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Detection and amplification systems for sensitive, multiple-target DNA and RNA in situ hybridization: looking inside cells with a spectrum of colors

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Abstract In situ hybridization (ISH) is a powerful technique for localizing specific nucleic acid sequences (DNA, RNA) in microscopic preparations of tissues, cells, chromosomes, and linear DNA fibers. To date, a wide variety of research and diagnostic applications of ISH have been described, making the technique an integral part of studies concerning gene mapping, gene expression, RNA processing and transport, the three-dimensional organization of the nucleus, tumor genetics, microbial infections, and prenatal diagnosis. In this review, I first describe the ISH procedure in short and then focus on the currently available non-radioactive probe-labeling and cytochemical detection methodologies that are utilized to visualize one or multiple different nucleic acid targets in situ with different colors. Special emphasis is placed on the procedures applying fluorescence and brightfield microscopy, the simultaneous detection of nucleic acids and proteins by combined ISH and immunocytochemistry, and, in addition, on the recent progress that has been made with the introduction of signal amplification procedures to increase the detection sensitivity of ISH. Finally, a comparison of fluorescence, enzyme cytochemical, and colloidal gold silver probe detection systems will be presented, and possible future directions of in situ nucleic acid detection will be discussed.

Introduction

In situ hybridization (ISH) has proved to be an important tool in molecular cell biology, genetics, and pathology for the detection and localization of specific nucleic acid sequences (DNA and RNA) within morphologically preserved chromosomes, cells, and tissues. Since its introduction three decades ago using radioisotopically labeled probes and autoradiographic probe detection (Gall and Pardue 1969; John et al. 1969), the ISH methodology has been optimized and improved continuously, with the crucial breakthrough being the introduction of non-radioactive probe labeling and detection formats in the late 1970s (Rudkin and Stollar 1977; Bauman et al. 1980). This took away basic shortcomings and practical inconveniences associated with the autoradiographic visualization procedure (for example, unstable probes, long probe detection periods, and poor spatial resolution) and has resulted in a great variety of applications of non-radioactive ISH in biomedical research and clinical diagnosis. Here, I will briefly describe the different steps that are essential for successful ISH, and subsequently focus on the multicolor detection and amplification systems that are currently utilized for sensitive ISH. By doing this, I apologize for the fact that some important ISH methodology and applications may have been excluded, since it is impossible to fully review all literature on this issue.

The ISH procedure

ISH is a multistep procedure, including: (1) preparation of the biological material, (2) nucleic acid probe selection and labeling, (3) hybridization with labeled probe, (4) cytochemical probe detection, and (5) microscopy and image analysis. Preparation of cells and tissue sections for ISH usually involves routine fixation to obtain maximum retention of the target nucleic acid sequences and maintain morphology, such as methanol/acetic acid (3:1) used for chromosome and cell preparations, 70% ethanol for single cell suspensions and touch preparations, methanol or acetone for cytological analysis, and 4% buffered (para)formaldehyde for histological sections. To prevent cells and tissue sections from floating during the ISH procedure, the material is attached to a

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Table 1 Choice of probe and probe label in relation to the copy number of target nucleic acid sequences in situ. (*BAC* Bacterial artificial chromosome, *PAC* P1-derived artificial chromosome, *PCR* polymerase chain reaction, *YAC* yeast artificial chromosome)

Target[a]	Copy number	Probe	Probe label[b]
RNA	High abundant	Oligonucleotide(s) Plasmid/PCR ssRNA/DNA	Fluorochrome[c] Enzyme[d] Hapten[e]
	Moderate/low abundant	Multiple oligonucleotides Plasmid/PCR ssRNA/DNA	Hapten[e]
DNA	High/middle repetitive	Oligonucleotide(s) Plasmid/PCR	Fluorochrome[c] Enzyme[d] Hapten[e]
	Unique	Plasmid/PCR Phage Cosmid Phage P1 BAC/PAC/YAC Padlock[f]	Hapten[e]

[a]Depending on their nucleic acid content (DNA or RNA) and copy number per infected cell, viruses can be detected as RNA or DNA sequences. Specific genomes and painting probes (for example, specific for one chromosome) can be considered as a collection of unique DNA sequences
[b]Probe labeling methods are shown in Table 2. In most cases fluorescence, enzyme cytochemical, and immunogold-silver detection procedures are used for the detection of labeled probes
[c]Fluorochrome: (diethylamino)coumarin, fluorescein, tetramethylrhodamine, Texas red, lissamine, cyanine dyes (for example, Cy3 and Cy5), Alexa dyes (for example, Alexa 488 and 568)
[d]Enzyme: alkaline phosphatase (APase), horseradish peroxidase (HRP)
[e]Hapten: biotin, digoxigenin, dinitrophenyl, estradiol, coumarin, fluorescein, rhodamine, Cy3, HRP (in combination with tyramide signal amplification for probe detection)
[f]In combination with the recently described rolling circle amplification method (Lizardi et al. 1998)

solid support, for example, glass slides that are coated with poly-L-lysine or aminopropyltriethoxysilane. Depending on the amount of cytoplasm and/or crosslinking of cellular proteins, pretreatments (for example, diluted HCl, detergents, such as saponin, SDS, and Triton X-100, and proteolytic enzymes including pepsin and proteinase K) are needed to permeabilize the cells, thereby unmasking the target nucleic acid sequences and facilitating access of the labeled nucleic acid probes and cytochemical detection molecules. Pretreatment steps should be optimized in such a way that a delicate balance is obtained between preservation of cell morphology and efficient ISH. More detailed descriptions of specimen preparation procedures for ISH applications on tissue sections, metaphase and interphase cell preparations, and linear DNA fibers can be found elsewhere (see, for example, Lichter et al. 1991; Johnson et al. 1991; Hopman et al. 1992, 1997b; Wiegant et al. 1992; Parra and Windle 1993; Haaf and Ward 1994; Leitch et al. 1994; Morey 1995; Pringle 1995; Alers and Van Dekken 1996; Dirks 1996; Hougaard et al. 1997; Lichter 1997; McNicol and Farquharson 1997; Hopman and Ramaekers 1998; Erdel et al. 1999; Görtz et al. 1999).

Three types of probes are mainly used for ISH, i.e. double-stranded DNA probes, single-stranded RNA (or DNA) probes, and oligonucleotide probes. During the last decade, the availability of easy-to-use and advanced molecular cloning techniques has led to the production and application of a large variety of DNA and RNA probes, also as a result of huge cloning and sequencing projects, such as the Human Genome Project. Hence, more and more probes have now become available from a number of commercial sources including, for example, probes recognizing highly repetitive sequences (of centromeric and telomeric regions), chromosome (arm) painting probes, and locus-specific genomic and cDNA probes. In addition, on the basis of DNA sequence data, oligonucleotides ranging in size from 18–50 nucleotides can be chemically synthesized using automated DNA synthesizers. The advantages and disadvantages of the different probe types with respect to stability, specificity, and sensitivity have been reviewed previously (see, for example, Matthews and Kricka 1988; Sambrook et al. 1989; Pollard-Knight 1990; Hopman et al. 1995, 1997b; Dirks 1996). Depending on the target nucleic acid (DNA or RNA) and the copy number of this sequence, a choice can be made with respect to probe and probe label (Table 1). To increase the ISH specificity and signal-to-noise ratio even more, the use of synthetic peptide nucleic acid (PNA; Egholm et al. 1993; Thisted et al. 1996; Taneja 1998; Hande et al. 1999) and padlock probes (Nilsson et al. 1997; Lizardi et al. 1998) has been suggested as an interesting alternative. PNA probes form much more stable hybrids with their target nucleic acid sequences than oligonucleotides, since they contain an uncharged polyamide backbone instead of a charged sugar phosphate backbone (in DNA/RNA), which results in the absence of electrostatic repulsion between probe and target. This allows more stringent washing conditions and thus reduces non-specific hybridization, making these probes excellent candidates for quantitative ISH studies. Padlock probes are oligonucleotides that can be ligated into circles upon nucleic acid target recognition and thus become topologically trapped by their target. As a conse-

Table 2 Enzymatic probe labeling methods frequently used for in situ hybridization (ISH)[a]. (*PRINS* Primed in situ labeling; NTP=ATP, CTP, GTP, UTP)

Label	Probe	Method
Hapten-dNTP[b]	dsDNA	Nick translation Random primed labeling PCR
Hapten-dNTP[b]	ssDNA	PCR/exonuclease
Hapten-NTP[c]	ssRNA	In vitro transcription
Hapten-ddNTP[d]	Oligonucleotides	3' Endlabeling
Hapten-dNTP[b]		3' Tailing
Hapten-dNTP[b]		PRINS

[a]Literature describing the different probe labeling methods mentioned in this table can be found elsewhere (see, for example, Sambrook et al. 1989; Hopman et al. 1995; Speel et al. 1995b; Gosden et al. 1998). Hapten-labeled nucleotides are commercially available from a number of companies, such as Boehringer (now Roche, Basel, Switzerland), Life Technologies (Rockville, Md., USA), Molecular Probes (Eugene, Ore., USA), NEN Life Science Products (Boston, Mass., USA), and Vysis (Downers Grove, Ill., USA)

[b]Hapten: amino group, biotin, (amino)digoxigenin, dinitrophenyl estradiol, (diethylamino)coumarin, fluorescein, tetramethylrhodamine, Texas red, lissamine, cyanine dyes (for example, Cy3 and Cy5), Alexa dyes (for example, Alexa 488 and 568). The amino group can be utilized to chemically couple enzymes, fluorochromes, or haptens

[c]Hapten: see b

[d]Hapten: biotin, (amino)digoxigenin, (diethylamino)coumarin, fluorescein, tetramethylrhodamine, Texas red, lissamine, cyanine dyes (for example, Cy3 and Cy5)

quence, these probes provide a high specific detection and low background, which enables the detection of single-base mismatches of centromeric repeat sequences.

In the last 15 years many hapten labeling methods have been developed, based on chemical or enzymatic modification of nucleic acid sequences (for reviews, see Matthews and Kricka 1988; Raap et al. 1989; Pollard-Knight 1990; Hopman et al. 1995). At the moment, enzymatic incorporation of nucleotides modified with biotin, digoxigenin, dinitrophenyl, estradiol (Van de Corput et al. 1997), or fluorochromes [for example, (diethylamino)coumarin, fluorescein, tetramethylrhodamine, lissamine, Texas red, cyanine dyes (for example, Cy2, Cy3, and Cy5), and Alexa dyes (for example, Alexa 488 and 568)] is usually preferred over chemical labeling techniques, since there are also a number of enzymatic probe labeling kits commercially available (Table 2). Nevertheless, the rate of enzymatic incorporation of labeled nucleotides into nucleic acids is often difficult to control and may vary depending on the fluorophore used. Therefore, chemical labeling systems may gain back ground (Van Belkum et al. 1994; Hopman et al. 1995), and improved, quick, reliable and simplified protocols have already been introduced by several commercial companies (for example, Kreatech, Amsterdam, The Netherlands, and Vector, Burlingame, Calif., USA). In addition, target nucleic acid sequences can be detected by means of the primed in situ (PRINS) labeling technique. A hybridized unlabeled DNA probe (oligonucleotide, cloned DNA, or PCR product) then serves as primer for in situ chain elongation, catalyzed by a DNA polymerase or reverse transcriptase, which incorporates labeled nucleotides (Table 2; Bains et al. 1993; Gosden and Lawson 1994; Hindkjaer et al. 1994; Speel et al. 1995a, 1996; Pellestor and Charlieu 1997; Gosden et al. 1998). However, until now this method has been shown to be less sensitive than ISH with a detection limit in the order of low-copy sequences.

After heat denaturation of probe and target nucleic acids (if necessary), hybridization of labeled probes and posthybridization washes are usually performed according to standard protocols for the different types of probes. (Post)hybridization mixtures contain formamide, salt, carrier RNA and DNA, and dextran sulfate, and stringent washings are used to remove non-specifically bound probe.

Next to improvements in specimen and probe preparation methods, the success of the ISH technique has been the result of the availability of a large variety of cytochemical detection methods (Speel et al. 1995b), as well as significant improvements in the quality of light microscopes and imaging systems, such as a charge-coupled device (CCD) camera, to evaluate the ISH results (Leitch et al. 1994; Du Manoir et al. 1995; Shotton 1995; Tanke et al. 1995; Schröck et al. 1996; Jonker et al. 1997). The combination of all these optimized individual ISH steps has recently led to a major milestone in the field of molecular cytogenetics, i.e., the delineation of each human or mouse chromosome in a different color (Schröck et al. 1996; Speicher et al. 1996; Lichter 1997; see also below). I will now provide a detailed update of the available non-radioactive detection systems for ISH, describe procedures for the combination of ISH with immunocytochemical detection of proteins and/or incorporated bromodeoxyuridine (BrdU) to simultaneously visualize genomic, phenotypic, and/or cell cycle parameters, review the possibilities of implementing catalyzed reporter deposition (CARD) signal amplification in ISH detection procedures, and, finally, compare fluorescence, enzyme cytochemical, and colloidal gold-silver procedures with respect to sensitivity, resolution, multiple-target probe detection, stability of generated ISH signals, and evaluation.

Cytochemical probe detection systems

Following probe hybridization, stringent washing steps, and blocking with reagents such as non-fat dry milk, bovine serum albumin (BSA), or normal serum to prevent non-specific binding of the detection reagents to the cells or to the glass slide, the sites of hybridization can be detected dependent on the type of label incorporated into the probe. The simplest way to visualize nucleic acid targets is to use fluorochrome-labeled nucleic acid probes, so that after ISH the site of hybridization can be localized directly by its fluorescence color (Wiegant et al. 1993, 1996; Dirks 1996; Schröck et al. 1996; Speicher et al. 1996; Femino et al. 1998; Egger et al. 1999; Hande et al. 1999). Alternatively, enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (APase) can be chemically conjugated to synthetic oligonucleotides, which makes it possible to localize the hybridization sites with enzyme precipitation reactions (see, for example, Nakane and Wilson 1975; Kiyama and Emson 1991). The use of these *direct* detection systems avoids background noise that can otherwise be induced by the application of cytochemical detection layers. In most cases, however, a higher detection sensitivity is needed than provided by these *direct* techniques, which can be achieved by applying cytochemical probe detection systems (= *indirect* detection; Table 3) or combining the use of HRP-labeled oligonucleotide probes with CARD signal amplification (see below). In principle, every probe labeling method can be combined with a fluorescence, enzyme cytochemical, or colloidal gold-silver visualization procedure. The presented detection systems vary from directly conjugated primary antibodies or labeled (strept)avidin (one-step detection) to more sensitive two- and three-step detection systems.

The sensitivity of each system will depend on both the number of fluorochromes, enzymes, or gold particles that are introduced by the cytochemical detection steps and the signal-to-noise ratio. For conventional fluorescence ISH, the amplification of fluorochrome-conjugated avidin, by means of biotinylated goat anti-avidin antibodies and repeated application of fluorochrome-conjugated avidin, has been shown to be the most sensitive detection system, resulting in six- to eightfold amplification in fluorescence intensity (Pinkel et al. 1986; Speel et al. 1992a). The avidin biotinylated enzyme complex (ABC) system can be considered the most sensitive system, in combination with conventional brightfield ISH using enzyme cytochemical precipitation reactions (Hsu et al. 1981). The signal-to-noise ratio will depend on the amount of non-specifically bound probe and detection molecules within cells, or on the glass slide (background), in relation to the signal intensity caused by the specific cytochemical networks accumulated on the hybridized probe. Treatments to reduce such non-specific binding include the use of acetic anhydride (Hayashi et al. 1978; Lawrence and Singer 1985), detergents (such as Triton X-100, Tween 20, and SDS), Ficoll, polyvinylpyrrolidone, heparin, and BSA (Terenghi and Fallon 1990), denatured and sonicated unlabeled genomic DNA and/or tRNA as carrier, and unlabeled avidin or streptavidin to block endogenous biotin within certain cell and tissue types (for example, liver and kidney; Naoumov et al. 1988; Komminoth and Werner 1997).

Cytochemical detection steps are usually performed for 20–30 min at 37°C. Antibody conjugates are incubated in 50–150 mM salt buffers [phosphate, phosphate-buffered saline (PBS) or TRIS/HCl] containing 2–10% normal serum and/or 0.5–5% blocking reagent (non-fat

Table 3 Cytochemical probe detection systems frequently used for ISH[a]. [*Ab* Antibody, *ABC* avidin biotinylated enzyme (HRP or APase) complex]

Label	Detection		
	First layer	Second layer	Third layer
Biotin	(Strept)avidin[b,c]		
Biotin	(Strept)avidin[b]	Biotin-labeled anti-(strept)avidin Ab	(Strept)avidin[b]
Hapten[d]	Anti-hapten Ab[b]		
Hapten	Mouse[e] anti-hapten Ab	Anti-mouse Ab[b]	
Hapten	Mouse anti-hapten Ab	Rabbit anti-mouse Ab[b]	Anti-rabbit Ab[b]
Hapten	Mouse anti-hapten Ab	Biotin-labeled anti-mouse Ab	ABC
Hapten	Mouse anti-hapten Ab	Hapten-labeled anti-mouse Ab	Anti-hapten Ab[b]

[a]Further amplification of ISH signals can be achieved by combining these detection systems with signal amplification using labeled tyramides (see Speel et al. 1999a)
[b]Fluorochrome: for example, (diethylamino)coumarin, fluorescein, tetramethylrhodamine, lissamine, Texas red, cyanine dyes (for example, Cy2, Cy3, Cy3.5, Cy5, Cy5.5, and Cy7), Alexa dyes (for example, Alexa 488 and 568), enzyme (HRP or APase) or colloidal gold particle. (1–20 nm)
[c]Optimal detection conjugate concentrations should be determined for one's own experiments. Usually the optimal dilutions of commercially available conjugates are indicated by the manufacturer. Antibody and (strept)avidin-biotin conjugates can be obtained from many companies, including Dako (Glostrup, Denmark), Jackson Immunoresearch (West Grove, Pa., USA), Molecular Probes, Roche, Sigma (St. Louis, Mo., USA), and Vector (Burlingame, Calif., USA)
[d]Hapten: biotin, digoxigenin, dinitrophenyl, estradiol, (diethylamino)coumarin, fluorescein, tetramethylrhodamine, Texas red
[e]Anti-hapten Ab raised in another species (for example, goat, rabbit, rat, sheep, and swine) can also be used as primary Ab in ISH detection schemes

Table 4 Fluorochromes often used for in situ probe detection and DNA counterstaining. (*PI* Propidium iodide; *DAPI* 4’,6-diamidino-2-phenylindole)

Fluorochrome[a]	Excitation optimum	Emission optimum	Fluorescence color
Probe detection			
Coumarin	349	448	Blue
Diethylaminocoumarin	426	480	Blue
Fluorescein	494	518	Green
Cy2	490	508	Green
Tetramethylrhodamine	555	580	Red
Cy3	554	568	Red
Lissamine	570	588	Red
Cy3.5	581	588	Red
Texas red	595	615	Red
Cy5	652	672	Far-red
Cy5.5	682	703	Far-red
Cy7	755	778	Far-red
DNA counterstain			
DAPI	358	461	Blue
Hoechst 33258	352	461	Blue
YOYO-1	491	509	Green
PI	535	617	Red

[a]For detailed information concerning fluorochrome spectra and additional less frequently used fluorochromes see, for example, Santisteban et al. 1992; Yu et al. 1994; Waggoner 1995; Haugland 1996

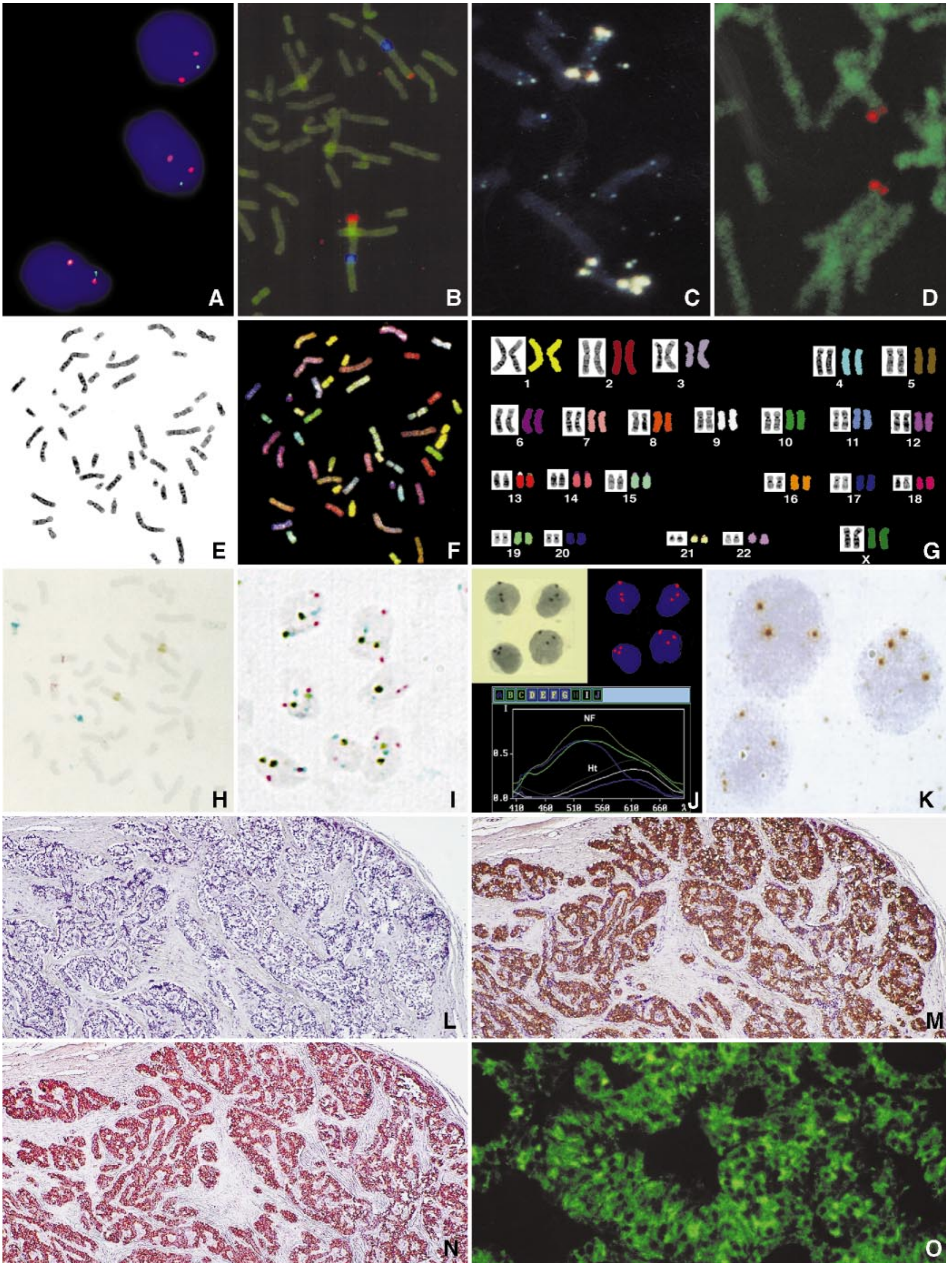
dry milk, casein, or commercially provided), whereas avidin conjugates are diluted in 0.6 M salt solution (4×SSC) containing 5% non-fat dry milk or casein as a blocking reagent. The detection of labeled nucleic acid probes by means of fluorescence, enzyme cytochemistry, and immunogold-silver cytochemistry will now be reviewed.

Fluorescence detection of probes

The use of fluorescence detection protocols for ISH provides a number of advantages, including easy and rapid detection of the fluorochrome-labeled probes, high sensitivity with low endogenous background, high resolution, multiple-target analysis with different fluorochromes, and the possibility to quantitate signal intensity. There are now a large number of fluorochromes available with high quantum yields and good spectral separation properties for use in *direct* (for example, coupled to nucleotides for probe labeling) and the more sensitive *indirect* (conjugated to (strept)avidin and antibody molecules) ISH detection procedures (Table 4). At the end of the cytochemical detection procedure, the cellular DNA can be stained with a DNA counterstain (Table 4), usually added to an anti-fading mounting medium containing anti-fading reagents, such as 1,4-diazabicyclo-[2,2,2]octane, p-phenylenediamine, or n-propyl gallate (for a comparison see, for example, Florijn et al. 1995). The embedding medium Vectashield (Vector) has been particularly recommended for fluorescence ISH. A fluorescence microscope, equipped with high-transmission multiple band-pass filters, is needed for slide evaluation and usually a digital CCD camera and image-processing software are used for pseudocolor presentation of the ISH signals (see above).

The development of a variety of optical filter sets has been connected with the availability of fluorochromes to spectroscopically separate multiple fluorochromes from each other over the limited spectral band width (approximately 350–800 nm) that exists for fluorescence imaging (Dauwerse et al. 1992; Haugland 1996; Speicher et al. 1996). As a result, the number of nucleic acid targets that can be detected in 4’,6-diamidino-2-phenylindole (DAPI)-stained chromosomes has been extended from 2 (fluorescein- and tetramethylrhodamine-labeled), in the late 1980s, to up to 27 and more to date. Most ISH applications, however, are still based on the use of two to three fluorochromes (blue, green, red), for example, for studying gene expression in relation to the cell cycle (Dirks 1996), the three-dimensional architecture of the cell nucleus (Kurz et al. 1996; Lamond and Earnshaw 1998), or in the area of molecular cytogenetics to map genes (Speel et al. 1998b), to visualize the intragenic structure of genes on DNA fibers (Erdel et al. 1999), to detect allelic loss in touch preparations of frozen tumor material (Fig. 1A; Görtz et al. 1999), or to identify and map DNA copy number changes in tumor genomes by comparative genomic hybridization (Kallioniemi et al. 1992; Forozan et al. 1997; Speel et al. 1999c). Using conventional fluorescence ISH procedures, the detection limit for target DNA sequences lies in the order of 1–5 kb and sometimes even smaller, on cell preparations and DNA fibers, and may reach the level of single mRNA molecules in the most optimal cellular model systems (Femino et al. 1998). On tissue sections, however, these detection sensitivities may not be reached, owing to factors such as accessibility and loss of target nucleic acids. In case a further increase of the ISH detection sensitivity or efficiency is needed, the fluorescence detection procedures can be extended with CARD signal amplification (see Fig. 1B and below).

To increase the number of detectable nucleic acid targets over the number of fluorochromes, two strategies have been developed, combinatorial labeling (Nederlof et al. 1989) and ratio labeling (Nederlof et al. 1992; Morrison and Legator 1997). In the combinatorial label-


A
B
C
D
1 2 3 4 5
6 7 8 9 10 11 12
13 14 15 16 17 18
19 20 21 22 X
E
F
G
NF
Ht
0.5
0.0
410 460 510 560 610 660
H
I
J
K
L
M
N
O

ing approach, each target sequence is recognized by probes labeled with a defined combination of fluorochromes, so that 2^n–1 targets can be visualized with n fluorochromes, for example, 15 targets with n=4 fluorochromes and, thus, in principle 31 targets with n=5 fluorochromes. With ratio labeling, the target sequences are discriminated by a combination of fluorochromes that are incorporated at different ratios into the probes. On the basis of the first approach and with the use of five fluorochromes, two different protocols have been reported to allow the simultaneous visualization of all 24 different human chromosomes in a different color, i.e., spectral karyotyping (SKY; Fig. 1E–G, Schröck et al. 1996) and multiplex fluorescence ISH (M-FISH; Speicher et al. 1996). SKY combines fluorescence microscopy, CCD imaging, and Fourier spectroscopy to measure the entire fluorescence spectrum for each pixel of the CCD chip, using an interferometer. After identification of the components of this spectrum, a specific color is assigned to all pixels in the image having identical spectra, resulting in the spectral classification of all chromosomes. M-FISH relies on digital images acquired separately for each of the five chromosomes, using a CCD camera. These images are analyzed by a dedicated software package, generating a composite image in which each chromosome is pseudocolored based on its fluorochrome composition. Painting all chromosomes in a single experiment simplifies chromosome karyotyping and provides the rapid diagnosis of chromosomal aberrations, such as the identification of chromosomal rearrangements, imbalanced material, and marker chromosomes in, for example, tumor specimens (Lichter 1997). In addition, the use of these techniques for interspecies hybridizations has already demonstrated interesting data, with respect to chromosome evolution between related species (O'Brien et al. 1997). Nevertheless, these techniques also have their limitations, with respect to resolution (the detection of small, cryptic aberrations) and the visualization of intrachromosomal rearrangements, and their usefulness in cases where metaphase chromosomes are difficult to prepare (for example, solid tumors) needs first to be established, for example, to detect diagnostically designed cocktails of locus-specific probes in multiple colors in more three-dimensionally shaped interphase cell nuclei.

Fig. 1 In situ hybridization (ISH) results with conventional probe detection systems (**A, C–J**) or catalyzed reporter deposition (CARD) signal amplification (**B, K–O**) on a touch preparation of frozen lung carcinoid tissue (**A**), metaphase preparations of human normal lymphocytes (**B–H**), formaldehyde-fixed and paraffin-embedded tissue sections of human bladder carcinoma (**I**), and pancreatic insulinoma (**L–O**) and interphase T24 cells (**J, K**). DNA counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI; **A**), YOYO-1 (**B**), thiazole orange (**D**), and hematoxylin (**H–N**), and probes were visualized with fluorescence microscopy (**A, B, D, F, G, O**), reflection contrast microscopy (**C**), brightfield microscopy (**E, H, I, K–N**), and spectral imaging (**J, G**). **A** Allelic loss of chromosome 11q13 as detected with a fluorescein-labeled 11q13-specific cosmid probe (two-step fluorescein detection) and a biotin-labeled centromere 11 probe (three-step rhodamine detection). **B** Double-target ISH with a biotin-labeled centromere 1 probe [avidin horseradish peroxidase (HRP)/AMCA-tyramide detection] and a digoxigenin-labeled 1p36 telomere probe (anti-digoxigenin HRP/rhodamine-tyramide detection). **C** Double-target ISH with two biotinylated 11p15- and 11q23-specific cosmid probes [three-step alkaline phosphatase (APase)-fast red detection, yellow reflection, no counterstaining]. **D** Detection of a biotinylated 11q23-specific cosmid probe (three-step APase-fast red detection). **E–G** Spectral karyotyping of a normal female, showing standard G-banding (**E**), red/green/blue display (24 combinatorially labeled chromosome painting probes with *direct* and one-step *indirect* detection; **F**), and spectral classification of chromosomes after pixel by pixel pseudocolor assignment (**G**). **H, I** Triple-target ISH with centromere 1-, 7-, and 17-specific probes labeled with biotin, digoxigenin, and fluorescein, respectively [one-step HRP-diaminobenzidine (DAB) and two-step APase-fast red and HRP-tetramethylbenzidine (TMB) detection; **H**], and centromere 1-, 15-, and 18-specific probes labeled with biotin, fluorescein, and digoxigenin, respectively (three-step APase-new fuchsin, two-step HRP-TMB and one-step HRP-DAB detection; **I**). **J** Spectral imaging of ISH with biotinylated centromere 7-specific probe (two-step APase-new fuchsin detection), showing raw spectral image in unrestored colors (*upper left*), classified spectral image in false colors (hematoxylin in blue, ISH signals in red; *upper right*), and reference absorption spectra of both hematoxylin and new fuchsin (*lower image*). **K** Detection of a digoxigenin-labeled 4p16-specific cosmid probe (anti-digoxigenin HRP/digoxigenin-tyramide/anti-digoxigenin HRP, HRP-DAB detection). **L–O** Insulin mRNA staining after ISH with an anti-sense digoxigenin-labeled oligonucleotide probe, anti-digoxigenin HRP detection, and HRP-DAB (**L**), digoxigenin-tyramide, anti-digoxigenin HRP, and HRP-DAB (**M**), trinitrophenyl-tyramide, anti-dinitrophenyl HRP, and HRP-DAB (**N**), and fluorescein-labeled tyramide visualization (**O**). [After Speel et al. 1992b (**D**), 1993 (**C**), 1994b (**H**), 1998a (**L–O**); Hopman et al. 1997a (**I, K**), 1998b (**B**); MacVille et al. 1997 (**E–G**) and unpublished results (**J**); Görtz et al. 1999 (**A**). Images **B, H, K** are reproduced with permission of the Journal of Histochemistry and Cytochemistry, and images **E–G, J** are courtesy of M. MacVille]

Despite the advantages of fluorescence detection of nucleic acid target sequences, it also has some disadvantages, including fading of the fluorescence signals upon exposure to light, the significant autofluorescence in, for example, formalin-fixed, paraffin-embedded tissue sections, difficulties of combining fluorescence ISH with routine histopathological stains for morphological examination, and, to a lesser extent, economical and practical considerations concerning the microscopic and imaging equipment needed. Therefore, other ways of probe detection are investigated, including time-resolved fluorescence microscopy and confocal scanning laser microscopy (Thompson et al. 1994; De Haas et al. 1999; Lockett et al. 1999). To obtain permanent preparations with non-fading ISH signals, enzyme cytochemical and immunogold-silver detection systems have been utilized.

Enzyme cytochemical detection of probes

The major advantages of cytochemical detection of hybridized probes with precipitating enzyme reactions include the stability of the resulting precipitate and thus the possibility of permanently storing cell and tissue preparations. The combination of these precipitates with routine stains, enabling the use of a standard brightfield microscope for the analysis, is an additional advantage, in particular in a setting where histopathological diag-

Table 5 Enzyme cytochemical reactions that can be applied to ISH[a,b] (for review see Speel et al. 1995b). (*GO* Glucose oxidase, ELF, enzyme-labeled fluorescence, *AEC* amino-ethylcarbazole, *BCIP* 5-bromo-4-chloro-3-indolyl phosphate, *DAB* diaminobenzidine, *HNPP* 2-hydroxy-3-naphthoic acid-2'-phenylanilide phosphate, *NA* not analyzed, *N-ASGR-P* naphthol-ASGR-phosphate, *N-ASMX-P* naphthol-ASMX-phosphate, *NBT*, 4-nitro blue tetrazolium salt, *PMS* phenozine methosulfate, *NBT/BCIP/INT* substrate, product name Dako, *TMB* tetramethylbenzidine)

Enzyme	Reagents	Embedding	Microscopy		
			Brightfield	Reflection-contrast	Fluorescence
APase	ELF-97 phosphate	Aqueous	NA	NA	+ (Yellow)
APase	N-ASMX-P+new fuchsin	Aqueous	Red	Yellow	+ (Red)
APase	N-ASMX-P+fast red TR	Aqueous	Red	Yellow	+ (Red)
APase	HNPP+fast red TR	Aqueous	Red	NA	+ (Red)
APase	N-ASMX-P+fast blue BN	Aqueous	Blue	Yellow	+ (Red)
APase	N-ASGR-P+fast blue BN	Aqueous	Green/gray	Red	–
APase	BCIP+NBT	Aqueous	Blue/purple	Orange/yellow	–
APase	NBT/BCIP/INT	Aqueous	Brown	White	–
HRP	H_2O_2+AEC	Aqueous	Red	Yellow	–
HRP	H_2O_2+Chloronaphthol	Aqueous	Purple	White/yellow	–
HRP	H_2O_2+DAB	Aqueous/organic	Brown	White	–
HRP	H_2O_2+TMB	Organic	Green	Pink/red	–
GO	PMS+NBT	Aqueous	Blue/purple	Yellow	–

[a]Commercial substrate kits are available from a number of companies, for example, Boehringer (Roche), Dako, Kirkegaard and Perry (Gaithersburg, Md., USA), and Vector

[b]Endogenous enzyme activity present in biological preparations can be inhibited or eliminated by a number of different treatments (reviewed by Speel et al. 1995b)

nostic analyses have to be performed. A number of enzymes that can catalyze the precipitation of a visible product at the hybridization site are now commercially available for use in *direct* (for example, coupled chemically to nucleic acids) or *indirect* [conjugated to (strept)avidin and antibody molecules] ISH detection procedures (Table 5). For optimal nucleic acid detection in situ, enzyme precipitation reactions are required that possess both a high sensitivity and precise localization properties. Moreover, rapid staining reactions resulting in stable reaction products with contrasting colors are preferred. So far, the most efficient results have been achieved with the enzymes HRP (molecular weight 40 kDa) and APase (molecular weight 100 kDa). The most frequently applied enzyme reactions for ISH are listed in Table 5, together with the colors of the precipitation products that can be observed in brightfield, reflection-contrast, and fluorescence microscopy (Graham et al. 1965; Graham and Karnovsky 1966; Nakane 1968; McGadey 1970; Malik and Daymon 1982; Gown 1988; Hoeltke et al. 1992; Speel et al. 1992b, 1994b; Kagiyama et al. 1993; Haugland 1996; reviewed in Speel et al. 1995b). It is recommended to fine tune the reaction time for each enzyme reaction by monitoring through the microscope or with image analysis. In case the biological material examined contains endogenous enzyme activity or pseudo-peroxidase activity (such as hemoglobin in erythrocytes), this must be blocked to prevent the formation of unacceptable background. Inhibition or elimination of these enzyme activities can be realized by a number of different treatments, including acetic acid, HCl, or levamisole treatment for endogenous APase (for example, in placenta and intestine; Ponder and Wilkinson 1981; Coggi et al. 1986; Kiyama and Emson 1991), and treatment with, for example, periodate and borohydride, sodium nitroferricyanide, phenylhydrazine, azide, and/or hydrogen peroxide, or HCl in case of endogenous HRP (for example, in erythrocytes, neutrophils, and macrophages; Straus 1971, 1972; Andrew and Jasani 1987; Chin-Yang et al. 1987; Speel et al. 1994b). In some cases, it may be better to change the enzyme system used, rather than expend effort to remove the endogenous enzyme activity. After performing the enzyme reactions and prior to embedding, the cell preparations can be lightly counterstained with, for example, hematoxylin and/or eosin, methyl green, neutral red, or nuclear fast red for brightfield microscopic analysis, and with fluorescent counterstains in the case of fluorescence microscopic analysis. Counterstaining can be omitted if reflection-contrast microscopy is utilized (see below). I will further focus on probe visualization by these three types of microscopy, and not consider the less frequently used dark-field, phase-contrast, or atomic force microscopic evaluation (see, for example, Heyting et al. 1985; Garson et al. 1987; Putman et al. 1993), electron microscopic visualization of HRP-diaminobenzidine (HRP-DAB) precipitates (Morey 1995), or enzyme reactions producing chemiluminescence for ISH probe detection (Lorimier et al. 1996).

Probe visualization by brightfield microscopy

The most appropriate enzyme reactions for ISH include the HRP-DAB, HRP-tetramethylbenzidine (HRP-TMB), APase-fast red, and APase-new fuchsin reactions producing well-contrasting brown-, blue-green-, and (the latter two) red-colored precipitates, respectively (Figs. 1H,I, 2H–J; Speel et al. 1994b; Hopman et al. 1997a). Furthermore, the APase–5-bromo-4-chloro-3-indolyl

phosphate/nitro blue tetrazolium salt (APase-BCIP/NBT) reaction is also known to be very sensitive (Garson et al. 1987), but the localization properties of this reaction for ISH do not appear as optimal as the reactions mentioned above. However, reduction of diffusion of the purple-colored enzyme precipitate from the site of production by means of viscous incubation media including, for example, polyvinyl alcohol, has resulted in discrete localization of this reaction product (Van Noorden and Vogels 1989; Kiyama and Emson 1991; De Block and Debrouwer 1993) as well as of the APase-fast red precipitate (Speel et al. 1992b, 1994a). Using conventional brightfield ISH, the detection limit for DNA target sequences on interphase and metaphase preparations appears to be in the order of 10–40 kb (Speel et al. 1995b; Davison et al. 1998; Slobbe-Van Drunen et al. 1998), whereas the sensitivity of mRNA detection lies in the order of the expression of housekeeping genes, or somewhat below. Higher sensitivities may be reached by combining an enzyme reaction with signal intensification methods, such as nickel-cobalt intensification (Adams 1981; Speel et al. 1998a) or silver amplification (Scopsi and Larsson 1986; Mullink et al. 1992) of HRP-DAB ISH signals, which have resulted in the detection of DNA sequences up to the level of 1 kb in metaphase preparations (Manuelidis and Ward 1984; Bhatt et al. 1988). Alternatively, CARD signal amplification with haptenized tyramides can be implemented in the detection procedure (see below), enabling the detection of single copy DNA (Fig. 1K) and low abundant mRNA sequences in cell and tissue preparations with high efficiency (Hopman et al. 1998b; Yang et al. 1999).

The simultaneous localization of two nucleic acid target sequences in cell and tissue preparations can be achieved by utilizing HRP and APase to produce two differently colored, contrasting precipitates (Hopman et al. 1986; Emmerich et al. 1989; Herrington et al. 1989; Mullink et al. 1989a; Kerstens et al. 1994). In addition, two APase or HRP reactions can be combined with each other for double-target ISH, using a mild acid treatment in between to inactivate the first enzyme and thus to avoid mixing of the precipitates during the second enzyme reaction (Speel et al. 1994b, 1995b). The introduction of the HRP-TMB reaction made it possible to develop a relatively fast triple-color brightfield ISH method for use on cell preparations (Speel et al. 1994b), again utilizing the mild acid treatment to avoid mixing of the precipitates of the two HRP reactions used (Fig. 1H,I). Embedding of the stained preparations in a thin BSA protein layer, crosslinked by formaldehyde, ensured permanent stabilization of the enzyme reaction products and optimal color contrast. This procedure has proven valuable for the detection of chromosome-specific, PRINS-labeled DNA sequences on chromosomes and frozen tissue sections (Speel et al. 1996), and the study of numerical chromosome aberrations in sperm cells (Martini et al. 1995), tumor cells, and routinely fixed, paraffin-embedded tissue sections (Speel et al. 1994b; Hopman et al. 1997a). On tissue sections, however, some modifications of the protocol were needed for optimal results. Firstly, the use of the APase-new fuchsin reaction was preferred over the APase-fast red reaction, since it was noted that the APase-fast red precipitate, which is unstable in organic solvents, sometimes partly dissolved during the HRP-TMB reaction, the reaction buffer of which contains 25% ethanol. Secondly, to ensure the stability of the HRP-TMB precipitate after performing the enzyme reaction, the specimens should only be washed with cold phosphate buffer pH 6, quickly counterstained with hematoxylin, and directly mounted in phosphate buffer or embedded in a crosslinked BSA layer. Alternatively, the slides can be counterstained prior to the HRP-TMB reaction. An explanation for the reduced stability of these enzyme reaction products in tissue sections may be the often lower color density of ISH signals in combination with a more open, three-dimensional structure of tissue sections, creating a higher chance for reagents to reach and dissolve the precipitates (Hopman et al. 1997a). It will be interesting to examine if the recent introduction of a number of commercially available HRP-TMB reaction kits, as well as a new, sensitive HRP reaction producing a red-colored precipitate stable in organic solvents (Vector), will be of help in this respect. The usefulness of this triple-staining technique for the visualization of single-copy nucleic acid sequences is currently under study.

It must be stressed that the distinction between the differently colored reaction products in brightfield microscopy is not always as clear as that between different fluorochromes in fluorescence microscopy (see, for example, Van der Loos et al. 1993), owing to the fact that enzyme precipitates often have broad absorption spectra. This can also hamper colocalization of differently colored ISH signals, although a combination of two well-distinguished precipitates (for example, red APase-fast red or new fuchsin and green HRP-TMB) with a different intermediate color may help in this respect. Furthermore, the utilization of specific filters can be beneficial for better color contrast, although in the case of small single-copy ISH signals color distinction may be a serious problem. One solution to this is the incorporation of a CARD signal amplification step in the procedure, to achieve a sufficient amount of reaction product for visualization (Fig. 1K). On the other hand, the spectral imaging technique (Schröck et al. 1996; MacVille et al. 1997) seems to be very useful, since preliminary data have already shown that absorption spectra for the enzyme precipitates used for ISH, as well as for hematoxylin, measured for each pixel in the image, can be clearly separated (Fig. 1J; MacVille et al., unpublished results). This may also lead to a further extension of colors for brightfield ISH, for example, by utilizing colored precipitates that are generated by other HRP chromophores, such as aminoethylcarbazole (Graham et al. 1965), chloronaphthol (Nakane 1968), or some new, commercially available reagents or APase chromophores (for example, BCIP/NBT; as listed in Table 5). Beta-galactosidase and glucose oxidase are not recommended for use in ISH due

to their high molecular weights and suboptimal precipitation properties, although they can be efficiently utilized for immunocytochemical detection of proteins in combination with ISH (see below). It is clear that such efforts will only be successful if factors, such as the number of different probe labels and detection protocols that can be applied simultaneously without crossreactivity, the influence of the enzyme inactivation procedures on the stability of the precipitates and nucleic acid probe labels, and the distinction of multiple colors, can be fulfilled.

Probe visualization by reflection contrast microscopy

In comparison with brightfield and fluorescence microscopy, the advantage of reflection contrast microscopy (RCM) is the high sensitivity and spatial information that can be obtained with, for example, the use of specifically localized, non-fading enzyme precipitates that exhibit reflectance properties (for review, see Prins et al. 1996; Ploem et al. 1997). Since the enzyme precipitate, the object, and its environment have different reflectance properties, a high-contrast image can be generated (Ploem 1975). Furthermore, no counterstaining is needed, since unstained cells and tissue sections can already be visualized on the basis of their reflectance properties. Especially in cases where difficulties arise with recording of faint staining of, for example, small nucleic acid target sequences, RCM detection is preferable over brightfield detection. Initial ISH studies used the reflection properties of the HRP-DAB reaction product and resulted in the efficient visualization of DNA targets, ranging from repetitive to single-copy sequences (10–40 kb), and mRNA sequences (Landegent et al. 1985; Ambros et al. 1986; Cornelese-Ten Velde et al. 1988; Multhaupt et al. 1989; MacVille et al. 1995). The application of other enzyme precipitation reactions for RCM detection, however, was hampered by the fact that most reaction products are only stable in aqueous-based mounting media (see Table 5) and, thus, are unstable in immersion oil, which is required in this procedure. In this respect, the application of a thin BSA layer crosslinked by formaldehyde, as described above, proved to be useful, since it prevented the enzyme precipitates from being dissolved in immersion oil. In this way, the specific reflection colors of several different precipitates could be observed by RCM (see Table 5; Fig. 1C; Speel et al. 1993). Only the HRP-DAB and HRP-TMB precipitates can be embedded directly (without the protein matrix) in immersion oil, since they are stable in organic-based mounting media (Table 5).

The combination of the HRP-DAB and APase-fast red reaction products enabled the localization of two nucleic acid targets simultaneously in the same cell preparation on the basis of the reflection colors white and yellow, respectively (Speel et al. 1993). Even triple-target ISH using the observed red reflection colors of the PO-TMB and APase-ASGR/fast blue precipitates (see Table 5) proved to be possible, although further optimization is needed to fully establish this tricolor detection approach.

The sensitive RCM technique has its own intrinsic types of artifacts, for example, dirt particles and small amounts of reaction product that are precipitated as a result of the non-specific binding of cytochemically introduced reagents. These artifacts are rarely seen in brightfield microscopy, but may give rise to interpretative problems during RCM analysis (Speel et al. 1993). Best results are obtained with thin cell preparations and semi/ultrathin tissue sections. The reflectance properties of, for example, the HRP-DAB and APase-fast blue reaction products have also been utilized for examination by confocal scanning laser microscopy (CSLM) in the reflection mode (Bianco and Boyde 1989; Robinson and Batten 1989, 1990; Deitch et al. 1990; Szarowski et al. 1992). However, in those cases no color information is obtained as a consequence of the monochromatic laser light.

Probe visualization with fluorescence microscopy

A number of APase precipitation reactions have been described to result in fluorescent reaction products (see Table 5; Raap 1986; Ziomek et al. 1990; Huang et al. 1992; Kagiyama et al. 1992; Murray and Ewen 1992), three of which have proven to be suitable for use in ISH. Two of these reactions are based on the coupling of an enzyme substrate with the diazonium salt, fast red TR (Larsson and Hougaard 1991; Harper et al. 1992; Speel et al. 1992b; Kagiyama et al. 1993; Table 5). The enzyme reactions result in accurately localized red fluorescent precipitates, enabling the detection of mRNA and both repetitive and unique DNA sequences (up to the level of 5 kb) in metaphase and interphase cell preparations. In comparison with the 2-hydroxy-3-naphthoic acid-2’-phenylanilide phosphate-fast red procedure (Kagiyama et al. 1993), the alternative naphthol-ASMX-phosphate-fast red staining reaction is rapid and produces a precipitate with a virtually permanent character and slow fading properties (Speel et al. 1992b, 1994c; Lizard et al. 1997). In combination with the APase-fast red precipitate, DNA is usually counterstained with blue fluorescent DAPI, green fluorescent YOYO-1, or a derivative of thiazole orange (see Fig. 1D; Speel et al. 1994a). The APase-fast red reaction, thus, can be utilized either in brightfield, fluorescence, or reflection contrast microscopy using the same reaction conditions. Optimal results for these applications can be achieved by varying the incubation time of this reaction. The APase-fast blue and APase-new fuchsin precipitates also show red fluorescent properties (Table 5), but are not recommended for fluorescence detection, owing to considerable diffusion of the reaction products. This hampers accurate fluorescence detection, but is not troublesome in brightfield microscopy.

Enzyme-labeled fluorescence (ELF) is an APase reaction introduced by Molecular Probes, yielding a yellow-green fluorescent precipitate (Haugland 1996). ELF sig-

nals develop in a few minutes and can be visualized upon UV excitation using a Hoechst/DAPI filter set, thus enabling the simultaneous localization of, for example, DAPI and ELF signals in one image. The ELF precipitate has been utilized successfully for the demonstration of mRNA, antigens, and endogenous APase in cells and tissue (Haugland 1996), as well as in single- and double-target ISH on cell preparations (Speel, unpublished results). The application of a thin protein layer as described above is also recommended for this precipitate to ensure its stability over a longer period of time.

Despite these developments on sensitive enzyme reactions producing strong fluorescent precipitates, it is to be expected that the CARD signal amplification method will be mostly applied to ISH in the near future, since this easy-to-use method is also based on an enzyme (HRP) reaction, with which a high number of, for example, fluorochrome-labeled tyramides, can easily be deposited in the direct vicinity of the hybridized probe, resulting in increased ISH detection sensitivity (see below).

Probe detection with colloidal gold and silver enhancement

Colloidal gold particles are available in different sizes (for example, 1–20 nm), complexed with avidin or antibody molecules, and can be visualized at both the light and electron microscopic level (Morey 1995; Thiry 1995; Puvion-Dutilleul and Puvion 1996). Since, in most cases, gold particles are too small for direct visualization with the brightfield microscope, the detection sensitivity can be increased by silver enhancement (Danscher 1981; Holgate et al. 1983; reviewed by Hayat 1993; Hacker et al. 1994; Lackie 1996). Colloidal gold catalyzes the reduction of silver ions (from silver lactate or silver acetate) to metallic silver, using hydroquinone as the reducing agent. Also the shell of metallic silver formed around the gold particle catalyzes this reaction, resulting in gray to black deposits when viewed with brightfield microscopy. Silver enhancement exhibits specificity, because it reveals only the colloidal gold label, unless carbon pigments, osmium, metallic gold or silver, or mercury sulfide is present in the tissue, which can act as a catalyst for the reduction of silver ions. In addition, it is not influenced by the presence of endogenous enzyme activities, and promotes accurately localized deposits without diffusion artifacts. However, longer reaction times may lead to conglomerates of silver, owing to coalescence of adjacent growing spheres. Therefore, ISH and detection procedures must be optimized to achieve reproducible labeling. For brightfield microscopy, the use of small gold particles (1–10 nm), which result in good access of the conjugates to the nucleic acid probes as well as low repulsion forces between the gold particles, in combination with silver enhancement is preferred for sensitive detection of DNA and mRNA sequences in cells and tissue sections (Hayat 1993; Hacker et al. 1994). The biological material can be counterstained and stored permanently, as described above for brightfield microscopic probe detection.

Gold particles, with or without silver intensification, have also been utilized to visualize mRNA, as well as antigens in cell preparations and thin tissue sections, by means of reflection contrast microscopy (Multhaupt et al. 1989; Prins et al. 1996; Ploem et al. 1997). In addition, with the use of CSLM in the reflection mode, mRNA transcripts have been localized in cell and tissue preparations, utilizing 1–5-nm gold particles with (Van den Brule et al. 1991) or without (Linares-Cruz et al. 1994) silver enhancement. Moreover, CSLM allowed the detection of rDNA sequences in plant tissue, using 1-nm gold conjugates and silver enhancement (Rawlins and Shaw 1990).

At the electron microscopic level, ISH signals can be localized at the highest spatial resolution (in the order of 0.1 nm), allowing the visualization of gold particles in association with the submicroscopic structure of cells. In general, 5–10-nm gold conjugates are mostly used, since they can be visualized *directly* (one detection layer) or *indirectly* (two or three detection layers) without the use of silver enhancement (Binder et al. 1986; Singer et al. 1989; Narayanswami and Hamkalo 1991; Thiry 1992; Egger et al. 1994; Morey 1995; Dirks 1996; Puvion-Dutilleul and Puvion 1996). Most authors agree that the *indirect* protocols are far more sensitive than the *direct* ones. Ultrasmall gold particles (0.8–1 nm) are useful if the accessibility of gold conjugates is a problem, but are difficult to visualize at the commonly used electron microscopic magnifications. In these cases, silver enhancement methods can be utilized (Narayanswami and Hamkalo 1991; Bassell et al. 1994; Hacker et al. 1994; Sibon et al. 1994; Lackie 1996), although silver enhancement of these small gold particles may lead to variations in reproducibility of size and outline of the grains (Egger et al. 1994). The sensitivity of colloidal gold detection is usually lower than for fluorescence or enzyme cytochemical detection of hybridized probes in situ, which may be a problem for localizing low-copy or unique nucleic acid sequences. Nevertheless, these ultrastructural nucleic acid detection procedures have contributed to a large extent to the current insight concerning the subcellular localization of mRNA transcripts, the functional subcompartmentalization of the cell nucleus, and the analysis of intracellular viral life cycles (Bassell et al. 1994; Morey 1995; Dirks 1996; Puvion-Dutilleul and Puvion 1996). The use of CARD signal amplification may help to further increase the detection sensitivity of ultrastructural ISH (Schöfer et al. 1997; see below).

Using a combination of conjugates with different gold particle sizes, it proved to be feasible to detect different nucleic acid target sequences in the material under study (McFadden et al. 1990; Narayanswami and Hamkalo 1991; Egger et al. 1994; Morey 1995; Dirks 1996). Furthermore, some degree of quantitation is possible in electron microscopy by counting the number of gold particles detecting ISH sites in different cellular areas. Posi-

tive and negative controls are extremely important in this respect, since the signal-to-noise ratio seems to vary considerably between different studies (Morey 1995). The size of gold clusters is an important variable to look at, since a false-positive signal tends to be composed of single particles rather than discrete clusters.

Combination of immunocytochemistry (ICC) and ISH

With ICC, specific information can be obtained regarding the presence or absence of proteins or antigens in cells and tissue sections, thus allowing to characterize (phenotype) cells (for example, their type of differentiation). Furthermore, the proliferative activity of cells can be determined by means of, for example, incorporated BrdU detection. In many instances, however, it would be advantageous to combine the results of ICC and ISH to examine the relationship between genetic and phenotypic parameters in the same biological material. Although this can be achieved by comparing the results of ICC and ISH carefully on serial sections, there are now a variety of protocols available to combine ICC and ISH efficiently on the same cell preparation or tissue section. These procedures have been utilized to simultaneously demonstrate the presence of mRNA and its protein product in one cell (see, for example, Larsson and Hougaard 1991; Harper et al. 1992; Heppelmann et al. 1994; Dirks 1996), to immunophenotype cells containing a specific chromosomal aberration or viral infection (see, for example, Porter et al. 1990; Van den Berg et al. 1991; Kibbelaar et al. 1992; Strehl and Ambros 1993; Weber-Matthiesen et al. 1993a; Zheng et al. 1993; Bridge et al. 1994; Knuutila et al. 1994; Leger et al. 1994; Robben et al. 1994; Speel et al. 1994a,b; Herbergs et al. 1996a), to facilitate evaluation of chromosomal aberrations in tissue sections by nuclear border staining of lamins prior to ISH (see, for example, Herbergs et al. 1996b), to determine cytokinetic parameters of tumor cell populations that are genetically or phenotypically aberrant (see, for example, Schutte et al. 1987; Balazs et al. 1991; Van Dekken et al. 1991; Ffrench et al. 1994; Speel et al. 1994a), and to study the three-dimensional organization of the cell nucleus (see, for example, Matera and Ward 1993; Zirbel et al. 1993; Junera et al. 1995; Xing et al. 1995; Jolly et al. 1997).

Requirements for an optimal combination of ICC and ISH include preservation of cell morphology and protein epitopes, accessibility of nucleic acid targets, no crossreaction between the different detection procedures, and good color contrast and stability of enzyme cytochemical precipitates and fluorochromes. Since several steps in the ISH procedure, including enzymatic digestion, postfixation, denaturation at high temperatures, and hybridization in formamide, may destroy antigenic determinants, a procedure starting with ICC and followed by ISH is preferred in most cases. In these procedures the visualization of proteins and nucleic acids have been achieved by utilizing either enzyme precipitation reactions, fluorochromes, or a combination of both.

Fluorochromes have been used mainly on acetone-fixed cell preparations, since the material can be mildly postfixed after detection of the antigen (usually with paraformaldehyde) and used directly for fluorescence ISH without any further pretreatment steps (Weber-Matthiessen et al. 1993a; Bridge et al. 1994; Leger et al. 1994). However, additional detection layers for both ICC and ISH signals are often necessary for clear visualization. In these procedures, enzymatic pretreatment after ICC has shown to be disadvantageous, since such a step dramatically destroys fluorescent ICC staining (Speel et al. 1994a). This combined fluorescence ICC/ISH procedure could also be successfully applied to cryostat sections (Weber-Matthiessen et al. 1993b).

Enzyme precipitation reactions have also been used efficiently for antigen staining in combined ICC/ISH procedures used on cell preparations and tissue sections. Enzyme precipitates that withstand the proteolytic digestion and denaturation steps used in the ISH procedure, include the APase-fast red (Zheng et al. 1993; Speel et al. 1994a,c; Herbergs et al. 1996a,b), APase-new fuchsin (Mullink et al. 1989b; Porter et al. 1990), APase-BCIP/NBT (Graham et al. 1991), HRP-DAB (Mullink et al. 1989b; Van der Loos et al. 1989; Strehl and Ambros 1993), and β-Gal-BCIG (Van den Brink et al. 1990; Robben et al. 1994; Speel et al. 1994b) precipitates. In these procedures, the pretreatment and denaturation steps needed for optimal ISH after ICC remove the antibody and enzyme detection layers from their targets, but the precipitate remains firmly localized. Hence, unwanted crossreaction between the detection procedures for ISH and ICC is prevented. However, appropriate controls should be incorporated to ensure that this is the case.

Antigen staining with the APase-fast red reaction has the advantage that a sensitive and high-resolution fluorescence approach can be used for both ICC and ISH on cell preparations and tissue sections (Zheng et al. 1993; Speel et al. 1994a,c; Herbergs et al. 1996a,b). This enzyme precipitate could be accurately localized (Fig. 2A), proved to be resistant to enzymatic pretreatment steps, and to be stable during the entire ISH procedure. This allowed the simultaneous cellular localization of a protein with APase-fast red and up to two DNA targets detected by fluorescein and/or coumarin, as has been utilized, for example, to prove the clonal origin of trisomy 7-positive colon adenoma cells (Fig. 2B; Herbergs et al. 1996a) and to detect chromosomal aberrations in cytokeratin-positive lung tumor cells (Fig. 2C; Speel et al. 1994a) or colon carcinoma cell area with high nuclear density (Fig. 2D,E; Herbergs et al. 1996b). Moreover, it proved feasible to localize a cellular antigen together with a DNA target and incorporated BrdU, enabling the assessment of phenotypic, genotypic, and cell cycle parameters in one cell (see Fig. 2F,G; Speel et al. 1994a). The HRP-DAB reaction has also been used for antigen staining prior to fluorescence ISH (Strehl and Ambros 1993). However, the HRP-DAB precipitate has been shown to make cells

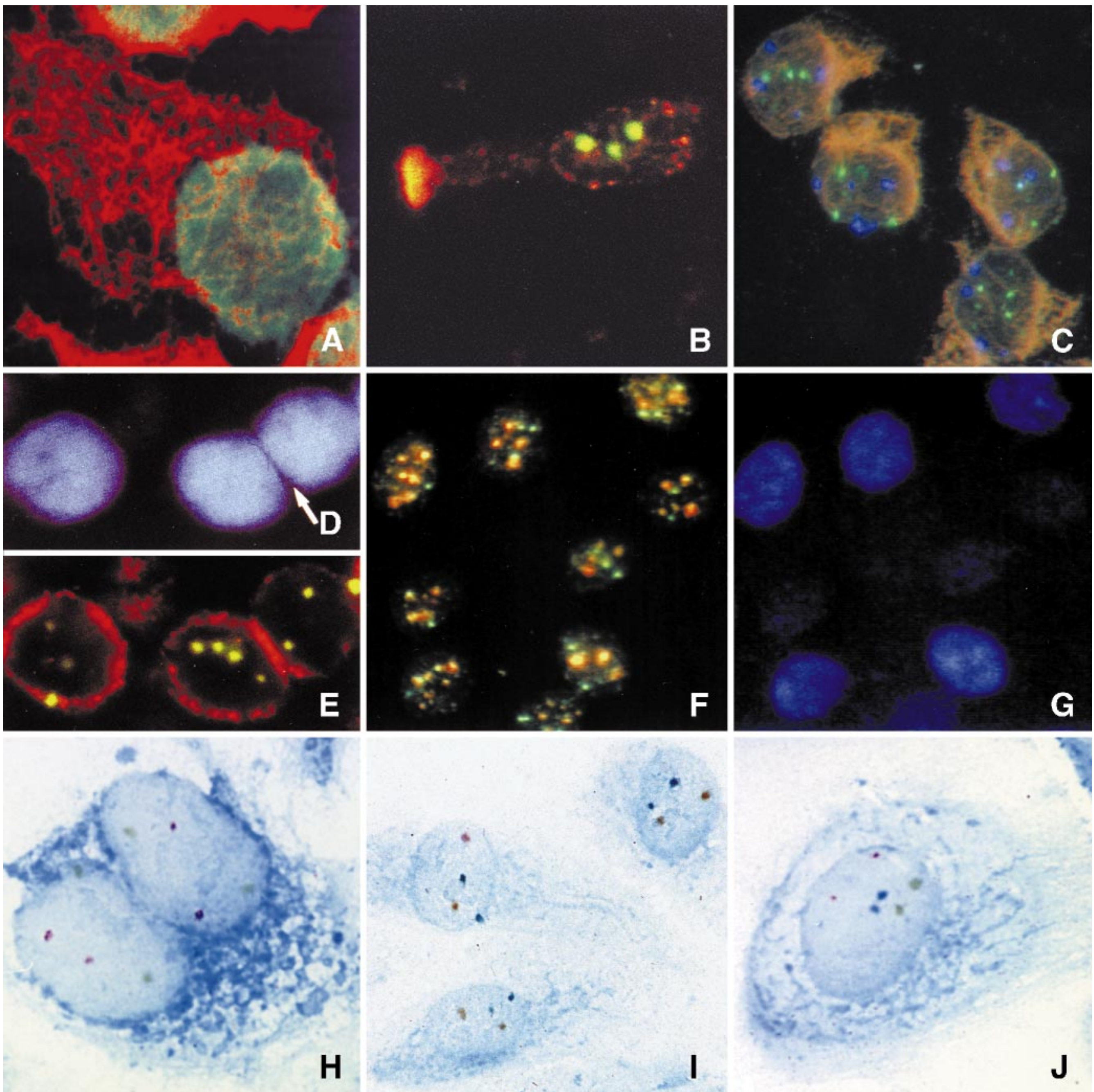


Fig. 2 Immunocytochemistry (ICC; **A**) and combined ICC/ISH (**B–J**) results on human malignant lung (EPLC 65; **A, C, F, G**), colon adenoma (**B**), and normal umbilical vein endothelial cells (**H–J**), and frozen tissue sections of colon carcinoma (**D, E**). DNA counterstaining was only performed in **A** with thiazole orange and in **D** with DAPI, and slides were visualized with fluorescence microscopy (**A–G**) or brightfield microscopy (**H–J**). **A** Visualization of the keratin cytoskeleton with two-step APase-fast red detection. **B** Trisomy 7 in a columnar cell visualized by villin staining (two-step APase-fast red detection) together with a fluorescein-labeled centromere 7-specific probe (two-step fluorescein detection). **C** Visualization of keratin cytoskeleton (two-step Apase-fast red detection) together with both a biotinylated centromere 1 and a fluorescein-labeled centromere 17-specific probe (three-step coumarin and *direct* detection, respectively). **D, E** Tumor cell nuclei (**D**) in a tissue area with tetrasomy 7, visualized by lamin staining (two-step APase-fast red detection) to recognize the nuclear borders (*arrow*) together with a fluorescein-labeled centromere 7-specific probe (two-step fluorescein detection; **E**). **F, G** Visualization of Ki67 antigen (two-step APase-fast red detection) together with a fluorescein-labeled centromere 7-specific probe (*direct* detection; **F**) and incorporated bromodeoxyuridine (three-step coumarin detection) to stain cells in S-phase of the cell cycle (**G**). **H–J** Visualization of the intermediate filament protein vimentin (two-step blue β-Gal-BCIG detection) together with biotinylated centromere 1 and digoxigenin-labeled centromere 7-specific probes [**H, I**; one-step HRP-DAB and two-step (**H**) APase-fast red or (**I**) HRP-TMB detection, respectively], or biotinylated centromere 1, digoxigenin-labeled centromere 7 and fluorescein-labeled centromere 17-specific probes (**J**; one-step HRP-DAB, and two-step APase-fast red and HRP-TMB detection, respectively). [After Speel et al. 1994a (**A, C, F, G**), 1994b (**H–J**); Herbergs et al. 1996a (**B**), 1996b (**D, E**). Images **C, F–J** are reproduced with permission of the Journal of Histochemistry and Cytochemistry, and images **B**, **D, E** are courtesy of J. Herbergs]

less accessible to ISH reagents and to shield the target DNA (Van der Loos et al. 1989; Strehl and Ambros 1993), so that fine tuning of the ICC staining reaction is required to ensure proper ISH results.

The triple-color brightfield ISH detection procedure (see above) can also be combined with ICC utilizing the β-Gal-BCIG reaction for antigen localization in advance (Fig. 2H–J; Bondi et al. 1982; Speel et al. 1994b), since the resulting precipitate also proved to be resistant to enzyme pretreatment steps and the ISH procedure. The color contrast between the blue β-Gal-BCIG and blue-green HRP-TMB reaction product, however, is relatively low (Fig. 2J). Therefore, a combination with HRP-DAB and APase-fast red or Apase-new fuchsin is recommended (see Fig. 2H; Speel et al. 1994b). It will be interesting to see if the introduction of spectral imaging (see above and Fig. 1J; MacVille et al. 1997 and unpublished results) may help to improve the analysis of cell and tissue preparations, for example, in cases where the precipitates used possess closely related absorption spectra or colocalize.

In several cases where mRNA targets have been simultaneously detected with proteins, the material under study is fixed in 4% buffered paraformaldehyde, permeabilized with detergents and/or proteolytic enzymes, then subjected to ISH, and finally to ICC (Larsson and Hougaard 1991; Harper et al. 1992; Trembleau et al. 1993; Heppelmann et al. 1994; Xing et al. 1995; Dirks 1996; Jolly et al. 1997). This is only effective if the antigenic determinants are preserved during the pretreatment steps and ISH procedure. An advantage of this sequence of treatments is the elimination of the risk of possible RNase activity, which might be present in the detection conjugates used for ICC (Egger et al. 1999). At the electron microscopic level, the combination of ISH with ICC is also applied in this sequence, using a combination of conjugates with different gold particle sizes (Singer et al. 1989; Bassell et al. 1994; Egger et al. 1994; Sibon et al. 1994; Morey 1995; Dirks 1996).

Amplification systems to increase the ISH detection sensitivity and efficiency

Although the detection sensitivity of conventional ISH procedures is approximately 1–5 kb of target DNA, and may reach the level of single mRNA molecules in the most optimal experimental cell systems, these sensitivities can not be reached for routine nucleic acid detection, as well as on tissue sections, due to factors such as accessibility and loss of target nucleic acids. As a consequence, ISH detection limits on tissue sections are rather in the range of 40 kb of target DNA and 10–20 copies of mRNA or viral DNA per cell (Höfler et al. 1986; Speel et al. 1995b). Hence, several strategies have been developed to improve the threshold levels as well as the efficiency of nucleic acid detection in situ, such as target and signal amplification methods (Table 6; for review see Komminoth and Werner 1997).

Target amplification systems

Target amplification methods combine an enzymatic nucleic acid amplification procedure with ISH to visualize specific amplified DNA or RNA sequences within cell and tissue preparations. So far, in situ PCR techniques have been mostly explored, since they are theoretically

Table 6 Possible approaches to amplify nucleic acid target sequences and (immuno)cytochemical detection signals in situ (after Speel et al. 1999a)

	Target	Reference
Nucleic acid target amplification		
In situ PCR	DNA	Haase et al. 1990; Nuovo 1992; Komminoth and Long 1993; Teo and Shaunak 1995; O'Leary et al. 1996
In situ reverse transcriptase-PCR	RNA	Nuovo 1992; Chen and Fuggle 1993; Komminoth et al. 1994; Mee et al. 1997
In situ self-sustained sequence replication	RNA	Zehbe et al. 1994; Höfler et al. 1995
PRINS and repeated/cycling PRINS	DNA	Gosden and Hanratty 1993; Terkelsen et al. 1993; Hindkjaer et al. 1994;Troyer et al. 1994; Speel et al. 1995a, 1996; Gosden et al. 1998
In situ transcription/PRINS	RNA	Tecott et al. 1988; Mogensen et al. 1991; Bains et al. 1993
Rolling circle amplification	Oligonucleotide (padlock probe)	Lizardi et al. 1998
Detection signal amplification		
Catalyzed reporter deposition (CARD; tyramide conjugates)		This review; Bobrow et al. 1989
Enzyme polymer system reagents		Pastore et al. 1995
Enzyme-labeled antibody-avidin conjugates		Van Gijlswijk et al. 1996a
End product amplification (anti-DAB antibody strategy)		Chen et al. 1996

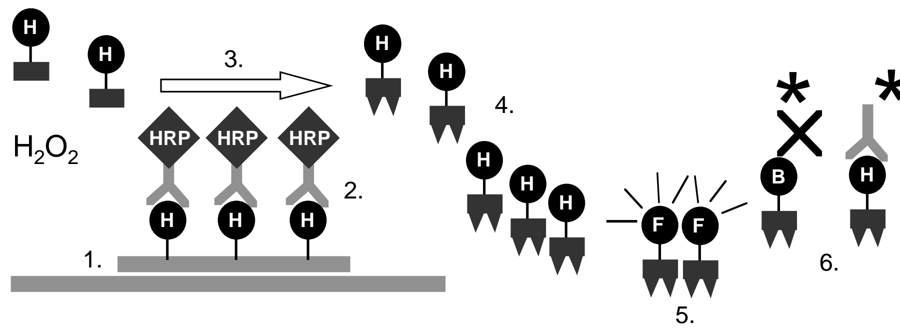


Fig. 3 CARD signal amplification for ISH. **1** Hybridization in situ with a hapten (H=biotin-, digoxigenin-, dinitrophenyl-, fluorochrome-)labeled probe. **2** Application of an anti-hapten antibody conjugated to HRP. **3** CARD signal amplification with hapten-labeled tyramine and H_2O_2. **4** Deposition of tyramide radicals to tyrosine moieties of proteins in situ in the vicinity of the site of synthesis. **5** Direct visualization of fluorochrome-labeled tyramides (hapten H=fluorochrome F). **6** Indirect visualization of biotin-labeled or hapten-labeled tyramides with, respectively, (strept)avidin or anti-hapten antibody conjugates labeled with fluorochromes or enzymes (*asterisks*). Enzyme activity can be visualized utilizing enzyme precipitation reactions (see Table 5; reproduced with permission of the Journal of Histochemistry and Cytochemistry, after Speel et al. 1999a)

straightforward [cell fixation, pretreatment to improve the accessibility of target nucleic acids and to avoid diffusion of PCR-generated amplificants, PCR, and *direct* or *indirect* (by ISH) detection of the amplified nucleic acid molecules] and seem to enhance the ISH detection sensitivity to a large extent. (Nuovo 1992; Teo and Shaunak 1995; O'Leary et al. 1996). In practice, however, several obstacles are encountered, including low amplification efficiency (restricted sensitivity), poor reproducibility (restricted specificity), and difficulties in quantification of the results (Höfler 1993; Komminoth and Long 1993). These findings are caused by a number of artifacts as a consequence of PCR amplification in situ, such as the inevitable diffusion of PCR products, inextricably bound up with the denaturation steps, from the site of synthesis inside and/or outside the cells, followed by the possible extracellular generation of amplificants. Furthermore, if labeled nucleotides are already incorporated in the produced nucleic acids during the PCR cycles, the generation of non-specific PCR products resulting from mispriming, from fragmented DNA undergoing "repair" by DNA polymerase ("repair" artifacts), or from priming of non-specific DNA or cDNA fragments ("endogenous priming" artifacts) will also give rise to positive staining (Sällström et al. 1993; Long and Komminoth 1997). Hence, multiple control experiments are essential to allow adequate interpretation of in situ PCR results, and the specificity of the amplified nucleic acids should preferably be checked with a consecutive ISH step. As a consequence of these clear disadvantages of in situ PCR, other amplification approaches have been explored during the last couple of years (Table 6).

A recently published, potential in situ target amplification method, named rolling circle amplification (RCA), allowed the detection of a DNA sequence of 46 bp on the basis of DNA polymerase-mediated amplification (by a rolling circle mechanism) of a single, circular padlock probe (described above) hybridized to DNA fibers or halo preparations. During RCA, BrdU is incorporated in the ssDNA product, which can be detected subsequently with a biotinylated anti-BrdU antibody and avidin-fluorescein (Lizardi et al. 1998). Although still in its infancy, the combination of padlock ISH combined with RCA may provide another means to increase the detection sensitivity of nucleic acids in situ in the near future, if applicable on more routinely processed cell and tissue preparations. CARD signal amplification has already shown its usefulness in this respect and will now be discussed.

CARD signal amplification

Of the detection signal amplification systems explored (Table 6), CARD signal amplification has proven to be the most promising, since it is an easy-to-use, rapid, sensitive, and efficient system that can be implemented in ISH detection protocols. It has been introduced by Bobrow et al. (1989) for use in immunoblotting and ELISA assays, and is based on the peroxidase-mediated deposition of haptenized tyramine molecules, binding to electron-rich moieties of proteins, such as tyrosine, at or near a site where peroxidase activity is localized (Fig. 3). Thus, if peroxidase-labeled probes are hybridized in situ, or haptenized probes that are detected with peroxidase conjugates, a lot of extra hapten molecules (coupled to tyramines) can be introduced at the hybridization site in situ by peroxidase-mediated tyramide deposition. Visualization of deposited tyramides can be either *directly* after the CARD reaction with fluorescence microscopy, if fluorochrome-labeled tyramides are used, or *indirectly* with, for example, fluorescence or brightfield microscopy, if biotin, digoxigenin, di- or trinitrophenyl are used as haptens, which can be detected subsequently by anti-hapten antibodies or (strept)avidin conjugates (in the case of biotinylated tyramides; see Table 3). Also coumarin, fluorescein, tetramethylrhodamine, and Texas red can be used as haptens, since specific antibodies against these fluorochromes are commercially available.

CARD signal amplification with biotinylated tyramides has been easily adapted for immunohistochemistry by Adams (1992), allowing an increase in sensitivity of up to 1000-fold (based on primary antibody dilution),

when compared with conventional ABC procedures (Adams 1992; Berghorn et al. 1994; Merz et al. 1995; Sanno et al. 1996; Von Wasielewski et al. 1997). In most cases, however, the increase in sensitivity seems to be in the range of 5- to 50-fold or even less. Hence, the optimal dilution for each primary antibody to be used in a signal amplification protocol needs to be determined. CARD signal amplification has also been applied to visualize antigens or incorporated BrdU in fluorescence and electron microscopy with increased sensitivity (Chao et al. 1996; Van Gijlswijk et al. 1997; Van Heusden et al. 1997; Mayer and Bendayan 1999), and has further been used in double stainings, with two unconjugated primary antisera raised in the same species (Shindler and Roth 1996), where one antibody could be applied at a concentration below the detection limit of fluorescently labeled secondary antibodies (used to detect the second antibody), but still sufficient for detection with CARD.

Since 1995, CARD has been further implemented in detection procedures for both DNA and RNA ISH on cell preparations and tissue sections, with reported improvements of the ISH sensitivity in the range of 2- to 100-fold. These approaches enabled the detection of DNA sequences up to the level of 1–5 kb (Kerstens et al. 1995; Raap et al. 1995; Macechko et al. 1997; Schmidt et al. 1997; Speel et al. 1997), the simultaneous localization of up to three different DNA sequences (repetitive as well as single-copy; Fig. 1B; Speel et al. 1997; Van Gijlswijk et al. 1997; Hopman et al. 1998a,b), and the detection of rRNA and mRNA, ranging from high to low abundance in cell and tissue preparations (Fig. 1L–O; Raap et al. 1995; Schmidt et al. 1997; Wanner et al. 1997; Speel et al. 1998a; Van de Corput et al. 1998; Yang et al. 1999). Moreover, CARD signal amplification can be implemented in diagnostic ISH procedures, which enabled, for example, the visualization of low- and single-copy human papillomaviruses in cell and tissue preparations (Poddighe et al. 1996; Zehbe et al. 1997). We have recently introduced CARD signal amplification to our diagnostic non-radioactive oligonucleotide ISH procedure, in order to increase the sensitivity of the assay and to shorten the overall turnaround time (Speel et al. 1998a, 1999b). This approach allows, for example, the detection of peptide hormone mRNA in tissue sections from routinely fixed, wax-embedded surgical samples within 1 working day, and makes the assay suitable for routine diagnostic purposes. Furthermore, it allows the use of DAB as a chromogen and, as a consequence, the application of conventional counterstains and the mounting of slides in xylene-based mounting solutions, making the procedure more acceptable to perform in a diagnostic setting (Fig. 1L–O).

To date, most of the protocols use biotinylated tyramides in the amplification step, which are commercially available (NEN Life Science Products) or can be synthesized in the laboratory (Bobrow et al. 1989; Adams 1992; Kerstens et al. 1995; Raap et al. 1995; Hopman et al. 1998b; Jacobs et al. 1998). However, the use of biotin can be disadvantageous in tissues with high amounts of endogenous biotin, such as liver or kidney, leading to low signal-to-noise ratios due to high background staining. It is, therefore, desirable to be able to rely on differently labeled tyramides, for example, with digoxigenin, di- or trinitriphenyl, or fluorochromes, which is now possible (Hopman et al. 1998b; Speel et al. 1998a; NEN Life Science Products). In addition, these different tyramide conjugates allow the signal amplification of multiple nucleic acid targets in situ or of a nucleic acid and a protein target, if applied consecutively (Speel et al. 1997; Hopman et al. 1998b).

Since with CARD signal amplification both specific and non-specific (background) ISH signals will be amplified, it is essential that non-specific probe binding and detection has to be avoided or kept at a minimum, in order to successfully apply this procedure. Therefore, probe hybridization, cytochemical probe detection, and CARD signal amplification always need to be optimized for one's own experiment. For this purpose, a *direct* or *indirect* (one- to three-step) detection system should be selected (Table 3) and combined with either a commercially available signal amplification kit [available from NEN Life Science Products as tyramide signal amplification kits or from Dako (Glostrup, Denmark) as Genpoint kit] or CARD signal amplification using an amplification buffer, such as PBS containing 0.1 M imidazole pH 7.6 and 0.001% H_2O_2, containing tyramide conjugates (Speel et al. 1997, 1998a, 1999b; Hopman et al. 1998b). To discretely obtain localized ISH signals of high intensity, parameters such as the number of cytochemical detection layers, the dilution of detection conjugates (usually the first detection layer can be diluted two- to tenfold further than in conventional detection systems), the tyramide concentration in the CARD amplification buffer, and the reaction time (at 37°C) need to be adjusted. In addition, probe concentrations may be diluted in combination with CARD signal amplification, which seems to be especially advantageous in cases where complex DNA probes are used (for example, chromosome painting, BAC, P1, or cosmid probes containing repetitive elements that need to be blocked by competitor DNA, such as Cot-I DNA; Speel et al. 1997; Van Gijlswijk et al. 1997). An even quicker but less sensitive way to detect nucleic acids in situ is the use of peroxidase-labeled oligonucleotide probes, which can be detected *directly* with signal amplification using fluorochrome-labeled tyramides (Van Gijlswijk et al. 1996b; Schmidt et al. 1997). Interestingly, Van de Corput et al. (1998) recently demonstrated that the use of multiple peroxidase-labeled oligonucleotide probes, in combination with biotinylated tyramide signal amplification and an *indirect* two-step fluorescence detection procedure, enabled the detection of low abundant mRNA with high efficiency.

Although the increase in ISH sensitivity by using CARD signal amplification is obvious from the literature, speculation about the obtained amplification factor is difficult. Moreover, since the tyramide deposition reaction runs very rapidly, minor differences in reaction time may lead to variations in the final signal intensities.

Nevertheless, an amplification factor of five- to-tenfold, or even higher, with preservation of distinct localization of ISH signals, seems to be a realistic indication for ISH procedures.

Comparison of ISH detection systems and future perspectives

Table 7 summarizes the similarities and differences between the currently available, most frequently used ISH detection systems. In comparison with the possibilities some 5 years ago (Speel et al. 1995b), considerable progress has been made with respect to the sensitivity and efficiency of ISH procedures, as well as the number of nucleic acid targets that can be localized simultaneously in situ. This is the result of the still continuous improvements of the individual steps in the ISH procedure (see Introduction), as well as the implementation of new detection and imaging methodologies, such as CARD signal amplification and spectral imaging.

In principle, every detection system now enables the reproducible light microscopic localization of DNA sequences up to the level of cosmid-sized single-copy probes, and high to moderately abundant mRNA sequences, in most types of biological material. In this respect, the use of CARD signal amplification may help to increase the ISH signal intensity and efficiency. In the more optimal experimental cell and tissue systems, the detection limit of target DNA is approximately 1–5 kb, which can be reached with fluorescence probe detection and CCD imaging, or with enzyme cytochemical or colloidal gold-silver probe detection systems in combination with CARD signal amplification and brightfield microscopy. Low abundant mRNA sequences, however, have only been visualized efficiently in cell and tissue preparations, with the use of a CARD signal amplification step in the ISH detection procedure (Van de Corput et al. 1998; Yang et al. 1999). This is also highly recommended to facilitate the analysis of single-copy DNA signals, for example, in the evaluation of chromosomal aberrations in cytological and histological specimens (visualization with low-magnification objectives), and might help advance the development of automated ISH spot counting by computer-assisted image generation and analysis (Krijtenburg et al. 1996; Netten et al. 1997). If carefully applied, the sensitivity of electron microscopic localization of nucleic acids in situ may also take advantage of CARD signal amplification, with only limited loss of resolution (Schöfer et al. 1997).

Another technique that shows promise for sensitive ISH is the combination of padlock probe hybridization with RCA to permit single-copy gene detection and allele discrimination in situ (Lizardi et al. 1998). So far, however, this system seems to work only with variable efficiency in so-called deproteinated halo preparations, and not as well

Table 7. Comparison of currently used fluorescence, enzyme cytochemical, and colloidal gold-silver detection systems for ISH including the implementation of CARD signal amplification (modified after Speel et al. 1995b). (*BFM* Brightfield microscopy, *CCD* cooled charge-coupled device camera, *CSLM* confocal scanning laser microscopy, *EM* electron microscopy, *RCM* reflection contrast microscopy)

	Fluorescence	Enzyme cytochemistry	Colloidal gold-silver
Sensitivity			
DNA	1 kb	1–5 kb	1–5 kb (BFM)
mRNA	Low abundant	Low abundant	Not explored
Resolution	High[a]	High (RCM)[a], reasonable (BFM, EM)[b]	Reasonable (BFM), high (EM, RCM)
	1 kb (linear DNA), 20–50 kb (interphase), 1–3 Mb (metaphase)	Not explored	Not explored
Multiplicity	>27 Targets[c]	≥3 Targets (BFM), 3 targets (RCM)	2 Targets (EM)
	Good color distinction	Reasonable/good color distinction[d]	Different gold particle sizes
Counterstains	DNA stains (DAPI, Hoechst, YOYO, PI)	Histochemical stains (hematoxylin, methyl green, nuclear fast red, neutral red). No counterstaining (RCM)	Histochemical stains (hematoxylin, methyl green, nuclear fast red, neutral red). No counterstaining (RCM)
Signal permanence	Limited (fading, unstable)[e]	Unlimited (no fading, stable)	Unlimited (no fading, stable)
Preparation storage	>1 Year	Permanent	Permanent
Evaluation/ quantitation	Fluorescence signal size and intensity (CCD, CSLM)	Brightfield color (intensity) and size, for example, spot counting	Grain counts (EM)

[a] Some loss of resolution on signal amplification
[b] Some diffusion of enzyme precipitates can occur
[c] Greater than three to five targets can be visualized on the basis of combinatorial or ratio-labeling of DNA probes
[d] Color distinction is dependent on a large signal size (for example, detection of repetitive sequences, or smaller sequences with the help of CARD signal amplification) or can be achieved with spectral imaging
[e] Some anti-fading reagents (for example, 1,4-diazabicyclo-[2,2,2]octane or Vectashield) can delay fading to a considerable extent

in intact nuclei. Nonetheless, if signals are generated with this technique, they seem to be highly specific, making it worthwhile to further optimize this amplification procedure for use in ISH. Alternatively, one might try to combine one or multiple CARD signal amplification steps, with padlock or PNA probe hybridization, to achieve this goal.

A disadvantage of these amplification methods is that they must be used sequentially to achieve multicolor detection, and that their use decreases the resolution that can be obtained with ISH. In this respect, it is generally accepted that electron microscopy provides the highest resolving power (in the order of 0.1 nm), which makes it possible to localize nucleic acid sequences at the ultrastructural, subcellular level. However, the ISH detection sensitivity in electron microscopic approaches is still too low compared to the light microscopic procedures. Thus, if not needed, mostly the latter procedures are utilized for ISH, of which fluorescence microscopy provides the highest resolution. The smallest physical distance between two different DNA target sequences that can be resolved by fluorescence ISH is dependent on the spatial resolution of the microscope used and on the degree of DNA condensation in the biological specimen under study (chromosomes, interphase nuclei, and DNA fibers). The resolution is in the order of 1–3 Mb on condensed DNA in metaphase chromosomes, 50–100 kb on less condensed DNA in interphase nuclei, and 1 kb (=0.32 μm on double-stranded DNA) on linear DNA fibers (Wiegant et al. 1992; Parra and Windle 1993; Erdel et al. 1999), which almost equals the maximal resolution of the conventional fluorescence microscope (0.2 μm). The spatial information obtained by enzyme cytochemical and colloidal gold-silver detection procedures, as well as with fluorescence approaches including CARD signal amplification, is logically less, owing to the increase of ISH signal size by the amplification reaction and/or some diffusion of the enzyme reaction products from the hybridization site. However, if applied under optimal conditions, the resolution that can be obtained with these procedures is very acceptable (see Figs. 1, 2). An important new development in the area of molecular cytogenetics is the increase in resolution that can be obtained by hybridizing two differentially labeled genomic DNA probes to slides consisting of an ordered set of defined nucleic acid clones (microarrays), instead of metaphase chromosomes (see, for example, Solinas-Toldo et al. 1997; Pinkel et al. 1998). Such a matrix-based comparative genomic hybridization procedure greatly enhances the resolution (from 1–3 Mb on metaphases to 40–100 kb or even lower on microarrays) and will simplify the analysis of genetic aberrations, which are prerequisites for a broad application of this technique as a diagnostic tool. The increasing availability of mapped clones as a result of the many genome sequencing projects and instrumentation to prepare and analyze microarrays will make this technique an especially important tool for the identification of genomic alterations in the near future. In addition, oligonucleotide and cDNA arrays can be utilized for RNA-based screening of gene expression in cells and tissues (Brown and Botstein 1999).

The most obvious difference between the detection methodologies summarized in Table 7 is the ability of the fluorescence approaches to detect up to 27 (and maybe more) DNA targets simultaneously (using spectral karyotyping or M-FISH), whereas the other procedures have been able to detect up to maximally three different targets. This has been the result of a number of factors, including the availability of a number of different fluorochromes that can be spectrally separated in the fluorescence microscope by high-contrast filters, the use of combinatorial and ratio-labeling approaches for probe modification, and the development of digital cameras and image analysis software to discriminate between the different ISH signals. The use of additional fluorochromes may contribute to an even further increase of the multiplicity of fluorescence detection. Despite the many advantages of the now available multicolor chromosome painting approaches, for example, to simplify chromosome karyotyping, to provide the rapid analysis of chromosome aberrations in cultured tumor cells, and to address the question of chromosome evolution between different species, their use has been limited to metaphase chromosomes, so that small deletions or duplications (less than the resolution of 1–3 Mb) and intrachromosomal rearrangements will not be detected. Thus, a new goal will be to utilize the probe-labeling strategies for multicolor approaches, for example, in which chromosomes can be banded additionally with probes in different colors or the telomeres of each chromosome can be labeled in different colors (Lichter 1997). Furthermore, it would be helpful to adapt these procedures in such a way, that cocktails of painting and/or locus-specific probes in multiple colors can be applied to more three-dimensionally shaped interphase cell nuclei, enabling the rapid interphase cytogenetic analysis of tumor samples, for example, for clinical diagnosis, and the extension of possibilities to study the three dimensional organization of the nucleus. Increasing the number of detectable mRNA targets, by making use of the combinatorial probe labeling strategy, is not an option when studying RNA expression in the same cell (Dirks 1996).

On the contrary, the often broad absorption spectra of enzyme precipitates have limited the number of targets that can be visualized simultaneously in brightfield microscopy to three or four. Furthermore, small nucleic acid targets are difficult to detect, on the basis of brightfield color contrast. A solution to this may be the implementation of CARD signal amplification in the detection procedure, to produce more intense ISH signals that can be easily detected on the basis of color contrast. On the other hand, preliminary data suggest that the spectral imaging technique may provide sufficient discriminating power to separate the absorption spectra of multiple enzyme precipitates and histological stains. This may allow the efficient detection of multiple repetitive and single-copy DNA sequences in situ, for example, to detect numerical and structural chromosomal aberrations in a diagnostic setting, and may even lead to a further extension of colors that can be used for brightfield ISH. The success of such efforts will also depend, of course, on the number of dif-

ferent probe labels and detection protocols that can be applied simultaneously without crossreactivity, and the influence of the enzyme inactivation procedures on the stability of the precipitates and nucleic acid probe labels.

In conclusion, this review describes the currently available non-radioactive ISH detection and amplification methodologies as well as procedures for simultaneous localization of proteins, nucleic acids, and/or incorporated BrdU in cell and tissue preparations. It is expected that the presently available spectrum of probe labels, probe detection, and signal amplification technologies, and image generation and analysis equipment will further improve the applicability and sensitivity of ISH in research and diagnosis, as well as promote the development of reproducible multiple-target nucleic acid detection in situ. Furthermore, the ever-increasing knowledge on cell cycle regulation, cancer initiation, and progression, and regulation processes that occur in embryonic development and differentiation of cells, will further stimulate studies examining the presence or absence of genes, gene transcripts, and proteins, as well as their nuclear and/or cytoplasmic topography. In this light, multiparameter analysis methods, such as the combined ICC/ISH procedures described in this review, will become more and more important, and these procedures will certainly profit by the currently available detection and amplification methods as well. Therefore, nobody should be any longer afraid of red, green, and blue, since it's time to look inside cells with a spectrum of colors!

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