

Combined DNA *in situ* hybridization and immunocytochemistry for the simultaneous detection of nucleic acid sequences, proteins, and incorporated BrdU in cell preparations

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The combination of *in situ* hybridization (ISH) and immunocytochemistry (ICC) enables us, *e.g.*, to simultaneously demonstrate mRNA and its protein product in the same cell, to immunophenotype cells containing a specific chromosomal aberration or viral infection, or to characterize cytokinetic parameters of tumor cell populations that are genetically or phenotypically aberrant. The factors that determine the success and sensitivity of a combination ICC and nonradioactive ISH procedure include:

- ▶ Preservation of cell morphology and protein epitopes
- ▶ Accessibility of nucleic acid targets
- ▶ Lack of cross-reaction between the different detection procedures
- ▶ Good color contrast
- ▶ Stability of enzyme cytochemical precipitates and fluorochromes

Since several steps in the ISH procedure (enzymatic digestion, post-fixation, denaturation at high temperatures, and hybridization in formamide) may destroy antigenic determinants, ICC usually precedes ISH in a combination procedure. A variety of such combined ICC/ISH procedures have been reported with either enzyme precipitation reactions (Mullink et al., 1989; Van den Brink et al., 1990; Knuutila et al., 1994; Speel et al., 1994b), fluorochromes (Van den Berg et al., 1991; Weber-Matthiesen et al., 1993), or a combination of both (Strehl & Ambros, 1993; Zheng et al., 1993; Herbergs et al., 1994; Speel et al., 1994a). The procedures can be subdivided into two groups, those which use fluorochromes for ICC, and those which use enzyme reactions.

Fluorochromes have been used mainly on acetone-fixed cell preparations, since the material can be mildly post-fixed (usually with paraformaldehyde) after antigen detection and used directly for fluorescence ISH without any further pretreatment (Weber-Matthiesen et al., 1993). However, amplification steps for both ICC and ISH signals are often necessary for clear visualization. In such cases, enzymatic ISH pre-treatment after ICC lowers fluorescent ICC staining dramatically (Speel et al., 1994a).

Enzyme precipitation reactions have also been used efficiently for combined ICC/ISH staining of proteins in cell preparations and tissue sections. Enzyme precipitation products that withstand the proteolytic digestion and denaturation steps used in the ISH procedure include:

- ▶ Several precipitates (Fast Red, New Fuchsin, and BCIP/NBT) formed by alkaline phosphatase
- ▶ The diaminobenzidine precipitate formed by horseradish peroxidase
- ▶ The BCIG (X-Gal) precipitate formed by β -galactosidase

In these cases, the digestion and denaturation steps of the ISH procedure remove the antibody and enzyme detection layers, but the precipitate remains firmly in place. The stability of the ICC precipitate thus prevents unwanted cross-reaction between the detection procedures for ISH and ICC.

Here we present a combined ICC/ISH procedure which describes compatible detection systems for fluorescence or brightfield microscopy (Table 1). Additionally, this procedure allows the localization of incorporated BrdU by fluorescence microscopy.

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The procedures given below are modifications of previously published procedures (Speel et al., 1994a, 1994b).

		For analysis by	
		Fluorescence microscopy	Brightfield microscopy
ICC²	Detection by	Anti-antigen Ab-APase	Anti-antigen- β -gal
	Visualization by	APase-Fast Red	β -Gal-BCIG
ISH	Probe	FITC- or AMCA-labeled nucleic acid	DIG- or biotin-labeled nucleic acid
	Detection and visualization by	Direct viewing	PO- or APase-labeled anti-DIG Ab or anti-biotin Ab plus PO-DAB, PO-TMB, or APase-Fast Red reaction
BrdU labeling	Detection by	AMCA- or FITC-labeled ³ anti-BrdU Ab	–
	Visualization by	Direct viewing	–


Table 1: Detection systems for combined ICC/ISH¹.

¹ Amplification steps may be necessary for detection of low amounts of antigen, nucleic acid target, or incorporated BrdU.

² Abbreviations used: Ab, antibody; AMCA, aminomethylcoumarin acetic acid; APase, alkaline phosphatase; BCIG, bromochloroindolyl-galactoside; BrdU, bromodeoxyuridine; DAB, diaminobenzidine; DIG, digoxigenin; FITC, fluorescein isothiocyanate; β -Gal, β -galactosidase; ICC, immunocytochemistry; ISH, *in situ* hybridization; PO, peroxidase; TMB, tetramethylbenzidine

³ The fluorochrome used in the BrdU visualization step should be different from the one used in the ISH visualization step.


I. Cell preparations and BrdU labeling

- 1 (Optional) To label cells, add BrdU (final concentration, 10 μ M) to the culture medium 30 min before harvesting the cells (Speel et al., 1994a).
- 2 Prepare cultured normal diploid cells or tumor cell lines (labeled or unlabeled) by one of the following methods:
 - ▶ Slide and coverslip preparations: Grow cells on glass slides or coverslips. Fix in cold methanol (-20°C) for 5 s, then in cold acetone (4°C) for 3×5 s. Air dry samples and store at -20°C .
 - ▶  *Alternatively, use other fixatives for the slide and coverslip preparations.*
 - ▶ Cytospins: Cytospin floating cells onto glass slides at 1000 rpm for 5 min. Air dry samples for 1 h at room temperature. Fix and store as with slide and coverslip preparations above.

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II. Detection of antigen by immunocytochemistry (ICC)


- 1 Incubate slides for 10 min at room temperature with PBS-Tween-NGS (PBS buffer containing 0.05% Tween 20 and 2–5% normal goat serum).
- 2 Incubate slides for 45 min at room temperature with an appropriate dilution of antigen-specific primary antibody in PBS-Tween-NGS.
- 3 Wash slides for 2 × 5 min with PBS containing 0.05% Tween 20.
- 4 Incubate slides for 45 min at room temperature with an appropriate secondary antibody conjugate. Use the following to decide which antibody conjugate to use:
 - ▶ If you wish to detect the ICC antigen, the ISH antigen, and (optionally) BrdU labeling by fluorescence microscopy, use a secondary antibody that is conjugated to alkaline phosphatase (APase).
 - ▶ If you wish to detect the ICC antigen and the ISH antigen under brightfield microscopy, use a secondary antibody that is conjugated to β-galactosidase (β-Gal).
- 5 Wash slides as follows:
 - ▶ 5 min with PBS containing 0.05% Tween 20.
 - ▶ 5 min with PBS.


 For amplification of the ICC signal, you may add a third antibody step after this wash. For details of possible antibodies to use in an amplified three-antibody detection procedure, see Table 1 of the article “Multiple target DNA in situ hybridization with enzyme-based cytochemical detection systems” on page 94 of this manual.
- 6 Visualize the antibody-antigen complexes according to either Procedure IIIA (for APase conjugates) or Procedure IIIB (for β-Gal conjugates).

III. Visualization of ICC antigen

IIIA. APase-Fast Red reaction (for producing a red precipitate visible under either fluorescence or brightfield microscopy)

- 1 Mix color reagent just before use:
 - ▶ 4 ml TM buffer [200 mM Tris-HCl (pH 8.5), 10 mM MgCl₂] containing 5% polyvinyl alcohol (PVA, MW 40,000; Sigma).
 - ▶ 250 μl TM buffer containing 1 mg naphthol-ASM_X-phosphate (Sigma).
 - ▶ 750 μl TM buffer containing 5 mg Fast Red TR salt (Sigma).
- 2 Overlay each sample with 100 μl color reagent and a coverslip.
- 3 Incubate samples for 5–15 min at 37°C.

 Monitor the enzyme reaction under a microscope and adjust the reaction time to keep the precipitate from becoming so dense that it shields nucleic acid sequences from the ISH detection step.
- 4 Wash samples 3 × 5 min with PBS.

 Do not dehydrate samples after washing.

IIIB β -Gal-BCIG reaction (for producing a blue precipitate visible under brightfield microscopy)

- 1 Mix color reagent:
 - ▶ 2.5 μ l 5-bromo-4-chloro-3-indolyl- β -D-galactoside (BCIG; X-Gal) stock solution [20 mg/ml BCIG (X-Gal) in N,N-dimethylformamide].
 - ▶ 100 μ l diluent (PBS containing 0.9 mM MgCl₂, 3 mM potassium ferricyanide, and 3 mM potassium ferrocyanide).
- 2 Overlay each sample with 100 μ l color reagent and a coverslip.
- 3 Incubate samples for 15 – 60 min at 37°C.
 - ⚠ *Monitor the enzyme reaction under a microscope and adjust the reaction time to keep the precipitate from becoming so dense that it shields nucleic acid sequences from the ISH detection step.*
- 4 Wash samples 3 \times 5 min with PBS.
 - ⚠ *If you wish, dehydrate the samples after washing.*

IV. Cell processing for *in situ* hybridization

- 1 Wash slides for 2 min at 37°C with 10 mM HCl.
- 2 Digest samples with pepsin as follows:
 - ▶ Overlay each sample with pepsin solution (100 μ g/ml pepsin in 10 mM HCl).
 - ▶ Incubate cell samples for 10 – 20 min at 37°C.
- 3 Wash samples for 2 min at 37°C with 10 mM HCl.
 - ⚠ *For cells stained with the β -Gal-BCIG reaction, you may (if you wish) dehydrate the slides after washing them.*
- 4 Post-fix samples for 20 min at 4°C with PBS containing 1% paraformaldehyde.
- 5 Wash samples as follows:
 - ▶ 5 min with PBS.
 - ▶ 5 min with 2 \times SSC.

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V. *In situ* hybridization (ISH)

- 1 Perform a standard ISH detection and visualization procedure on the slides as described elsewhere in this manual. Use either:
 - ▶ Fluorescence-based procedures (FITC, AMCA) (when APase-conjugated antibodies were used for ICC).
 - ▶ Enzyme-based procedures (PO-DAB, PO-TMB, APase-Fast Red) (when β -Gal-conjugated antibodies were used for ICC).

Example: For a detailed description of enzyme-based ISH procedures, see “Multiple target DNA in situ hybridization with enzyme-based cytochemical detection systems” on page 107 of this manual.

- 2 Include appropriate controls in the ISH procedure to ensure the lack of cross-reaction between ICC and ISH.

! *The ICC precipitate remains firmly in place during ISH. The stability of the ICC precipitate usually prevents unwanted cross-reaction between the detection procedures for ISH and ICC.*

VI. Fluorescence detection of BrdU (optional)

- 1 After performing the APase-Fast Red ICC (Procedure IIIA) and fluorescence ISH (Procedure V) reactions on BrdU-labeled cells, incubate the cells for 45 min at room temperature with a specific anti-BrdU antibody.

- 2 Wash slides for 2 \times 5 min with PBS containing 0.05% Tween 20.

- 3 Incubate samples with a secondary antibody that is conjugated to a fluorochrome not used in the ICC or ISH reactions.

Example: Use an alkaline-phosphatase conjugated antibody and the APase-Fast Red for ICC; a FITC-conjugated antibody for ISH; and an AMCA-conjugated antibody for BrdU detection.

! *If necessary, use an amplified three-antibody detection procedure as outlined in the article “Multiple target DNA in situ hybridization with enzyme-based cytochemical detection systems” on page 94 of this manual.*

- 4 Before embedding, wash slides as follows:

- ▶ 2 \times 5 min with PBS containing 0.05% Tween 20.
- ▶ 5 min with PBS.

- 5 Include appropriate controls to ensure that the BrdU detection step does not cross-react with ISH detection.

VII. Embedding

For fluorescence microscopy (for detection of APase-Fast Red ICC, fluorescence ISH, and BrdU):

Embed specimens in a Tris-glycerol mixture [1:9 (v/v) mix of 0.2 M Tris-HCl (pH 7.6) and glycerol] containing 2% DABCO (Sigma) and (optionally) 0.5 µg/ml blue DAPI (Sigma).

For brightfield microscopy (for detection of β-Gal-BCIG ICC and enzyme-based ISH):

Depending on the enzyme reactions used, embed specimens in one of the following:

- ▶ Aqueous, organic, or protein embedding medium, as described in the article “Multiple target DNA *in situ* hybridization with enzyme-based cytochemical detection systems” on page 94 of this manual.
- ▶ Entellan (Merck) organic embedding medium and immersion oil (Zeiss) (for peroxidase-DAB or peroxidase-TMB reactions only).
- ▶ Tris-glycerol mixture [1:9 (v/v) mix of 200 mM Tris-HCl (pH 7.6) and glycerol] (for peroxidase-DAB or phosphatase-Fast Red reactions only).

Results

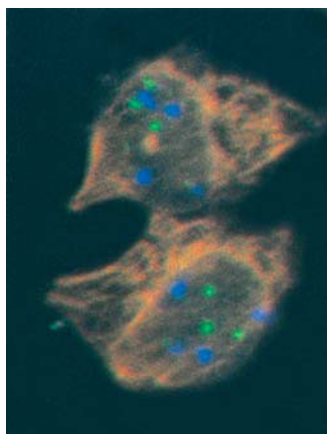


Figure 1: Combined ICC and fluorescence ISH on lung tumor cell line EPLC 65, showing cytokeratin filaments, chromosome 1, and chromosome 17. The cytokeratins (red) were visualized with a monoclonal anti-cytokeratin antibody (Ab), an alkaline phosphatase-conjugated goat anti-mouse Ab, and the APase-Fast Red reaction. The centromere of chromosome 1 (blue) was visualized with a biotinylated probe, avidin-AMCA, a biotinylated goat anti-avidin Ab, and avidin-AMCA. The centromere of chromosome 17 (green) was visualized directly with a FITC-labeled probe.

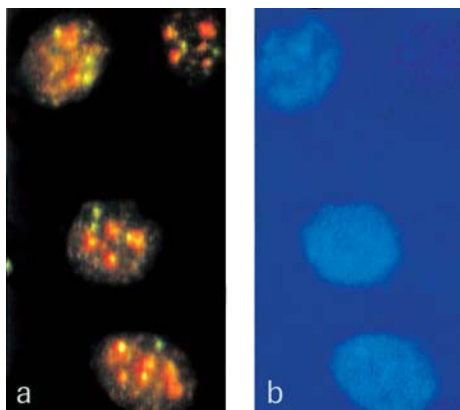


Figure 2: Combined ICC and fluorescence ISH on EPLC 65 cells, showing the nuclear proliferation marker Ki67, chromosome 7, and incorporated BrdU.

Panel a: The Ki67 antigen (red) was visualized with a rabbit anti-Ki67 Ab, alkaline phosphatase-conjugated swine anti-rabbit Ab, and the APase-Fast Red reaction. The centromere of chromosome 7 (green) was visualized directly with a FITC-labeled probe.

Panel b: Incorporated BrdU (blue) was visualized with monoclonal anti-BrdU Ab, biotinylated horse anti-mouse Ab, and avidin-AMCA.

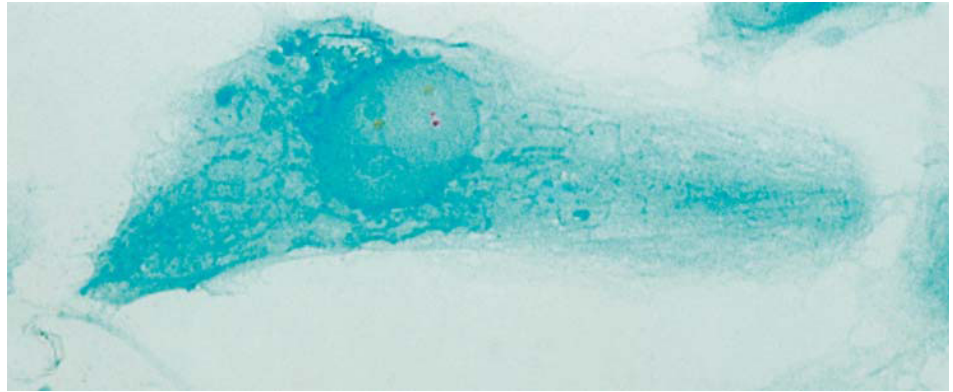


Figure 3: Combined ICC and enzyme-based ISH on a human umbilical vein endothelial cell, showing the intermediate filament protein vimentin, chromosome 1, and chromosome 7. Vimentin (blue) was visualized with monoclonal anti-vimentin Ab, β -galactosidase-conjugated goat anti-mouse Ab, and the β -galactosidase-BCIG reaction. The centromeres of chromosome 1 (biotinylated probe, brown) and chromosome 7 (digoxigenin-labeled probe, red) were visualized with avidin-peroxidase, rabbit anti-digoxigenin Ab and alkaline phosphatase-conjugated swine anti-rabbit Ab, the APase-Fast Red reaction, and the PO-DAB reaction. Samples were embedded in a Tris-glycerol mixture, but were not counterstained. Slides were viewed by brightfield microscopy.

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Reagents available from Roche Applied Science for this procedure

Reagent	Description	Cat. No.	Pack size
Pepsin	Aspartic endopeptidase with broad specificity	10 108 057 001	1 g
Digoxigenin-11-dUTP, alkali-stable[∇]	Tetralithium salt, 1 mM solution	11 093 088 910	25 nmol (25 µl)
		11 558 706 910	125 nmol (125 µl)
		11 570 013 910	5 × 125 nmol (5 × 125 µl)
Fluorescein-12-dUTP	Tetralithium salt, 1 mM solution	11 373 242 910	25 nmol (25 µl)
Tetramethyl-rhodamine-5-dUTP	Tetralithium salt, 1 mM solution	11 534 378 910	25 nmol (25 µl)
Biotin-16-dUTP	Tetralithium salt, 1 mM solution	11 093 070 910	50 nmol (50 µl)
DNase I	Lyophilizate	10 104 159 001	100 mg
DNA Polymerase I	Nick Translation Grade	10 104 485 001	500 units
		10 104 493 001	1000 units
Anti-Digoxigenin-AP*	Fab Fragments from sheep	11 093 274 910	150 U (200 µl)
Fast Red	1 tablet contains 0.5 mg naphthol substrate, 2 mg Fast Red chromogen and 0.4 mg levamisole (inhibitor of endogenous alkaline phosphatase activity)	11 496 549 001	20 tablets

[∇] EP Patent 0371262 and US 5,198,537 owned by Roche Diagnostics GmbH.

* The labeling of nucleic acids with DIG is covered by EP patents 0 324 474 and 0 371 262 as well as the following US patents 5.344.757, 5.35 4.657 and 5.702.888 owned by Roche Diagnostics GmbH.

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