixation) are not usually used on cells that will need to be permeabilized (for examination of intracellular antigens).

Getting started: Procedures for immunofluorescence microscopy

This section describes procedures for indirect and direct immunofluorescence staining of epitope-tagged proteins in cultured mammalian cells or in yeast.

Caution: *These procedures must be optimized empirically for each individual experimental system (tagged protein, cell in which protein is expressed, tag-specific antibody, secondary antibody). Use the parameters listed in these procedures (antibody concentrations, incubation times, wash conditions, and so forth) only for initial experiments or for determining optimal ranges for each parameter. Pay particular attention to those procedural steps marked with a **Note** or **Caution** statement, since these are critical steps that must be optimized if the procedure is to produce meaningful results.*

Note: *The products indicated in colored type are available from Boehringer Mannheim. For detailed ordering information on these and related products, see the Boehringer Mannheim Product Ordering Guide, Section 5B of this manual.*

I. Detecting a tagged protein in cultured mammalian cells

Note: *This procedure is a modification of a method obtained from Dr. D.J. Dietzen of the Washington University School of Medicine. It was developed to detect a c-myc-tagged protein in stably transfected human embryonic kidney (293) cells with Anti-c-myc tag-specific antibody and goat Anti-Mouse IgG (H&L), F(ab')₂-fluorescein secondary antibody. The procedure will need to be optimized for detection of other tagged proteins or for other types of mammalian cells.*

Starting material: To perform this procedure, you must have added a suitable epitope tag sequence to a cDNA that encodes the protein to be visualized, cloned that tag-target protein construct into a mammalian expression vector (for instance, according to Algrain *et al.* [1993] or Dietzen, Hastings and Lublin [1995]), and transfected an appropriate strain of cells with the expression vector.

Note: *For more information on adding epitope tags to DNA and getting the tagged protein into cells, see Chapter 2 of this manual.*

IA. Preparing the cells

- 1 Culture the transfected cell line in an appropriate supplemented medium, using standard cell culture procedures.
- 2 Harvest the cells according to the following guidelines:

| If the tagged protein is expressed in the cell | Then harvest cells |
|--|---|
| Transiently | After the cells begin expressing the tagged protein |
| Stably | During the exponential growth phase |

- 3 Prepare cell sample in a sterile 4-chamber slide (Nunc) as follows:
 - ▶ Coat each chamber of the slide with 30 μ l of serum or poly-L-lysine (0.1 g/100 ml) solution, to ensure good cell adhesion.
 - ▶ Plate 10^5 cells in one chamber of the slide.

Note: *Chamber slides will hold up to 4 different 1 ml cell samples. Alternatively, you can grow cells on a sterile, coated glass coverslip or slide.*
- 4 Incubate cells at 37°C overnight in standard culture media under a 5% CO₂ atmosphere.

- 5 Using a pipette, gently wash cells twice with phosphate-buffered saline (PBS), pH 7.4.

IB. Fixing and permeabilizing the sample

- 1 Fix cultured mammalian cells by one of the following methods:

- ▶ Incubate cells for 10 min at 4°C with PBS containing 4% (v/v) paraformaldehyde.

Note: We have successfully used this fixation method on human embryonic kidney cells (strain 293).

OR

- ▶ Incubate cells for 10 min at 4°C with PBS containing 0.5% paraformaldehyde.

OR

- ▶ Incubate cells in cold methanol for 5 min at 4°C.

OR

- ▶ Incubate cells in cold acetone for 3–5 min at –20°C.

Caution: The best fixation method varies with cell type and properties of the tagged protein. Initially, try several of the above methods to determine which gives optimal results.

Note: Use methanol or acetone fixation only for analysis of cell surface antigens. Generally, methanol- and acetone-fixed cells should not be permeabilized.

- 2 Gently wash fixed cells 3 times with PBS (5 min per wash).
- 3 Do one of the following:

| If you are studying | Then go to |
|------------------------|--------------------------|
| Cell surface antigens | Procedure IC. |
| Intracellular antigens | Step 4 of this procedure |

- 4 To permeabilize cells, do the following:
 - ▶ Incubate slide for 3 min at 4°C in PBS containing 0.1% Triton X-100.
 - ▶ Wash slide quickly in 0.1% Triton X-100.
- 5 Go to Procedure IC.

IC. Staining the sample with tag-specific antibody

Note: Perform all incubation steps in this procedure at room temperature (RT) in a humidified chamber. An easy way to make a humidified chamber is to place a moistened paper towel in the bottom of a shallow plastic box that is large enough to hold the slides. Cover the box with a lid during the incubations.

- 1 Place slide in a humidified chamber.

- 2 Block nonspecific antibody binding sites by doing the following:

- ▶ Prepare Blocking Solution (PBS containing bovine serum albumin [BSA, 0.5 g/100 ml]).

Caution: Use only a highly purified BSA preparation (such as Fraction V or fatty acid free). Less purified BSA contains immunoglobulin G (IgG) and will cause high background staining.

- ▶ Incubate cells with Blocking Solution for 15 min.

- ▶ Decant the Blocking Solution from the cells.

Caution: Do not rinse cells.

- 3 Cover the cells with a suitable concentration (prepared in Blocking Solution) of tag-specific antibody:

| If you are using | Then use this concentration in the incubation |
|--|---|
| Anti-c-myc | 1–10 µg/ml |
| Anti-HA, Anti-HA-fluorescein, or Anti-HA-rhodamine | 1–10 µg/ml |
| Anti-VSV-G | 1–10 µg/ml |

Note: Use the antibody concentrations listed here for initial experiments. Determine the optimal concentration of tag-specific antibody experimentally by treating duplicate samples with different concentrations of the antibody.

- 4 Incubate slide for 30 min at RT.

Note: Longer incubations may be required for detection of some epitope-tagged proteins. For initial experiments, try 30 min, then lengthen the incubation if the staining is not optimal.

- 5 Wash slide 3 times (5 min per wash) with PBS.
- 6 Depending on the tag-specific antibody you used in Step 3 above, do one of the following:

| If you used | Then go to Procedure |
|--|----------------------|
| Anti-c-myc, Anti-HA or Anti-VSV-G | I D |
| Anti-HA-fluorescein or Anti-HA-rhodamine | I E |

ID. Detecting antibody-antigen complexes with secondary antibody

Note: Perform all incubation steps in this procedure at RT in a humidified chamber.

- 1 Cover each sample with a suitable dilution (prepared in Blocking Solution) of secondary antibody:

| If you are using this secondary antibody | Then use this dilution of antibody in the incubation |
|--|--|
| Anti-Mouse IgG (H&L), F(ab') ₂ -fluorescein | 1:10–1:300 |
| Anti-Mouse IgG (H&L), F(ab') ₂ -rhodamine | 1:20–1:500 |

Caution: Use the antibody concentrations listed here only for initial experiments with the fluorescein-conjugated or rhodamine-conjugated Anti-Mouse IgG (H&L), F(ab')₂ antibodies. Other secondary antibodies should be used at the dilutions recommended by their manufacturer.

Note: Determine the optimal dilution of secondary antibody as follows: Prepare several identical samples; incubate them with the same amount of primary antibody; then incubate with different dilutions of secondary antibody.

- 2 Incubate slide for 30 min at RT.

Note: Longer incubations may be required for detection of some antibody-antigen complexes. For initial experiments, try 30 min, then lengthen the incubation if the staining is not optimal.
- 3 Wash samples 3 times (5 min/wash) with PBS.
- 4 Do one of the following:

| If you want to | Then |
|--------------------------------|---|
| Stop at this point | (a) Store the slide in the dark at 4°C or at –20°C*. (b) View slide (Procedure IE) within 12–72 h. |
| View stained slide immediately | Go to Procedure IE. |

***Note:** Stained slides are more stable at –20°C than at 4°C.

IE. Viewing the stained sample

- 1 Remove the walls between the chambers on the slide.

Note: The walls of the 4-chamber slide are held on with adhesive and may easily be removed to allow all the chambers to be prepared at the same time.
- 2 Prepare all samples on the slide for viewing as follows:
 - ▶ Add a small drop of aqueous mounting media.

Caution: Be sure to use a mounting media (for example, Aqua-Mount from Lerner Laboratories) that does not interfere with fluorescence microscopy.

- ▶ Carefully apply a coverslip.

Caution: Avoid trapping air bubbles under the coverslip.
 - ▶ Blot off excess mounting media.
 - ▶ Seal coverslip to slide with nail polish.
- 3 Examine samples at 60x magnification under a fluorescence microscope fitted with appropriate filters:
 - ▶ Maximum emission wavelength for samples stained with fluorescein-conjugated antibody is 523 nm.
 - ▶ Maximum emission wavelength for samples stained with rhodamine-conjugated antibody is 570 nm.
 - 4 Take photographs of the slide with a scope-mounted camera.
 - 5 Store the completed slide at –20°C in the dark.

II. Detecting a tagged protein in yeast

Note: This procedure is a modification of a method obtained from Dr. Susan Michaelis of The Johns Hopkins University School of Medicine. Dr. Michaelis has used it to directly detect an HA-tagged protein in *Saccharomyces cerevisiae* with Anti-HA-fluorescein. The procedure will need to be optimized for detection of other tagged proteins or for other types of yeast.

Starting material: To perform this procedure, you must have added a suitable epitope tag sequence to an expression vector that encodes the protein to be visualized and transformed an appropriate strain of *S. cerevisiae* with the expression vector. For details on the construction of the vectors needed and transformation of yeast cells, see Berkower, Loayza and Michaelis (1989).

IIA. Preparing the yeast

- 1 Inoculate yeast cells expressing epitope-tagged protein from a petri dish into 5 ml of an appropriate liquid growth medium and grow (overnight) to saturation at 30°C on a rotating wheel.
- 2 Prepare a log-phase culture of yeast as follows:
 - ▶ Dilute saturated culture 1000-fold into 10 ml of fresh liquid growth medium.

Note: A 1000-fold diluted culture will require about 20 h to reach log phase if the doubling time is about 2 h.
 - ▶ Grow diluted culture at 30°C on a rotating wheel.

- ▶ Monitor the cell count in the culture with a hemacytometer.
- ▶ Harvest cells when the cell count is $1\text{--}2 \times 10^7$ cells/ml (log phase).

Note: Monitor the absorbance at 600 nm (A_{600}) of the culture in parallel with the cell count. Once you have correlated cell count to A_{600} , you may monitor future cultures solely by absorbance.

- 3 Place a suspension containing about 10^8 log-phase cells in a 15 ml screw-top centrifuge tube.
- 4 Centrifuge the tube for 5 min in a table-top centrifuge to pellet the cells.
- 5 Pour off liquid and resuspend the cells in 5 ml Phosphate Buffer (0.1 M potassium phosphate, pH 6.5).
- 6 Fix the cells as follows:
 - ▶ Vortex the tube to keep the cells in suspension.
 - ▶ While vortexing the cells, add 600 μ l of 37% formaldehyde, drop-by-drop, to the tube.
 - ▶ Incubate the cells on a wheel at 30°C for 40 min.
 - ▶ Centrifuge the tube for 5 min in a table-top centrifuge to pellet the cells.
- 7 Wash the cells with 5 ml Phosphate Buffer as follows:
 - ▶ Resuspend the cells in buffer by vortexing.
 - ▶ Centrifuge the tube for 5 min in a table-top centrifuge to pellet the cells.
- 8 Wash the cells as follows:
 - ▶ Once more with Phosphate Buffer (as in Step 7)
 - ▶ Once with 5 ml Phosphate-Sorbitol Buffer (0.1 M phosphate, pH 6.5; 1.2 M sorbitol)

IIB. Spheroplasting the yeast

- 1 Resuspend the cells in 1 ml Phosphate-Sorbitol buffer.

Note: The sorbitol helps to stabilize the cells during the spheroplasting procedure.
- 2 To the 1 ml of resuspended cells, add 10 μ l 2-mercaptoethanol and 10 μ l of stock (10 mg/ml) Zymolyase 100T (ICN).
- 3 Incubate the cells at 30°C for about 40 min or until 70%–80% of the cells form spheroplasts (that is, lose their cell wall).

Caution: Do not let the cells incubate with zymolyase for too long or the cells will completely disintegrate. The time needed for spheroplasting will vary with the lot of Zymolyase used, the strain of yeast used, and the stage of cell growth before harvest. Always monitor the formation of spheroplasts under a

phase-contrast microscope. Cells with a cell wall will be phase bright; spheroplasts will be phase dark.

- 4 Wash spheroplasts once as follows:
 - ▶ Resuspend spheroplasts gently in 5 ml Phosphate-Sorbitol Buffer by pipetting the cell-buffer mixture up and down.

Caution: Never vortex spheroplasts.
 - ▶ Centrifuge the tube in a table-top centrifuge for 5 min to pellet spheroplasts.
 - ▶ Remove the buffer by aspiration.
- 5 Resuspend spheroplasts gently in 1 ml Phosphate-Sorbitol Buffer by pipetting the cell-buffer mixture up and down.
- 6 Do one of the following:

| If you want to | Then |
|-----------------------------|-----------------------------|
| Store the cells | Store spheroplasts* at 4°C. |
| Stain the cells immediately | Go to Procedure IIC. |

*Note: Fixed, spheroplasted yeast are stable for several days.

IIC. Staining the sample with tag-specific antibody

- 1 Coat a 4-well slide as follows:
 - ▶ Add about 30 μ l poly-L-lysine (0.1 g/100ml) solution to each well of the slide.
 - ▶ Incubate for 10 s.
 - ▶ Remove the excess solution by aspiration.
 - ▶ Wash the wells 3 times with distilled H_2O .
 - ▶ Air dry the slide.
- 2 For each sample, add 30 μ l of resuspended spheroplasts to each of two wells of the coated slide.

Caution: Leave the other two wells of the slide empty and always use any one slide for duplicate samples only, since there is a danger of carryover from one well of a slide to the other.
- 3 Incubate the slide for 15 min in a humidified chamber at RT while the cells adsorb onto the slide.

Note: An easy way to make a humidified chamber is to place a moistened paper towel in the bottom of a shallow plastic box that is large enough to hold the slides. Cover the box with a lid during the incubations.
- 4 Remove the unadsorbed cells by aspiration.
- 5 Wash slides at RT as follows:
 - ▶ Cover slides with PBST (0.04 M K_2HPO_4 ; 0.01 M KH_2PO_4 ; 0.15 M NaCl; 0.1% Tween 20; 10 mg/ml BSA; 0.1 g/100 ml sodium azide) in a Coplin jar.
 - ▶ Incubate for 15 min.
 - ▶ Remove and discard the wash buffer.

- 6 To each sample well on the slide, add 30 μ l of a suitable concentration of tag-specific antibody (prepared in PBST):

| If you are using | Then use this concentration in the incubation |
|---|---|
| Anti-c-myc | 1–10 μ g/ml |
| Anti-HA | 1–10 μ g/ml |
| Anti-HA-fluorescein or Anti-HA-rhodamine | 1–10 μ g/ml |
| Anti-VSV-G | 1–10 μ g/ml |

Note: Use the antibody concentrations listed here for initial experiments. Determine the optimal concentration of tag-specific antibody experimentally by treating identical samples with different concentrations of the antibody.

- 7 Incubate slides overnight at RT in a humidified chamber.
Caution: For fluorescein- rhodamine- or other fluorochrome-conjugated antibody, perform this incubation in the dark (for instance, under an aluminum foil cover).
- 8 Wash wells 4 times with PBST (as in Step 5).
- 9 Depending on the tag-specific antibody you used in Step 6 above, do one of the following:

| If you used | Then go to Procedure |
|---|----------------------|
| Anti-c-myc, Anti-HA or Anti-VSV-G | II D |
| Anti-HA-fluorescein or Anti-HA-rhodamine | II E |

IID. Detecting antibody-antigen complexes with secondary antibody

Note: Perform all incubation steps in this procedure at RT in a humidified chamber.

- 1 To each sample well on the slide, add 30 μ l of a suitable dilution (prepared in PBST) of secondary antibody:

| If you are using this secondary antibody | Then use this dilution of antibody in the incubation |
|---|--|
| Anti-Mouse IgG (H&L), F(ab') ₂ -fluorescein | 1:10–1:300 |
| Anti-Mouse IgG (H&L), F(ab') ₂ -rhodamine | 1:20–1:500 |

Caution: Use the antibody concentrations listed here only for initial experiments with the fluorescein-conjugated or rhodamine-conjugated Anti-Mouse IgG (H&L), F(ab')₂ antibodies. Other secondary antibodies should be used at the dilutions recommended by their manufacturer.

Note: Determine the optimal dilution of secondary antibody as follows: Prepare several identical samples; incubate them with the same amount of primary antibody; then incubate with different dilutions of secondary antibody.

- 2 Incubate the slides at RT for 2–4 h in a dark, humidified chamber.
Caution: Incubation time is critical. Do not incubate the slides for less than 2 h nor more than 4 h.
- 3 Wash the slides 4 times with PBST (as in Step 5, Procedure IIC).
- 4 (Optional) Allow the slides to air dry at RT in the dark (for instance, under aluminum foil).
Note: The procedure works even if the slides are not completely dry.
- 5 Go to Procedure IIE.

IIE. Viewing the stained sample

- 1 Prepare the slides for viewing as follows:
- ▶ Add a small drop of aqueous mounting media to cover the wells.
Caution: Be sure to use a mounting media (for example, Aqua-Mount from Lerner Laboratories) that does not interfere with fluorescence microscopy.
 - ▶ Carefully apply a coverslip.
Caution: Avoid trapping air bubbles under the coverslip.
 - ▶ Blot off excess mounting media.
 - ▶ Seal slides with nail polish.
- Caution:** Store slides in the dark (for instance, under aluminum foil) at -20°C when not using them.
- 2 Examine samples at 60x magnification under a fluorescence microscope fitted with appropriate filters:
- ▶ Maximum emission wavelength for samples stained with fluorescein-conjugated antibody is 523 nm.
 - ▶ Maximum emission wavelength for samples stained with rhodamine-conjugated antibody is 570 nm.
- 3 Take photographs of the slide with a scope-mounted camera.
- 4 Store the completed slides at -20°C in the dark.