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Multi-colour brightfield in situ hybridisation on tissue sections

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Abstract We describe the brightfield microscopical detection of multiple DNA target sequences in cell and tissue preparations. For this purpose, chromosome-specific DNA probes labelled with biotin, digoxigenin or fluorescein were simultaneously hybridised and detected by enzyme cytochemistry using two horseradish peroxidase (PO) reactions and one alkaline phosphatase (APase) reaction. For triple-colour detection on single cell preparations, the combination of the enzyme precipitates PO/diaminobenzidine (DAB, brown colour), APase/fast red (FR, red colour) and PO/tetramethylbenzidine (TMB, green colour) resulted in an accurate detection of DNA targets. Embedding of the preparations in a thin cross-linked protein layer further stabilised the enzyme reaction products. For in situ hybridisation on tissue sections, however, this detection procedure showed some limitations with respect to both the stability of the APase/FR and PO/TMB precipitates, and the sequence of immunochemical layers in multiple-target procedures. For this reason, the APase/FR reaction was replaced by the APase/new fuchsin (NF, red colour) reaction and the washing steps after the PO/TMB reaction were restricted to the use of phosphate buffer pH 6.0. Furthermore, to improve the efficiency of the ISH reaction, APase/NF was applied in an avidin-biotin complex detection system and, to avoid target shielding in the triple-target ISH, the third primary antibody was applied prior to the second enzyme cytochemical reaction. These adaptations resulted in stable, well contrasting brown, red and green coloured precipitates. After quick haematoxylin counterstaining, the tissue preparations were directly mounted in phosphate buffer and, optionally, embedded in the cross-linked protein layer.

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Introduction

In situ hybridisation (ISH) has become an important tool for the detection of specific nucleic acid sequences (DNA and RNA) in morphologically preserved chromosomes, cells and tissue sections (Lichter and Cremer 1992; Raap et al. 1992; Bentz et al. 1994; Joos et al. 1994). The use of fluorescence ISH (FISH) procedures provides a number of advantages, including easy and rapid detection of fluorochrome-labelled probes, high sensitivity with low endogenous background, high resolution, multiple-target analysis with different fluorochromes and the possibility of quantitating the signal intensity (Raap et al. 1992; Tanke et al. 1995). To increase the detection sensitivity, nucleic acid probes can also be labelled with haptens and localised indirectly using antibody or avidin molecules conjugated with fluorochromes (for review see Speel et al. 1995).

The use of enzyme cytochemical precipitation is another means to detect nucleic acids in situ, which can be combined with different types of microscopy (Emmerich et al. 1989; Mullink et al. 1989; Speel et al. 1993). Using brightfield microscopy, the major advantage of a cytochemical detection with enzymes is the stability of the precipitate, which allows the permanent storage of the cell preparations. Moreover, the use of a standard brightfield microscope for the cell analysis is an additional advantage, in particular in a setting where routine analysis has to be performed. The detection of target nucleic acid sequences in situ requires the use of enzyme precipitation reactions that possess both high sensitivity and precize localisation properties. Moreover, rapid staining reactions resulting in stable reaction products with contrasting colours are preferred. These criteria imply that only certain enzymes are used in ISH proedures, such as horseradish peroxidase (PO) and alkaline phosphatase (APase) (Hopman et al. 1986, 1991; Emmerich et al. 1989; Kerstens et al. 1994). For these enzymes, a variety of substrates have been tested that produce well contrasting brown, green, red and blue coloured precipitates (for review see Speel et al. 1995).

Recently, we reported the development of a rapid triple-colour ISH detection procedure for the simultaneous detection of three chromosome-specific DNA sequences by brightfield microscopy in interphase nuclei and metaphase preparations (Speel et al. 1994). A combination of three hybridised DNA probes, labelled with either biotin, digoxigenin or fluorescein, could be localised simultaneously, since the cytochemical detection layers used were unrelated and did not interfere with each other. Distinct localisation of the DNA probes was accomplished within 3 h by consecutive application of three sensitive enzyme cytochemical reactions, i.e. PO/diaminobenzidine (DAB), APase/fast red (FR) and PO/tetramethylbenzidine (TMB). For this purpose, several steps in the detection protocol appeared to be essential to obtain optimal stability and colour contrast of the enzyme precipitates used. These included: (1) the addition of sodium tungstate to the PO/TMB reaction to stabilise the resulting green precipitates; (2) the irreversible inactivation of the first PO with 0.01 M HCl to enable a second PO reaction; and (3) the embedding of the cell preparations in a thin protein layer.

In the underlying study we tested the triple-colour ISH detection procedure on formalin-fixed, paraffin-embedded tissue sections using a bladder carcinoma as a model system. Different immunochemical detection systems were combined and evaluated for optimal ISH results. Furthermore, the use of the PO/TMB and APase/FR reactions was studied in detail for the application of ISH on tissue sections.

Materials and methods

Cell and tissue processing

Cell preparations from a 70% ethanol suspension of the human transitional cell carcinoma line T24 (DNA index 1.6; trisomic for the centromeres of chromosomes 1 and 7, tetrasomic for the centromere of chromosome 15; Bubeník et al. 1973), as well as steps necessary for removal of cytoplasm to improve DNA probe and conjugate penetration (pepsin digestion), were performed as previously described (Hopman et al. 1988; Speel et al. 1992).

The formalin-fixed and paraffin-embedded tissue of a bladder cancer was processed as follows (Hopman et al. 1991). A 4- to 6-μm thick paraffin section was stretched on distilled water at 40°C and picked up on a coated glass slide. The slide was air dried and baked overnight at 56°C. The section was deparaffinised in 100% xylene (3 × 10 min), washed with 100% methanol (2 × 5 min) and immersed in 1% H_2O_2 in 100% methanol (30 min) to block the endogeneous POs. After a wash with 100% methanol (2 × 5 min), the slides were air dried and immersed in 1 M NaSCN at 80°C for 10 min to improve the efficiency of the proteolytic digestion step. After washing with H_2O (20×5 min), the slides were incubated for 15 min at 37°C in 4 mg pepsin (porcine stomach mucosa, 2500–3500 U per mg protein) per ml 0.2 M HCl. The slides were rinsed in H_2O (5 times dip wash), PBS (5 times dip wash) and dehydrated in 70%, 90%, and 100% ethanol (3 min each). After dehydration the probes were applied.

DNA probes and labelling procedures

The probes for the (peri)centromeric regions of the human chromosomes 1 (pUC 1.77), 7 (p7t1), 15 (D15Z1) and 18 (L1.84) have been described by Cooke and Hindley (1979), Waye et al. (1987), Higgins et al. (1985) and Devilee et al. (1986), respectively. The DNA probes were labelled with biotin-11-dUTP (Enzo Diagnostics, New York, USA), digoxigenin-11-dUTP, or fluorescein-12-dUTP (Boehringer, Mannheim, Germany) in a standard nick-translation reaction and used in single-, double-, or triple-target ISH procedures.

ISH procedure

The DNA probes described above were used at a concentration of 0.4 ng/μl (pUC 1.77) or 1 ng/μl (p7t1, D15Z1,L1.84) and hybridised in different combinations in a hybridisation buffer containing 60% formamide, 2×SSC (0.3 M NaCl, 30 mM sodium citrate), pH 5.0, 10% dextran sulphate, 0.2 μg/μl herring sperm DNA as carrier DNA and 0.2 μg/μl yeast tRNA as carrier RNA. Ten microlitres of buffer was added to each slide under a coverslip (20×20 mm). Denaturation was performed on the bottom of a metal box in a water bath at 70°C for 3 min for T24 cells and 5 min at 80°C for tissue sections and hybridisation was performed overnight at 37°C. The slides were washed twice for 5 min at 42°C with 60% formamide, 2×SSC, pH 5.0, containing 0.05% Tween 20, followed by two 5-min washes with 2×SSC, pH 7.0, at 42°C and a 5-min wash with 4×SSC, pH 7.0, containing 0.05% Tween 20 (Buffer A) at room temperature (RT).

Cytochemical detection procedures

To reduce background staining in the cytochemical detection procedures, the slides were preincubated with 4×SSC, pH 7.0, containing 5% non-fat dry milk (Buffer B) for 10 min at 37°C, followed by dipping in Buffer A. For all the detection procedures, the avidin conjugates were diluted in Buffer B, and all the antibody conjugates were diluted in PBS containing 0.05% Tween 20 (Buffer C) and 2% normal goat serum. After each incubation step of 20–30 min at 37°C, the slides were rinsed twice in Buffer A [avidin conjugates, avidin-biotin complex (ABC) system] or Buffer C (antibody conjugates).

The following antibodies, antibody conjugates and affinity systems were used for the detection of:

1. Biotin-labelled probes. PO-conjugated avidin (1:50 dilution; Dako, Glostrup, Denmark) or monoclonal mouse anti-biotin (1:100; Dako) in combination with biotin-conjugated horse anti-mouse (HAMBio, 1:200; Vector, Brunschwig Chemie, Amsterdam, The Netherlands) and streptavidin biotinylated-APase complex (ABC, Boehringer).
2. Digoxigenin-labelled probes. PO-conjugated sheep anti-digoxiginin Fab fragments (1:100; Boehringer) or monoclonal mouse anti-digoxin (1:2000; Sigma, St Louis, USA) in combination with APase-conjugated goat anti-mouse IgG (1:50; Dako) or HAMBio and the ABC detection kit.
3. Fluorescein isothiocyanate (FITC)-labelled probes. PO-conjugated sheep anti-FITC (1:100; Amersham, Life Science, Little Chalfont, UK) or polyclonal rabbit anti-fluorescein (1:2000; Dako) in combination with PO-conjugated swine anti-rabbit IgG (1:100; Dako).

Detection of enzyme activity

PO/DAB reaction (brown colour; Graham and Karnovsky 1966)

Mix just before use: 1 ml DAB (Sigma) in PBS (5 mg/ml stock), 9 ml PBS/0.1 M imidazole, pH 7.6 and 10 μl 30% H_2O_2. Incubate slides with 0.1–1.0 ml for 5–15 min at 37°C. Wash 3×5 min with PBS and dehydrate optionally. Coverslip with an aqueous-based mounting medium (e.g. PBS/glycerol, 1:9 v/v) or organic-based mounting medium (e.g. Entellan; Merck, Darmstadt, Germany).

PO/TMB reaction (green colour; Speel et al. 1994, 1996)

Dissolve 100 mg sodium tungstate (Sigma) in 7.5 ml 100 mM citrate-phosphate buffer, pH 5.1, and bring back the pH afterwards to pH 5.0–5.5 with 37% HCl. Dissolve, just before use, 20 mg dioctyl sodium sulphosuccinate (Sigma) and 6 mg TMB (Sigma) in 2.5 ml 100% ethanol at 80°C. Mix both solutions and add 10 µl 30% H_2O_2. Incubate slides with 0.1–1.0 ml for 1–5 min at 37°C. Wash 3×1 min with ice-cold 0.1 M phosphate buffer pH 6.0 and dehydrate optionally. Coverslip with an organic-based mounting medium or immersion oil.

As an alternative to this reaction, the TrueBlue Peroxidase Substrate kit (Kirkegaard and Perry, Laboratories, Gaithersburg, USA) was used, which also contains TMB as the substrate. Slides were incubated with 0.1–1.0 ml substrate reagent for 10 min. Slides were washed for 1–5 min in Milli Q, counterstained with haematoxylin, washed again for 5 min in Milli Q and dehydrated through an alcohol series. Samples were mounted in an organic-based mounting medium.

APase/FR reaction (red colour; Speel et al. 1992)

Mix just before use: 4 ml 0.2 M TRIS-HCl, pH 8.5, containing 10 mM $MgCl_2$ and 5% polyvinyl alcohol (PVA, molecular weight 40 000; Sigma), 250 µl buffer without PVA containing 1 mg naphthol-ASMX-phosphate (Sigma) and 750 µl buffer without PVA containing 5 mg FR (TR salt; Sigma). Incubate slides with 0.1–1.0 ml reaction mixture for 5–15 min at 37°C, and wash 3×5 min with PBS. Coverslip with an aqueous-based mounting medium.

APase/new fuschin (NF) reaction
(red colour; Malik and Daymon 1982)

Mix just before use: 4.69 ml 0.2 M TRIS-HCl, pH 8.5, containing 10 mM $MgCl_2$ and 5% PVA, 250 µl buffer without PVA containing 1 mg naphthol-ASMX-phosphate (Sigma) and 60 µl of a mixture of 30 µl NF in 2 M HCl (40 mg/ml stock) and 30 µl $NaNO_2$ in Milli Q (40 mg/ml) that has been incubated for 2 min at RT. Incubate slides with 0.1–1.0 ml for 5–15 min at 37°C. Wash 3×5 min with PBS and dehydrate optionally. Coverslip with an aqueous-based mounting medium.

In multiple-target ISH with different PO reactions, the PO activity still present after the first PO reaction was inactivated by incubation of the slides in 0.01 M HCl for 10 min at RT. All sections were slightly counterstained with haematoxylin. If aqueous and organic dissolving precipitates were present on the slides, these were washed after haematoxylin staining with 0.1 M phosphate buffer pH 6.0, dehydrated, air dried and embedded. Briefly, the slides were smeared with 50 µl of a mixture of 20 mg/ml bovine serum albumin (Sigma) in Milli Q and 2% formaldehyde and air dried. Then, the slides could be analysed directly or mounted optionally in an aqueous- or organic-based mounting medium, or in immersion oil, with similar results.

Brightfield microscopy

Photomicrographs were made on a Leica DM RBE microscope equipped with the Metasystem Image Pro system (black-and-white CCD camera; Sandhausen, Germany). Images were captured using green, red and blue filters and processed for sharpness in Adobe Photoshop 3.0 (120 pixels/inch).

Results

To develop a triple-target ISH detection procedure for brightfield microscopy, different combinations of precipitating PO reactions and APase reactions were tested in cell and tissue preparations. The individual enzyme reactions have been previously described in detail and can be applied in routine ISH on single cells (cell lines and peripheral lymphocytes). For the applicability of these reactions on tissue sections, however, several adaptations were needed both in the cytochemical detection protocol as well as in the choice of the enzyme substrates.

Single-target ISH

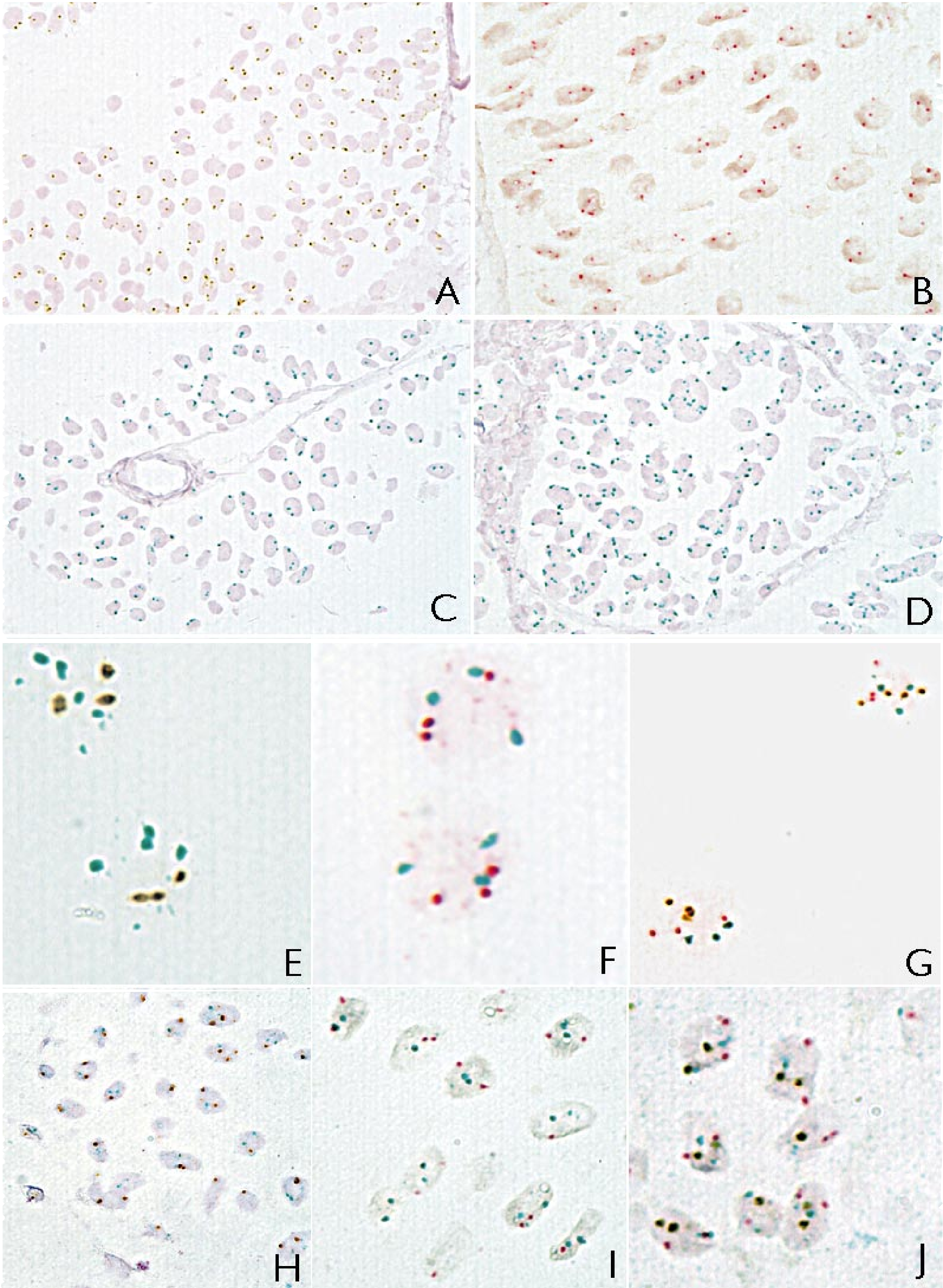
For single-target ISH with chromosome-specific probes, four PO and six APase cytochemical reactions have been described. The PO/DAB and PO/TMB reactions were shown to be the most accurate and sensitive for PO cytochemistry, whereas the enzyme reagents, N-ASMX-P/FR and N-ASMX-P/NF, were the best choices for the APase cytochemistry. These enzyme reactions allow the production of strong brown, green and red precipitates in cell preparations, which can be combined with a light blue/purple haematoxylin counterstaining. Comparison of these enzyme reactions showed that, with respect to detection sensitivity, substrate turnover and colour contrast, the PO/TMB reaction can be considered as the best choice, followed by the PO/DAB and the APase/FR or NF reaction. With respect to the stability of the precipitates during the entire detection protocol, the PO/DAB product proved to be more stable than the products of APase/FR or NF and PO/TMB. For single-target ISH on tissue sections, all enzyme reactions could be utilised with high efficiency (Fig. 1A–D).

To obtain coloured ISH signals with comparable staining intensity and high contrast by all enzyme reactions on tissue sections, the PO/TMB reaction needed only to be combined with a one-layer detection system and the PO/DAB reaction with a one- or two-layer detection system. The APase/FR or NF required the combination of a three-layer ABC system. Table 1 summarises the systems for biotin, digoxigenin and FITC probe detection that have been used in this study.

Although the PO/TMB reaction was successfully applied to ISH on tissue sections, as has been reported before (Speel et al. 1996), the stability of its precipitate

Table 1 Immunochemical detection systems for brightfield in situ hybridisation (ISH). [*Bio* biotin, *Dig* digoxigenin, *FITC* fluorescein isothiocyanate, *PO* peroxidase, *indirect* peroxidase or alkaline phosphatase-conjugated second antibody, *ABC* biotinylated secondary antibody and avidin biotinylated enzyme (peroxidase or alkaline phosphatase) complex]

Probe label	First layer	Second and third layer
Bio	Mouse anti-Bio Avidin–PO	Indirect; ABC
Dig	Mouse anti-Dig Sheep anti-Dig–PO	Indirect; ABC
FITC	Rabbit anti-FITC Sheep anti-FITC–PO	Indirect; ABC


A
B
C
D
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F
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H
I
J

Table 2 Sequence of probe detection steps including cytochemical enzyme reactions in multiple-target ISH on cell lines and paraffin-embedded tissue sections. In the case of double-target ISH, only two labels were detected using either the indicated steps or an immunochemical detection system selected from Table 1. (*Dig* digoxigenin, *FITC* fluorescein isothiocyanate, *Bio* biotin, *MADig* mouse anti-digoxigenin, *GAMAPase* alkaline phosphatase-conjugated goat anti-mouse, *APase/FR* alkaline phosphatase fast red reaction, *RAFitc* rabbit anti-FITC, *SWARPO* peroxidase-conjugated swine anti-rabbit, *PO/DAB* peroxidase diaminobenzidine reaction, *Av-PO* peroxidase-conjugated avidin, *PO/TMB* peroxidase tetramethylbenzidine reaction, *SHADigPO* peroxidase-conjugated sheep anti-digoxigenin, *MABio* mouse anti-biotin, *HAMBio* biotinylated horse anti-mouse, *ABC* avidin-biotinylated alkaline phosphatase complex, *APase/NF* alkaline phosphatase new fuschin reaction)

	Probe label	Subsequent incubation/detection steps						
		1	2	3	4	5	6	7
Cells	Dig	MADig	GAMAPase	APase/FR[a]				
	FITC	RAFitc	SWARPO		PO/DAB[b]			
	Bio					AvPO	PO/TMB[c]	
Sections	Dig	SHADigPO	PO/DAB[b]					
	FITC	RAFitc		SWARPO				PO/TMB[c]
	Bio			MABio	HAMBio	ABC	APase/NF	
Time (min)		40	80	120	130	170	180	190

[a] Instead of fast red, new fuchsin could also be applied

[b] The PO activity still present after the first PO reaction was inactivated by incubation of the slides in 0.01 M HCl for 10 min at room temperature

[c] In all cases, the PO/TMB reaction was performed last, since the resulting precipitate proved to be unstable in aqueous solutions with a pH above pH 6.0 (e.g. Milli Q and PBS)

was sometimes shown to be limited. This was in spite of the precautions that have been recommended before (on single-cell preparations), such as sodium tungstate stabilisation, short washing procedures and organic-based embedding. In some experiments we noticed that the strong green ISH signals formed during the PO/TMB reaction, as monitored by means of the brightfield microscope, disappeared partially or completely after the subsequent washing, counterstaining and mounting steps. To limit dissolution of the PO/TMB precipitate during washing steps, counterstaining and embedding, it was found essential to carry out the washing steps only with 0.1 M phosphate buffer pH 6.0, in which the reaction product is stable. Furthermore, the haematoxylin counterstaining should be very short (a few seconds) and the embedding after dehydration should be in organic-based media or in phosphate buffer only, since combinations with glycerol dissolves the precipitate. Fig. 1C, D shows examples of a properly performed ISH PO/TMB reaction on normal and aberrant cells, respectively.

As an alternative to the PO/TMB reaction, we tested the TrueBlue Peroxidase Substrate kit, which is also based on a TMB precipitation reaction. The resulting precipitate was claimed to be stable and even to survive aqueous washing steps. However, without our additional precautionary steps (see above), this reaction product demonstrated the same stability features as the PO/TMB precipitate on tissue sections.

◀ **Fig. 1** Brightfield microscopical detection of (**a–d**) single-target, (**e, f, h, i**) double-target and (**g, j**) triple-target in situ hybridisation on paraffin-embedded tissue sections of a bladder carcinoma (**a–d**, **h–j**) and T24 cells (**e–g**). The tissue sections were faintly counterstained with haematoxylin, DNA probes used were: pUC 1.77-biotin (Bio) (**a, c–j**), p7t1-digoxigenin (Dig) (**b**); in (**e–g**) pUC 1.77-Bio was hybridised in combination with either pLC11A-Dig (**e**), p7t1-Dig (**f**), or p7t1-Dig and D15Z1-fluorescein isothiocyanate (FITC) (**g**), L1.84-Dig (**h, i**), L1.84-Dig and D15Z1-FITC (**j**). Subsequent incubation steps were performed as indicated in Tables 1 and 2. **a–d** Detection of the centromeric regions of chromosome 1 with peroxidase (PO)/diaminobenzidine (DAB) (brown) (**a**), chromosome 7 with alkaline phosphatase (Apase)/new fuschin (NF) (red) (**b**), and chromosome 1 with PO/tetramethylbenzidine (TMB) (green) (**c, d**). **e–g** Multi-colour detection of chromosomes 1 with PO/DAB and 11 with PO/TMB (**e**), chromosomes 1 with PO/TMB and 7 with APase/NF (**f**), and chromosomes 1 with PO/TMB, 7 with APase/NF and 15 with PO/DAB (**g**). **h–j** Multi-colour detection of chromosomes 1 with PO/DAB and 18 with PO/TMB (**h**), chromosomes 1 with APase/NF and 18 with PO/TMB (**i**), chromosomes 1 with APase/NF, 15 with PO/TMB and 18 with PO/DAB (**j**)

Double-target ISH

Table 2 summarises the detection procedures that we used for multiple-target ISH in single cells and tissue sections. In the case of a double-target ISH, the probes could be detected as indicated in Table 2 or with an immunochemical detection system selected from Table 1. In all cases, the PO/TMB reaction was the last reaction. Figure 1E, F shows the results of a double-target ISH in T24 cells with PO/DAB and PO/TMB, and APase/FR and PO/TMB, respectively. The PO/TMB reaction was always performed at the end of the detection procedure to ensure the stability of the reaction product, whereas the combination of PO/DAB and PO/TMB required an inactivation of the first applied PO activity (see Table 2). In addition, both FR and NF could be used as the trapping agent in the APase reaction, leading to strong red ISH signals on single cells (Fig. 1F). In the case of tissue section hybridisations, however, the APase/FR precipitate was shown to have a limited stability when com-

bined with the PO/TMB reaction. Since this is most likely caused by the low percentage of alcohol present in the PO/TMB reaction buffer, we decided to use NF for the following experiments on tissue sections. An additional advantage of the use of this enzyme reagent is that now, after ISH, the sections can be dehydrated. Figure 1H, I illustrates the results of double-target ISH on normal cells in tissue sections combining the PO/DAB and PO/TMB, and APase/NF and PO/TMB reactions, respectively. For this purpose, the detection sytems were used as presented in Table 2. Depending on the number of detection layers needed, the entire detection procedure could be performed within 2–3 h.

Triple-target ISH

The modifications that were required in the double-target ISH experiments were also incorporated in the triple-target ISH detection procedure. Table 2 summarises an example of a triple-colour detection within 3 h. Figure 1G, J shows the results of these detection procedures on T24 cells and a tissue section (normal cells), respectively. The efficiency of the ISH on the cells was shown to be very high since, in almost all cells, multiple coloured signals could be seen. On the tissue sections, however, the efficiency of subsequent probe detection in a triple-target ISH experiment was shown to decrease after each enzyme reaction. For this reason, in the detection protocol for tissue sections, the third primary antibody was applied prior to the second enzyme reaction (see Table 2). This reduces the shielding of the DNA target (third target) by the precipitate of the enzyme cytochemical reaction for the detection of the second DNA target. The efficiency decreased with the increasing number of enzyme reactions. If the first chromosomal targets could be visualised as intense ISH signals, the second and third targets could also be detected clearly (Fig. 1J). If the first targets, however, could only be visualised as weak ISH signals (mostly after extended enzyme reaction times), than often only the second target was still detectable, whereas the third target was not.

Discussion

We report here the application of a triple-colour brightfield ISH detection procedure on paraffin-embedded tissue sections combining three sensitive cytochemical reactions, i.e. the PO/DAB, Apase/NF and PO/TMB reactions. For this purpose, a combination of three repetitive DNA probes, labelled with either biotin, digoxigenin or fluorescein, was hybridized simultaneously to different in situ DNA targets and detected with three unrelated detection systems. Distinct localisation of the DNA probes in cell nuclei was accomplished within 3 h by consecutive application of these three enzyme precipitation reactions, resulting in clearly contrasting and discernible brown (PO/DAB), red (APase/NF) and green (PO/TMB) ISH signals.

In comparison with brightfield ISH on single cells, however, several adaptations in the original detection protocol were needed. These included: (1) the use of the APase reagent new fuchsin instead of fast red; (2) the performance of a series of critical processing steps to retain the green PO/TMB reaction product in tissue cell nuclei; and (3) introduction of the third primary antibody prior to the second enzyme cytochemical reaction to minimise shielding of the third DNA target.

The choice of the APase/NF reaction as a substitute for the APase/FR originated from the finding that the APase/FR precipitate dissolved in the PO/TMB buffer which contains 25% ethanol. This implies the instability of the APase/FR reaction product in organic solvents, such as ethanol. The reason why the APase/FR reaction product was found to be much more stable in combination with PO/TMB on single cells is therefore unclear (Speel et al. 1994, see below). However, the use of the APase/NF reaction has the additional advantage that tissue sections can now be dehydrated without the possibility of losing coloured ISH (see also Kerstens et al. 1994).

Although the green PO/TMB reaction product could be efficiently stabilised with sodium tungstate in multicolour ISH on tissue sections, some critical notes should be emphasised To enable routine application of the PO/TMB reaction on tissue sections, the specimen should be only washed with cold phosphate buffer pH 6.0, quickly counterstained with haematoxylin, and directly mounted in phosphate buffer or optionally embedded in a cross-linked protein layer. Ignoring these requirements often resulted in the PO/TMB reaction products being dissolved to a large extent. This limited stability of the precipitates becomes much more evident on tissue sections and has not yet been solved. Future research will be needed to examine this phenomenon further. The substrate TrueBlue is also not the answer to this problem, although the supplier claims a permanent stability of the reaction product. They also noticed the limited stability of the TMB substrate and reported the stabilisation of the precipitate with heavy metals. We do not know if their reaction product did not contain these ions.

We can only speculate why the APase/FR and PO/TMB products in tissue sections have a reduced stability compared to ISH on cell suspensions. These reactions products proved to be rather stable in thin preparations (single cells) while in the tissue sections the product dissolved during the short processing steps such as counterstaining with haematoxylin and embedding of the specimen in a cross-linked protein layer. A possible explanation could be that the stability of the PO/TMB reaction product is dependent on the colour density and low hydration content of the precipitate after dehydration. These conditions can perhaps be easily satisfied in a thin cell preparation, such as the single cells, but not in tissue sections. Moreover, the structure of a tissue section is more open for diffusion.

So far, only the principle of triple-colour ISH on tissue sections has been explored, based on the previously

selected colours of brown, green and red. Additional experiments are needed to be performed to test the routine applicability of triple-target ISH on tissue sections.

A further extension of colours could be explored by utilising coloured precipitates that are generated by other PO chromophores, such as aminoethyl carbazole (Graham et al. 1964) and chloronaphthol (Nakane 1968), or by other APase reactions using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. In addition, combined PO and APase reactions can be applied, resulting in mixed colours as described for immunocytochemistry by Claassen et al. (1986). In this respect, the efficacy of the mild acid treatment step to inactivate PO activity may be of help.

We showed here the modification of a multi-colour ISH detection procedure for the simultaneous detection of two and three chromosome-specific repetitive DNA sequences by brightfield microscopy in paraffin sections. So far, only the enzymatic reactions have been optimised for application in multi-color ISH reactions. To improve the detection sensitivity and imaging of brightfield ISH signals, several new developments need to be incorporated in the procedure. This will allow the visualisation of locus-specific target sequences (in the range of 20–100 kb) with our multi-colour ISH approach. On the one hand, the number of enzyme molecules at the target site needs to be increased to achieve a sufficient amount of coloured reaction product for visualisation. A signal amplification system which fulfills this requirement is the catalysed reporter deposition system, which results in a PO-mediated deposition of hapten- and fluorochrome-labelled tryamides. The first (F)ISH results with this system have already demonstrated that the sensitivity can be further increased (Kerstens et al. 1995; Raap et al. 1995; Speel et al. 1997; A.H.N. Hopman et al. manuscript in preparation). On the other hand, novel types of immunochemcial detection systems and microscopes or imaging techniques will be needed to improve colour contrast in the case of small DNA target sites (e.g. for cosmid probes) or complex colour mixtures. As an example, the recently introduced spectral imaging technique enables the separation of multiple fluorochromes on the basis of their spectra (Schroeck et al. 1996). We hope that spectral imaging also can be applied to brightfield microscopical analyses. Preliminary data have already shown that absorption spectra of the dyes (TMB, NF and DAB) measured for each pixel in the image can be clearly separated. Developments at the levels of both amplification systems and microscopy will have their impact on brightfield microscopical evaluation of tissue section hybridisations.

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