

Ultra-sensitive FISH using peroxidase-mediated deposition of biotin- or fluorochrome tyramides

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We describe a detection principle for indirect fluorescence *in situ* hybridization (FISH) methods that with only one or two antibody layers dramatically improves FISH signal intensities. The method uses as a first layer an anti-hapten immunoglobulin [or (strept)avidin] conjugated to peroxidase. The quintessence of the method is the use of fluorochrome- or biotin labelled tyramides as peroxidase substrates to generate and deposit many fluorochrome or biotin molecules close to the *in situ* bound peroxidase. These may either be directly evaluated under the fluorescence microscope or after another incubation with fluorochrome-labelled (strept)avidin.

INTRODUCTION

Fluorescence *in situ* hybridization (FISH) techniques have been established over the past decade for a variety of molecular genetic and cell biological applications (for review see 1).

Although direct FISH techniques have been developed lately (2,3), indirect FISH is still often the method of choice, because of its greater sensitivity. Mostly, biotin and digoxigenin-(d)UTP are used to hapten-modify the DNA or RNA probe and after *in situ* hybridization, detection of the haptens is accomplished by immunocytochemical means.

For biotin detection, the method described by Pinkel *et al.* (4) is most frequently used, because it permits ample possibilities for signal amplification by repeated rounds of incubation with biotinylated anti-(strept)avidin and fluorochrome conjugated-(strept)avidin. For digoxigenin-based FISH, multi-immunological layers are used as well to enhance signals (2). The number of immunological amplification rounds possible before the noise level becomes unacceptable is, however, generally limited to two or three. Hence, dependent on the size of the target, the quality of the probe, the specimen and the fluorescence microscope, the signal-to-noise ratio may be such that a specific signal is still only faintly or not detectable.

Here we describe a detection principle for indirect FISH methods using only one or two antibody layers, which permits ultra-sensitive FISH. The method uses as a first layer an anti-hapten immunoglobulin [or (strept)avidin] conjugated to peroxidase. The method is based on the use of a fluorochrome- or a biotin labelled peroxidase substrate to generate and deposit many fluorochrome or biotin molecules close to the *in situ* bound antibody-peroxidase conjugate(s). Fluorochrome-labelled deposits can be visualized directly by fluorescence

microscopy. To detect the deposited biotin molecules, (strept)avidin-conjugates are used in a second layer.

The peroxidase substrates used in this study are biotin-, fluorescein, tetramethylrhodamine and aminomethylcoumarin acetic acid conjugates of tyramine. The phenolic part of these tyramine derivatives reacts with peroxidase to produce highly reactive intermediates that interact with any electron-rich moiety leading to immobilization of biotin or fluorochromes at and in the immediate vicinity of the immunoperoxidase binding site. The principle has been developed originally for ELISA (5) and has been applied immunocytochemically by Adams *et al.* (6), who first demonstrated the unprecedented sensitivity of the above sketched detection strategy.

RESULTS

Use of biotin-tyramide in FISH

Figure 1 shows results obtained with biotin and digoxigenin labelled repeat probes for the heterochromatic block on the q arm of the Y chromosome and the centromere of the X chromosome. Standard hybridization conditions (60% formamide/2SSC at 37°C) and biotin-tyramide reactions were used. The total signal intensity is extremely high at the major binding sites. Note that, in contrast to our conventional methods, even the low affinity binding of the X alphoid probe to the centromeres of some other chromosomes is clearly detected by the new method. Also the heterochromatic Y probe appears to have additional binding sites on an autosome.

The diffusion of the biotin-tyramide reaction intermediates to some extent compromises resolution, especially for the

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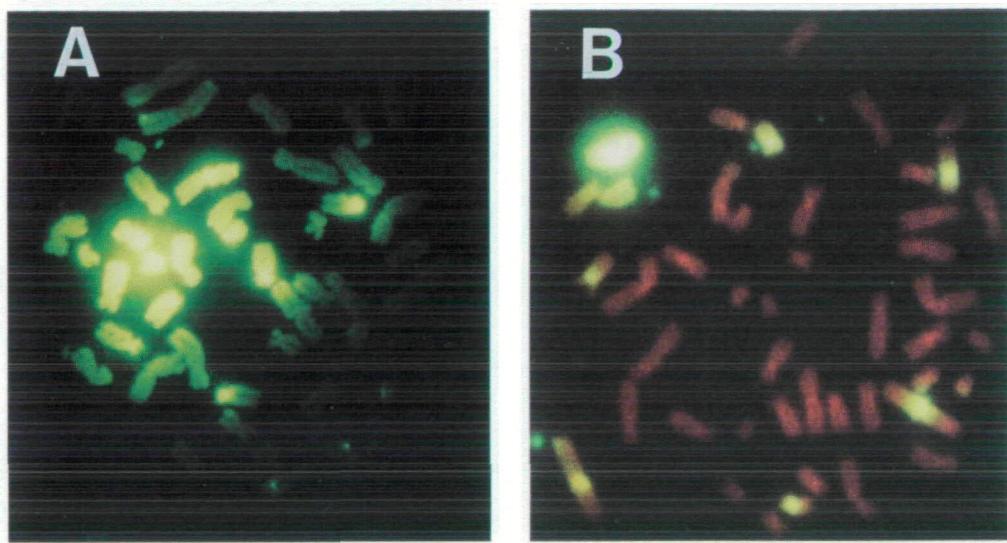


Figure 1. Biotin-tyramide based detection of the digoxigenin-labelled probe pY3.4 for the heterochromatic block on chromosome Yq. Note the 'halo' of fluorescein (**A**). Biotin-tyramide based detection of biotin-labelled alphoid DNA probe pBamX5 for cenX (**B**). Note the minor binding sites, which are not visible using conventional immunological detection. Hybridization- and post-hybridization stringency conditions for these repeat probes were 60% formamide/2SSC at 37°C.

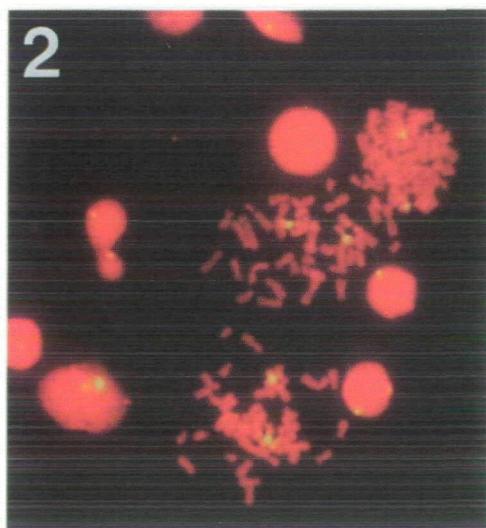


Figure 2. Biotin-tyramide based detection of the digoxigenin-labelled alphoid DNA probe for chromosome 17. To taper the visualization reaction, the standard biotin-tyramide substrate was reduced 5-fold further and the incubation lasted only 3 min. Concentration of probes and antibodies were not changed.

major binding sites of centromere probes. Figure 2 shows for a centromere probe for chromosome 17 that, by reducing the tyramide substrate concentration and the reaction time each 5-fold, improved resolution can be obtained under otherwise identical hybridization and immunological conditions. For FISH experiments with different resolution and sensitivity demands, a different optimal balance needs to be established. As shown in Figure 3, a good resolution, while preserving high signal intensity, can be obtained with the standard biotin-tyramide reaction using cosmid-sized targets.

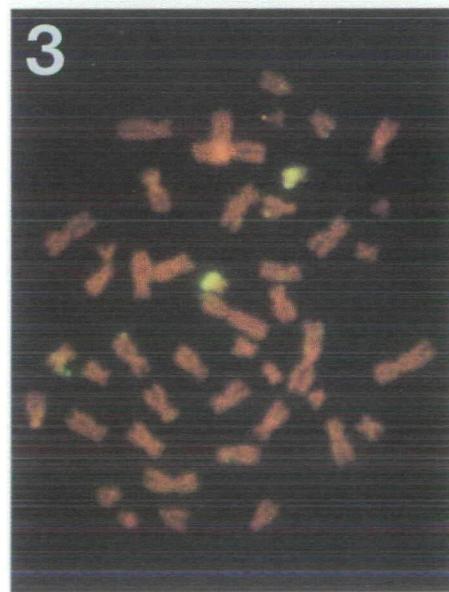


Figure 3. Biotin-tyramide based detection of a biotin-labelled cosmid probe for the adenosine deaminase gene on chromosome 22.

The targets described above can all be visualized with conventional immunological detection technology as well. Clearly, very high FISH signal intensity can now be obtained predicting that targets at the conventional detection limits should be easily visible with tyramide-based methodology. The following RNA-FISH experiment underpins this point.

Using a 440 bp PCR-biotin labelled probe, the expression of the external transcribed spacer of A-type ribosomal RNA of *Plasmodium berghei* is at the border of detectability with

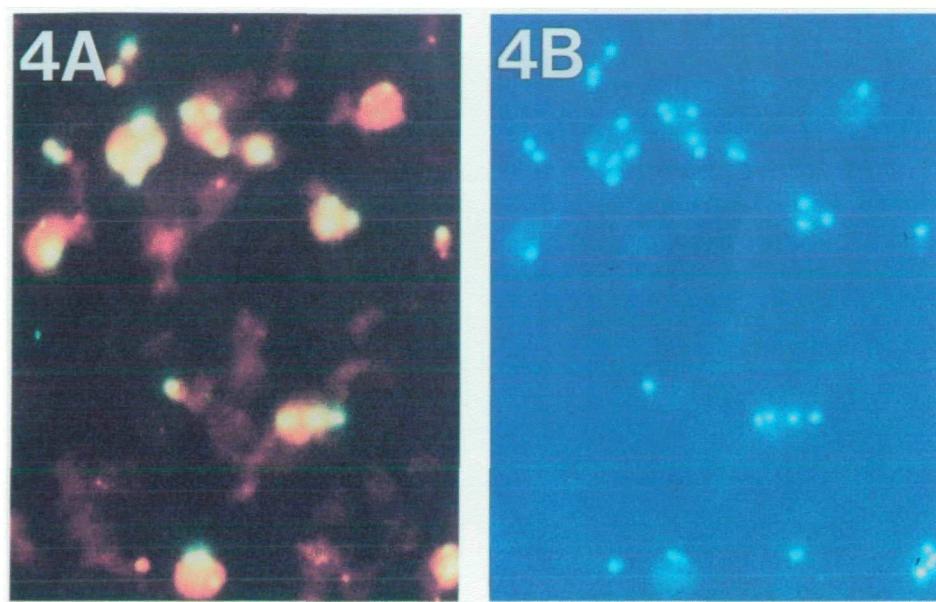


Figure 4. Biotin-tyramide based detection of RNA expression of an external transcribed spacer of A-type ribosomal DNA of *Plasmodium berghei* in blood smears of infected mice with a biotin-labelled PCR probe. (A) the fluorescein image; (B) the DAPI image. The red objects are remnants of mouse erythrocytes.

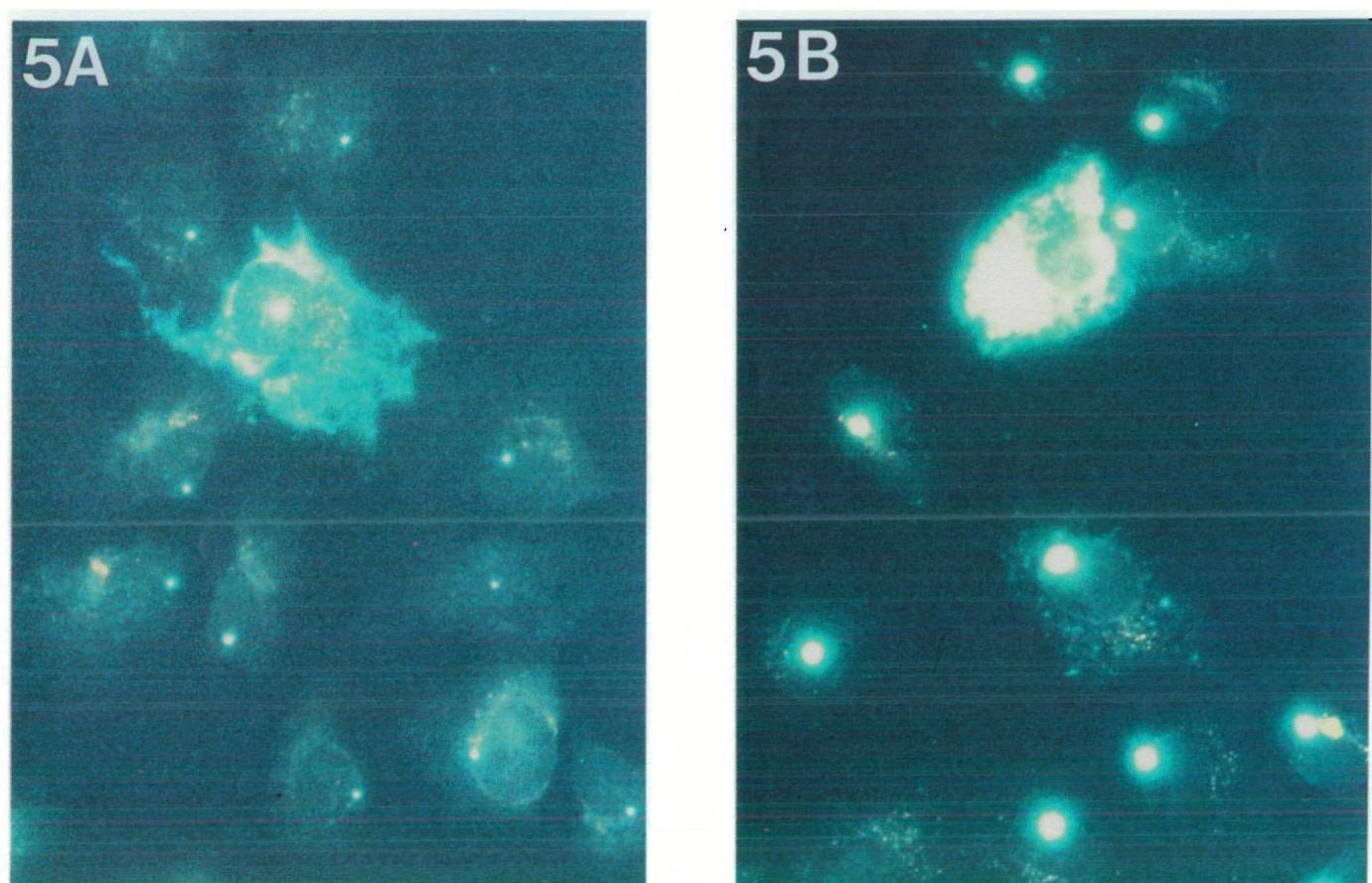


Figure 5. Comparison of conventional (A) and biotin-tyramide based (B) detection of HCMV-IE mRNA in rat 9G cells using the digoxigenin-labelled genomic pSS probe (3). Cytoplasmic expression of HCMV-IE mRNA is heterogenous (7,8). A cytoplasmic mRNA positive cell is present in both A and B, next to non-expressing cells. The cells were denatured prior to RNA-FISH leading to a nuclear DNA signal in all cells. Note the difference in the intensities of the nuclear DNA signals between biotin-tyramide based and conventional detection.

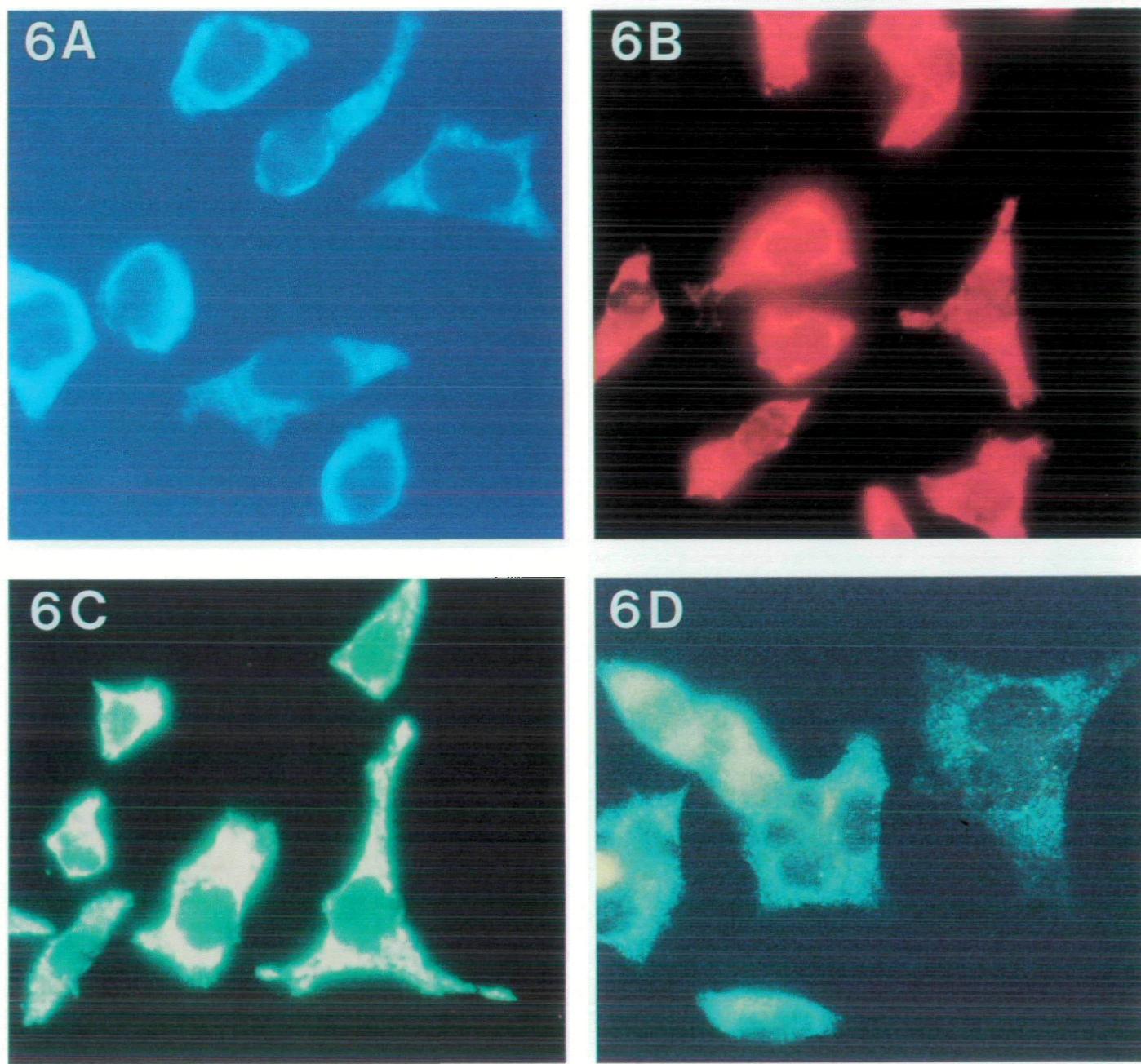


Figure 6. Aminomethyl coumarin acetic acid-, rhodamine- and fluorescein- tyramide based detection of HEF-mRNA in HeLa cells using a digoxigenin-labelled cDNA (A,B and C, respectively). The signal obtained with our conventional fluorescein detection is presented in D.

conventional methods. A total of five immunological layers was necessary to reach sufficient signal to decide upon presence or absence of a specific RNA-FISH signal in the parasites. With the standard biotin-tyramide detection format, the RNA-FISH signal was easily detected indeed, in a low background (Fig. 4).

We have also used the biotin-tyramide detection strategy for other RNA-FISH with success. For example, the RNA expression of the (transfected) gene encoding the Human Cytomegalovirus Immediate Early transcription unit (HCMV-IE) in rat9G cells (7) is easily detected and the signal is much stronger than obtained by the conventional detection (Fig. 5).

Use of fluorochrome-labelled tyramides

The results of Figures 6 and 7 illustrate the use of fluorochrome-labelled tyramides in RNA- and DNA-FISH. By RNA-FISH, housekeeping gene transcripts such as human elongation factor (HEF) can easily be seen in the cytoplasm of HeLa cells with very good signal-to-noise ratios, using all fluorochromes tested. Negative controls (RNA FISH with the pSS probe of Figure 5 and a no probe FISH) showed the same autofluorescence levels as present in the cells before RNA-FISH. Following photomicrography at identical exposure times as for the HEF-RNA FISH (3–5 s), such controls lead to photomicrographs showing only faintly fluorescing cells (not shown).

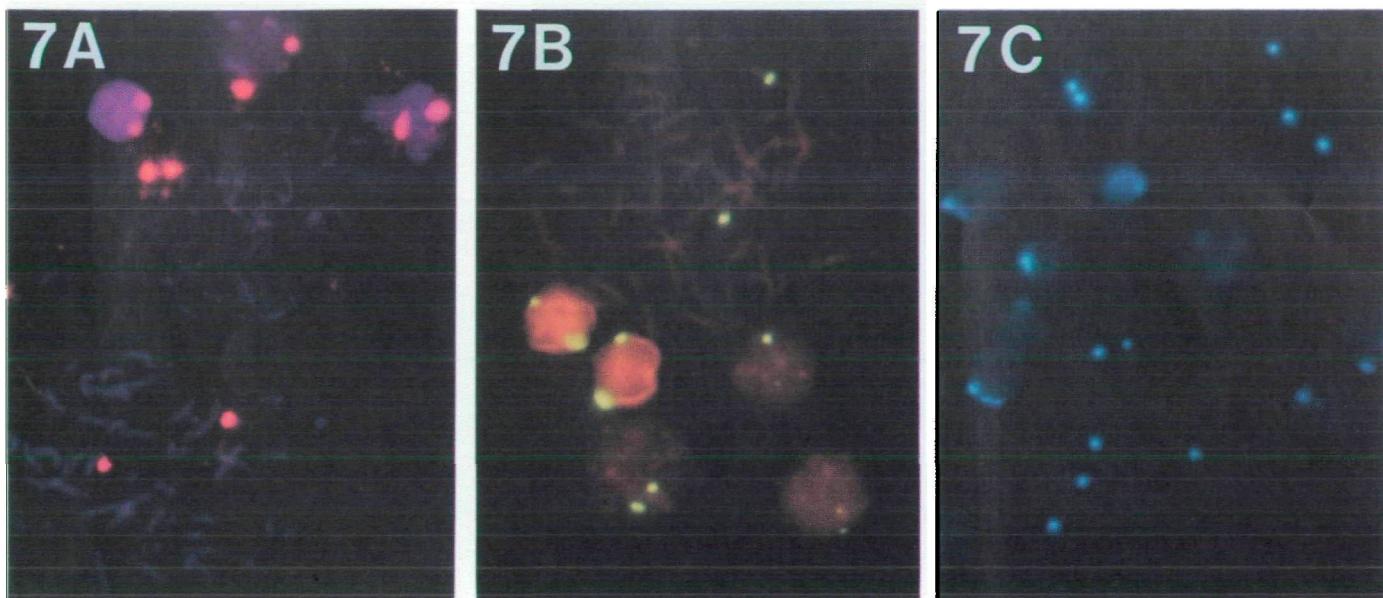


Figure 7. Aminomethyl coumarin acetic acid-, rhodamine- and fluorescein- tyramide based detection of cen17 in human metaphase chromosomes using a digoxigenin probe (A, B and C respectively).

Also centromeric alphoid DNA probes are easily detected with this rapid fluorochrome tyramide methodology (Fig. 7). We found that any of the three fluorochrome-tyramides provides much better sensitivity than our conventional protocols. Of these the tetramethylrhodamine tyramide proved to provide the highest sensitivity.

DISCUSSION

We present here our first results with haptene-labelled DNA probe detection based on biotin- and fluorochrome conjugated tyramides. Each first layer peroxidase-conjugate molecule generates many covalently immobilized fluorochrome or biotin molecules, which are detected in the fluorescence microscope directly, or after an additional incubation with a (strept)avidin-conjugate. Multiple FISH with tyramide based detection has been attempted with success (results not shown).

The photomicrographs illustrate clearly that the tyramide conjugate-based detection formats provide FISH signal intensities far higher than those obtainable with conventional methods and that background on the glass surface is extremely low. This low background is probably a consequence of the low binding capacity of the glass surface for peroxidase-activated tyramide reaction intermediates which diffuse beyond the chromosomal or cellular matrix.

Currently we are in the process of quantitating the amplification factors by digital imaging microscopy. The automatically determined photomicrographic exposure times may give a first, semi-quantitative indication. For example, the exposure time for HEF-RNA-FISH images are 10 s or less using biotin-tyramide, while about 60 s are needed for a much less bright result using the conventional method (Fig. 5). This suggests that the difference in sensitivity is at least one order of magnitude and possibly more.

Through the very mechanism of signal generation, involving freely diffusible highly reactive intermediates, the resolution of tyramide-based detection is less than the one of conventional techniques for visualizing haptenized probes. However, extensive optimization of resolution has not been performed yet. For example, the use of electron-rich (i.e. tryptophane- or tyrosine-rich) protein coats may improve resolution. The choice between conventional or tyramide-based detection should obviously be made in relation to the spatial resolution and sensitivity required by the molecular (cyto)genetic or cell biological question under study.

The lowest detection limits are still being explored. In this respect we note that the limiting factor for detecting very small targets is not only determined by the sensitivity of the immunocytochemical detection system, but also by the hybridization efficiency *in situ*.

In the 25 years that have passed since the first description of *in situ* hybridization (9), several methodological breakthroughs have established FISH as a widely used research and diagnostic tool providing high sensitivity, resolution and multiplicity. The tyramide-based detection method described here will further increase detection sensitivity of FISH and consequently increase its application. The combination of low-background with extremely high sensitivity of detection, may permit very short unique sequence detection *in situ*.

MATERIALS AND METHODS

In situ hybridization

Nick-translation of DNA with biotin- or digoxigenin dUTP, the DNA *in situ* hybridization to human chromosome spreads, the RNA *in situ* hybridization to HeLa, rat-9G cells and *Plasmodium berghei* and conventional immunological detection was according to our published procedures (2,3,8,10).

Biotin- and fluorochrome tyramide based detection

After *in situ* hybridization, slides were incubated with either streptavidin-peroxidase (Vector) or anti-digoxigenin-peroxidase (Boehringer Mannheim). Following washes, the biotin-tyramide substrate solution was incubated for 15 min at room temperature. The biotin-tyramide substrate solution was prepared as follows. A stock biotin-tyramide solution of 1 mg/ml (kindly provided by NEN-DuPont) was diluted 1:1000 in 0.2 M Tris-HCl, 10 mM imidazole at pH 8.8 after which H₂O₂ was added to a final concentration of 0.01%. After the enzyme reaction slides were washed, incubated with streptavidin-FITC, washed and mounted in antifading agent (Vectashield from Vector) containing diamidino phenyl indole (DAPI) or propidium iodide (PI).

The three fluorochrome-labelled tyramides were synthesized by mixing 25 µl 150 mM tyramine (Aldrich) in dimethylsulphoxide with 50 µl 150–160 mM N-hydroxysuccinimide esters of the fluorochromes (Boehringer Mannheim). They were used without further purification. Stocks were made 1 mg/ml in tyramine. The peroxidase reaction with the fluorochrome-labelled tyramides was for 10 min at room temperature with the stocks diluted 1:1000 in 0.2 M Tris-HCl, 10 mM imidazole, 0.01% H₂O₂ at pH 8.8.

Microscopy and photography

Photomicrographs (slides) were taken with a Leica DM microscope equipped with appropriate filter sets and an automatic exposure timing device. Colour photomicrographs were made from these slides under standard conditions to allow semi-quantitative comparison.

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