

No 9: Immunofluorescence - detection of cellular antigens



[Back to overview](#)

Vimentin filaments in the cell skeleton of mesodermal cells

When browsing through the leading technical journals for cell biologists, our own cells Susi Pensa and Adi Herence frequently come across photographs of cells in spectacular colours.

Looking at herself, Susi sees that her dress is predominantly grey and that her colleague Adi is also well dressed but not in any way conspicuous. "Adi, how do these cells in the journals get these nice green and red outfits?", asks Susi.

"Don't you read the adverts, Susi?", answers Adi and explains: "You can buy fairly expensive antibody molecules which bind highly specifically to cellular molecules. If you have such a molecule on your cell body, this antibody can bind to you".

"But this still doesn't make me beautifully coloured", replies Susi, and Adi continues: "To make this antibody binding visible, you need a second antibody. In contrast to the first antibody, the second antibody doesn't bind to cellular molecules but specifically to the molecules of the first antibody. In addition, fluorescing dyes are bound to the molecules of the second antibody; when these are irradiated with light of a certain wavelength, they give off light and become coloured. There are fluorescing dyes which emit green light, e.g. fluorochrome FITC, or those that emit red light, e.g. Texas red or rhodamine.

This process is called immunofluorescence and it is used for the highly specific detection of cellular molecules".

"Here's an example", continues Adi. "Being a cell of mesodermal origin, my skeleton comprises the protein vimentin. There are now antibody molecules produced by mouse B cells and hence mouse-typical and which at the same time bind highly specifically to vimentin: This is then called antivimentin from mouse. If my cell membrane is rendered permeable by treatment with alcohol/glacial acetic acid, these antibodies bind specifically to the vimentin in my cell skeleton. The secondary antibody binds to the antivimentin antibody - this happens because they have been made capable of binding specifically to mouse antibodies by treatment with rabbit B cells. This results in a so-called sandwich. As the fluorescing dye FITC is attached to all secondary antibodies, I would appear in a dazzling green suit if irradiated with blue light".

"This is now becoming clear", says Susi. "Only those cells carrying the corresponding molecule can be made to light up. As long as I have no vimentin in my cell skeleton I will have to remain a grey mouse. And if you are treated with tetanus toxin antibodies, you will also remain uncoloured; because tetanus toxin is present on nerve cells and not on cells of mesodermal origin". - "Correct", confirms Adi.

Protocol for carrying out immunofluorescence on cells

Preparation:

- Wash buffer: PBS - phosphate buffer containing 0.5 % BSA
 - Antibody diluted in PBS containing 10% BSA according to manufacturer
- First antibody:**
 Anti-vimentin from mouse
 Anti-tetanus toxin from mouse (or another irrelevant antibody) for specificity control

Secondary antibody: Anti-mouse immunoglobulin from rabbit, FITC coupled.

- Fixing solution: 95% ethanol, 5% glacial acetic acid, -20 °C
- Embedding agent: 1 g polyvinyl alcohol dissolved in 7 ml PBS is reacted with 10 mg of p-phenylene diamine, adjusted to pH 8 and 3 ml glycerol (fluorescence microscopy quality) added. The phenylene diamine reduces the fading of the fluorescence during illumination when microscoping. Careful pH adjustment and storage at -20°C are important for maintaining the quality of the preparation.
- Copper glasses, 2 cm² surface, fat-free, washed, sterilised
- Sterile 24-well cell culture
- Cover glass storage area, e.g. the rim of a 24-well plate
- Humid chamber
- Tweezers
- Cannulas, bent appropriately for removing cover glasses.

Procedure:

Place 4 cover glasses (two per test) in each well of a 24-well plate using tweezers and wet them with medium. Inoculate the cells, e.g. rat tail fibroblasts onto the cover glasses in the usual cell densities and cultivate for one to two days until the desired cell density has been achieved.

For the immunofluorescence experiment, remove one cover glass after the other using the prepared cannula and tweezers and place them, cell side upwards, on the rim of a 24-well plate. Cover the cells immediately with 45 µl medium. Remove as many cover glasses as required for the experiment and place them next to each other: 4 are required.

The procedure requires much patience and steady nerves; for the cover glass must not break, must not fall and cells must not be allowed to dry out.

Pick-up a cover glass with tweezers and wash it by immersing for 30 seconds in washing buffer.

Back on the shelf, the cells should be fixed: 100 µl of fixing solution stored at -20 °C should be applied to the cells and then incubated for 10 minutes at -20 °C in a freezer.

Remove the fixing solution by thoroughly washing (three times, for 30 seconds). Coat both cover glasses with 45 µl of the primary antibody anti-vimentin or anti-tetanus toxin (specificity check). Incubate the cells for 30 minutes at room temperature in the humid chamber.

Wash the cover glasses once and coat all four with 45 µl of the FITC-coupled secondary antibody anti-mouse immunoglobulin. Due to the light sensitivity of the fluorescing dye, the 30-minute incubation should be carried out in a darkened humid chamber at room temperature.

The cells should be washed again and subsequently embedded for microscopic analysis: The embedding agent should be dropped onto a slide and the cover glass, cell side downwards, allowed to fall, free of bubbles, onto the drop. Excess embedding agent should be removed with an absorbent pad by wiping the cover glass. The edges should be fixed using nail varnish; this keeps the cover glass in position and prevents the cells from drying out.

Evaluation under a fluorescent microscope shows, in cells of mesodermal origin, the vimentin cell skeleton as a network of green fibres; the cover glasses treated with control antibody should not show any green fluorescence in the cells.

Modifications:

If molecules of the cell surface are to be shown, fixing should be carried out after the secondary antibody incubation instead of at the beginning of the experiment.

Source:

Cell & Tissue Culture: Laboratory Procedures
Editors: A. Doyle, J.B. Griffiths and D.G. Newell
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[Back to overview](#)