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ORIGINAL INVESTIGATION

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High resolution multicolor fluorescence in situ hybridization using cyanine and fluorescein dyes: rapid chromosome identification by directly fluorescently labeled alphoid DNA probes

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Abstract We tested DNA probes directly labeled by fluorescently labeled nucleotides (Cy3-dCTP, Cy5-dCTP, FluorX-dCTP) for high resolution uni- and multicolor detection of human chromosomes and analysis of centromeric DNA organization by in situ hybridization. Alpha-satellite DNA probes specific to chromosomes 1, 2, 3, 4 + 9, 5 + 19, 6, 7, 8, 10, 11, 13 + 21, 14 + 22, 15, 16, 17, 18, 20, 22, X and Y were suitable for the accurate identification of human chromosomes in metaphase and interphase cells. Cy3-labeled probes had several advantages: (1) a high level of fluorescence (5–10 times more compared with fluorescein-labeled probes); (2) a low level of fluorescence in solution, allowing the detection of target chromosomes in situ during hybridization without the washing of slides; and (3) high resistance to photobleaching during prolonged (1–2 h) exposure to strong light, thus allowing the use of a high energy mercury lamp or a long integration time during image acquisition in digital imaging microscopy for the determination of weak signals. For di- and multicolor fluorescence in situ hybridization (FISH), we successfully used different combinations of directly fluorophorated probes with preservation of images by conventional microscopy or by digital imaging microscopy. FluorX and Cy3 dyes allowed the use of cosmid probes for mapping in a one-step hybridization experiment. Cyanine-labeled fluorophorated DNA probes offer additional possibilities for rapid chromosome detection during a simple 15-min FISH procedure, and can be recommended for basic research and clinical studies, utilizing FISH.

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Introduction

Detection of nucleic acids by fluorescence in situ hybridization (FISH) is now a valuable approach in basic and applied human genetics, including studies of chromosome structure and function, genome mapping, and clinical cytogenetics (for a review, see Lichter and Cremer 1992). Numerous improvements in this technique have been made over the last few years, allowing multiple probe visualization and analysis. The introduction of fluorophore-labeled nucleotides permits the direct production of fluorescent DNA probes, and, in combination with alternative probe labeling and digital imaging microscopy, allows multicolor high resolution detection of target DNA sequences in metaphase and interphase chromosomes, including decondensed chromatin or DNA fibers (Wiegant et al. 1991, 1992; Ried et al. 1992 a, b; Heng et al. 1992; Parra and Windle 1993; Haaf and Ward 1994). Directly flurophorated DNA probes offer several additional advantages for FISH studies because of (1) the elimination of secondary detection steps and the visualization of hybridization results in a "one-step" procedure; (2) very low background fluorescence; and (3) increased potential for multicolor and quantitative probe detection (Ballard and Ward 1993). Several nucleotide triphosphates labeled with different fluorophores, including fluorescein, rhodamine, and aminomethyl coumarin acetate, are available for the preparation of directly fluorophorated DNA probes. However, fluorophore-labeled probes have commonly only 10%–15% of signal strength compared with biotin- or digoxigenin-labeled probes; they are also prone to photobleaching during exposure to strong light and are quenched by many counterstains. Therefore, for many applications, biotin- and digoxigenin-labeled probes may be preferable (Ried et al. 1992 a, b; Ballard and Ward 1993).

Cyanine dyes are also useful as fluorescent labels for biological macromolecules (Mujumdar et al. 1993). Cyanine 3 dye provides significantly brighter fluorescence than any other fluorophore, including fluorescein, tetramethylrhodamine, and Texas Red (Wessendorf and Brelje 1992). Cyanine-dye-labeled nucleotide triphosphates, such as Cy3-dCTP and Cy5-dCTP (Biological Detection Systems, Pittsburg, Pa.) are available for non-radioactive labeling of DNA probes. These new fluorophores have not been extensively used in molecular cytogenetic studies. Therefore, the potentials of these fluorophores have not been fully realized. In this report, we have tested a number of alphoid and site-specific DNA probes, directly labeled by fluoresceinated nucleotides (Cy3-dCTP, Cy5-dCTP, FluorX-dCTP) for uni- and multicolor FISH.

Materials and methods

Cell material

Metaphase spread and interphase nuclei were prepared from phytohemagglutinin-stimulated blood lymphocytes obtained from karyotypically normal individuals, patients with trisomy 21, and cultured lymphoblastoid and Hela cell lines by using standard techniques of colcemid treatment, hypotonic treatment, and methanol/acetic acid fixation. Cell suspensions and preparations were stored in 80% methanol at –20° C and were re-fixed before use. Uncultured amniocyte cells were fixed as described by Soloviev et al. (1995). Slides with fixed sperm nuclei were supplied by Prof. M. R. Guichaoua (Hopital de Conception, Laboratoire de Biologie de la Reproduction, Marseille, France), Frozen samples of colorectal tumors were provided by Dr. M. Muleris (Institute Curie, Section de Biologie, Paris, France) and processed by standard techniques, utilizing collagenase treatment and methanol/acetic acid fixation. Samples of buccal smears were collected from a scraping of the inside of the cheek and fixed immediately with methanol/acetic fixative.

DNA probes

A battery of original chromosome enumeration probes, developed at Cytogenetics Laboratory, National Research Center of Mental Health, Moscow, Russia, and including alphoid DNA probes specific to chromosomes 1, 2, 3, 4, 5, and 19; 6, 7, 8, 10, 11, 12, 13, and 21; 14 and 22; 15, 16, 17, 18, 20, 22, X, and Y, was used. Alphoid plasmid probes were as described earlier (Yurov 1984; Alexandrov et al. 1986 a, b, 1988, 1991, 1993 a, b; Varsanova et al. 1986, 1990, 1994; Mashkova et al. 1994; Plattner et al. 1993; Yurov et al. 1986, 1987, 1988, 1991). The alphoid probes specific to chromosomes 2 (cos alpha2, No21–21), 11 (cos alpha11, No22–35), 13 and 21 (cos alpha21–13, No21A-1), 14 and 22 (cos alpha14–22, No22–4), 15 (cos alpha15, No22–12), 16 (cos alpha16, No22–19), 17 (cos alpha17, No22–55), 20 (cos alpha20, No22–35), X (cos alphaX, No22–42), Y (cos alphaY, No22-B12), and ribosomal DNA probes specific to nucleolus organizer regions (NORs) of all acrocentric chromosomes (cos R, No13–r34) are human alphoid genomic fragments with sizes ranging from 15 to 35 kb, cloned in Lowrist cosmid vectors; these will be described elsewhere. The cosmid probe for the detection of NORs was isolated by cross-hybridization with human ribosomal RNA in a chromosome-13-specific cosmid library, supplied by Dr. N. Jankowski (Moscow). This probe is highly specific to NORs of all human acrocentric chromosomes. Cosmid probes site-specific for chromosome 21 were as described by Soloviev et al. (1995). Cot 1 DNA (Gibco BRL) was used as a probe for pericentromeric heterochromatic regions of all human chromosomes; it gave more intense hybridization signals in chromosomal regions 1q1, 9q1, 15p1, 16q11–13, and Yq12.

In situ hybridization

The DNA probes were labeled using a standard nick translation procedure with Cy3-dCTP, Cy5-dCTP or FluorX-dCTP, produced by Biological Detection Systems. Usually, 1 μg DNA for each probe was labeled in 20 μl nick-translation mixture, containing (1) 20 μ*M* each dATP, dGTP, dTTP and 25 μ*M* Cy3-dCTP for Cy3-labeled probes; (2) 20 μ*M* dATP, 20 μ*M* GTP, 25 μ*M* bio-11-dUTP (Sigma) and 25 μ*M* Cy3-dCTP for both biotin- and Cy3-labeled probes; (3) 20 μ*M* each dATP, dGTP, dTTP, dCTP and 100 μ*M* Cy5-dCTP for Cy5-labeled probes; (4) 20 μ*M* each dATP, dGTP, dTTP, dCCT and 50 μ*M* FluorX-dCTP for fluorescein-labeled probes. Labeled DNA samples were ethanol precipitated in the presence of a 20-fold excess of sonicated salmon sperm DNA as the carrier. Precipitates were dissolved in 200 μl hybridization mixture of 50% formamide (Fisher) 2 × SSC (0.3 *M* NaCl, 0.03 *M* trisodium citrate), 10% dextran sulfate (Pharmacia), at a concentration of approximately 5 ng/μl (stock solution of probes ready for use) and stored at –20° C for at least 1 year, viz., the duration of these experiments.

In situ hybridization and probe detection

In situ hybridization was performed as described in detail previously for isotopic in situ hybridization (Yurov 1984; Yurov et al. 1987). Slides with fixed cells (blood lymphocytes and amniocytes) were treated with 0.07 N NaOH, 2 × SSC for 30 s for chromosomal DNA denaturation, dehydrated in 70%, 96%, 100% ethanol solutions for 2 min each, and air-dried. Slides with interphase tumor cells, spermatozoa nuclei, or buccal epithelium cells were treated in 2 N NaOH, 2 × SSC for 2–3 min without application of pronase or pepsin treatment, dehydrated in ethanol, and air-dried.

DNA probes at a concentration of 1–2 ng/μl in hybridization solution were denatured at 70° C for 5 min, placed in ice, and then applied (7.5–10 μl probes for a 22 × 22 mm coverslip) to each slide. For dural- and three-color hybridization, DNA probes were mixed in equal proportions (5 μl each probe at a concentration of 5 ng/μl for each probe). Hybridization was usually performed at 42° C overnight, although clear hybridization signals were seen after 30–60 min of hybridization. The slides were washed in 50% formamide, 2 × SSC at 42–45° C, three times for 2 min, and rinsed in 0.1–2 × SSC for 5 min.

Detection of biotin-labeled probes was performed as previously described (Pinkel et al. 1986) by the use of a layer of fluorescein-avidin (Sigma) or, alternatively, Cy3-avidin (Biological Detection Systems), and one or two steps of amplification with biotinylated goat anti-avidin (Vector Laboratories), if necessary. Slides were mounted in antifade solution (0.2% p-phenylenediamine, Sigma; in 80% glycerol, 20 m*M* TRIS-HCl, pH 8.0), and 200 ng/ml DAPI (4′, 6-diamidino-2-phenylindole-2HCl) as counterstaining for the Cy3 dye or 400 ng/ml propidium iodide plus DAPI for fluorescein.

Control of hybridization efficacy and specificity

In experiments when Cy3-labeled probes were hybridized separately or mixed with Cy5-, FluorX- or biotin-labeled probes, the step of microscopic control for hybridization efficiency was performed without the detaching of coverslips and washing of slides. Cy3-labeled probes usually have a low level of fluorescence in solution, and hybridization signals are clearly seen, through the cover-glass and hybridization mixture, by means of a 40 × objective. If hybridization efficiency was not sufficient by visual inspection, it was possible to continue hybridization for a longer time or to rehybridize the slides with a new sample of DNA probe after repeated denaturation of chromosomal DNA (in 70% formamide, 2 × SSC at 70° C for 2 min followed by fixation of slides in 70%, 96%, 100% ethanol solutions for 2 min).

Directly fluorophorated probes could be analyzed immediately before (through the coverslip) or after the post-hybridization washing procedure, if necessary. Post-hybridization washing was performed to remove minor or non-specific hybridization signals in 50% formamide, 2 × SSC at 42–45° C for 15–30 min, with microscopic analysis of the results and repetitive washing steps.

Rapid FISH protocol with Cy3-labeled alphoid DNA probes

Cy-3 fluorophorated alphoid DNA probe (7.5–10 µl probe at a concentration of 1–5 ng/µl in a hybridization mixture of 50% formamide, 2 × SSC, 10% dextran sulfate) was placed directly on the slide with fixed cells, covered by a coverslip (22 × 22 mm), and denatured together with target chromosomal DNA at 72° C for 5–10 min. The hybridization results could be seen after 1–2 min of cooling the slides at room temperature and directly through the coverslips by using a conventional fluorescent microscope, equipped with an immersion-oil objective (40 ×) and the appropriate Cy3-dye filter set. The hybridization could be continued at room temperature for 5–10 min., if necessary, before post-hybridization washing procedures. Moreover, the slides could be stored in a dark box for up to 24 h at room temperature (for more intense hybridization signals) or for several weeks in a refrigerator before washing, counterstaining with DAPI in antifading solution and microscopic analysis.

In order to eliminate non-specific cross-hybridization of alphoid DNA probes to the centromeric regions of many chromosomes, the slides were washed at 72° C in 0.1–0.2 × SSC for 2–5 s (up to 5 min, if it was necessary to reduce background or non-specific hybridization signals). Alternatively, the slides could be washed in 50% formamide, 2 × SSC at 42–45° C according to standard washing procedures.

Microscopy

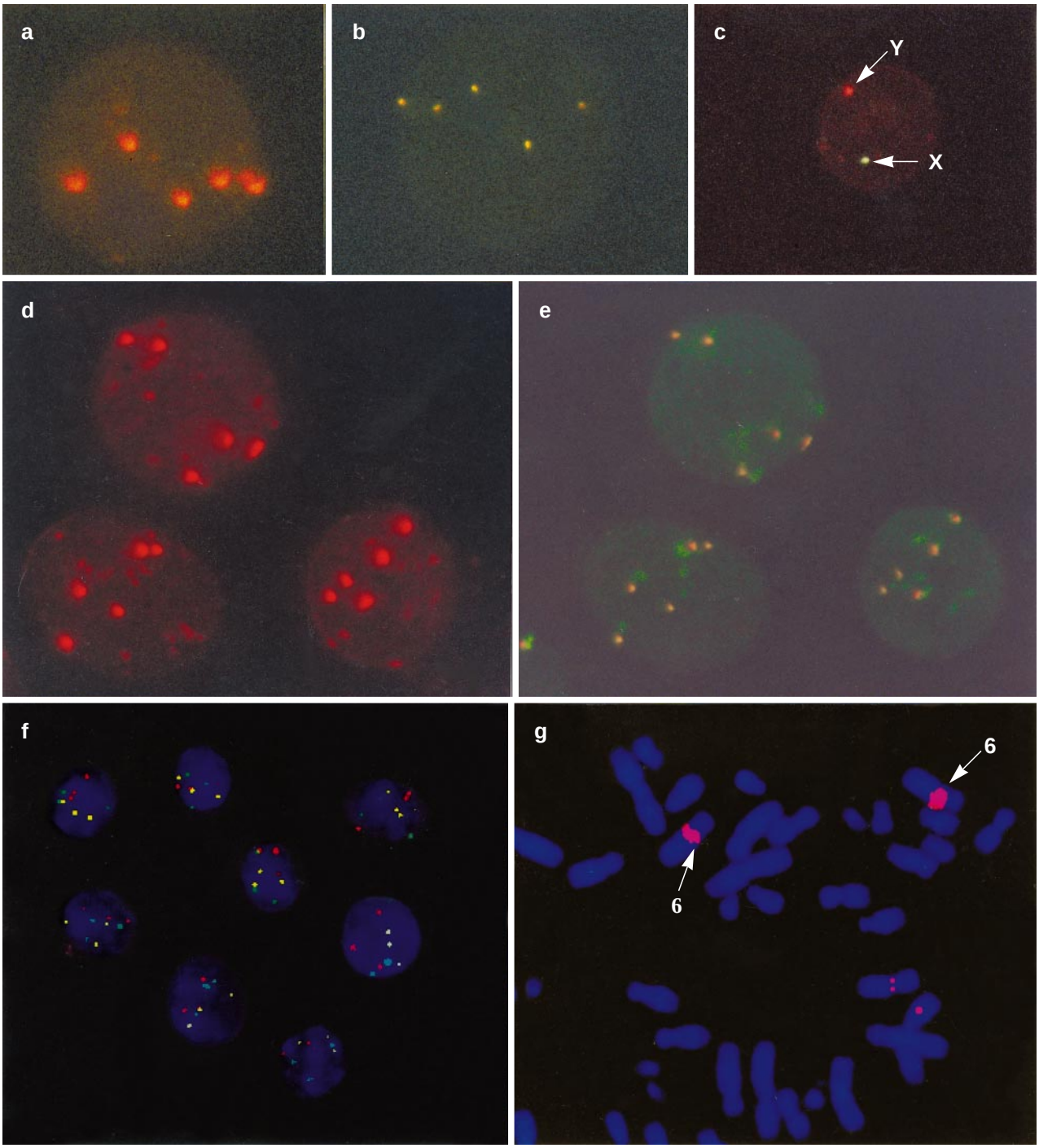
For epifluorescence microscopy, a Leitz Orthoplan photomicroscope equipped with a 100 W lamp was used with the following filter sets (Leica Mikroskopie und Systeme): A (No. 513596) for DAPI fluorescence; I3 (No. 513719) or GR (No. 513821) for propidium iodide fluorescence and fluorescein isothiocyanate (FITC) signals; GR (No. 513821) for both fluorescein and cyanine signals; N2 (No. 513609) for cyanine signals. All images were observed with the Plan-Neofluotar 40 ×/1.30 or 63 ×/1.30–0.60 oil immersion lens. Microphotographs were taken with Agfachrome 1000 RS or Fujichrome p1600 D color-slide films. Multicolor digital images were recorded using a cooled charge couple device (CCD) camera (Photometrics, Tuscon, Ariz., USA) and the software package "BDS Image" (now as ONIPIPE-P, "Oncor" Imaging Systems) at the laboratory of Biological Detection Systems.

Results

In order to perform "one-step" uni-color FISH, we used a battery of alphoid probes specific to all human chromosomes (see above), labeled by Cy3-dCTP or FluorX-dCTP (Fig. 1 a, c, d, g). Analysis of metaphase chromosomes and interphase nuclei, in cytological preparations of lymphocytes, amniocytes, tumor cells, spermatozoa, and buccal epithelium, showed that Cy3- and FluorX-fluorophorated alphoid DNA accurately identified target human chromosomes by "standard" and rapid in situ hybridization protocols (Table 1). The optimal time of hybridization was approximately 5–10 min for Cy3-labeled and 30–60 min for FluorX-labeled DNA probes, used at a concentration of 1–5 µg/µl. Low copy number alpha satellites from chromosomes 1, 2, 20, 22, and Y needed a prolonged hybridization of 0.5–1 h or the use of a more concentrated probe (10–20 µg/µl).

In additional experiments, we used DNA probes labeled in equimolar proportion with both Cy3-dCTP and biotin-11-dUTP, followed by the application of FITC-avidin (Fig. 1 b) or Cy3-avidin (Fig. 1 e) for the detection of biotin labels. The sensitivity of FISH with Cy3-labeled probes was significantly higher in comparison with Cy5, FluorX or biotin labels. The same interphase nuclei with trisomy 21, hybridized with a cosmid probe specific to chromosomes 13 and 21, could be labeled by both Cy3-dCTP (orange signals with the filter set N2 for rhodamine; Fig. 1 a) and biotin-11-dUTP (yellowish-green signals of FITC-avidin with filter set I3 for fluorescein; Fig. 1 b). The intensity of Cy-3 fluorescence was 5–10 times higher than that for fluorescein, as registered at similar exposure times (15 s for each) during microphotography. The Cy3-signals were extremely stable during prolonged microscopic analysis of up to 1–2 h. Whereas the FITC or FluorX signals were quenched after only 2–3 min of exposure to strong light. Taking into account the high stability of Cy3 dye against photobleaching, the documentation of Cy-3-labeled probes by microphotography or image acquisition using a CCD camera can be performed over a

Fig. 1 a–g Fluorescence in situ hybridization with Cy3-, FluorX-, and Cy5-labeled DNA probes. **a** Detection of chromosomes 13 and 21 in interphase nuclei of a patient with trisomy 21, by both Cy3- and biotin-labeled alphoid DNA probe cos-gen-s-13, 21 after 4 min hybridization; five *bright red-orange* Cy3-dye signals, detected by a filter set for rhodamine. **b** The same nucleus as in **a**, but after application of one layer of FITC-avidin for the detection of the biotin label, using a filter set for fluorescein; five *green-yellow* signals with intensities approximately 5–10 times less than for Cy3 dye. **c** Dual-color detection of chromosome Y (Cy3-labeled chromosome-Y-specific DNA probes gen-s-Y-alpha – in *red*) and chromosome X (FluorX-labeled chromosome-X-specific probe pYAM10–40 in *green*) in an interphase nucleus of an individual with karyotype 46,XY; hybridization for 30 min. **d** Three interphase nuclei of a patient with trisomy 21 (Down syndrome) showing five *red* signals (filter set for rhodamine only) after 5 min hybridization with chromosome-13-specific and chromosome-21-specific alphoid DNA probes (cos-alpha-13-12), labeled with Cy3-dCTP. Several minor hybridization sites are seen in addition to five intense signals, as this micrograph was made through the coverslip before post-hybridization washing of the slide. The Cy3-labeled probe co-hybridizes with FluorX-labeled ribosomal DNA probe cos13-r34, which is not seen by this filter set. **e** The same three nuclei as in **d**, but after washing in 0.2 × SSC at 70° C for 30 s and analysis by a filter set for both rhodamine and fluorescein. The hybridization signals in chromosomes 13 and 21 are composed of two regions, colored *red* by the Cy3 dye (label for alphoid DNA) and *green* for FluorX (label for iboisomal DNA); this gives *yellow* in overlapping areas of pericentromeric regions in interphase chromosomes 13 and 21. NORs of acrocentric chromosomes 14, 15, and 22 are *green* only with the FluorX-labeled ribosomal DNA probe. **f** Multicolor detection of chromosomes 3 *(purple)*, 8 *(white)* and X *(yellow)* by alphoid DNA probes labeled by Cy3, Cy5, and FluorX dyes, respectively, after overnight hybridization with Hela interphase nuclei (*blue* DAPI fluroescence), pseudocolored by using digital imaging microscopy. **g** Detection of chromosome 6 by Cy3-labeled alphoid DNA probe alphaRI-12 in a normal metaphase plate (overnight hybridization), by using digital imaging microscopy



long time. Therefore, this fluorophore may by especially useful for the documentation of very weak signals or the quantitative determination of hybridization intensity by confocal laser microscopy.

In experiments with dual-color FISH, we tested different combinations of probes, specific to chromosomes 13 and 21 (probes cos alpha21, 13, No21-A1), 18 (pBRHS13 or pYAM9–60), X (pYAM10–40 or cos alphaX, No22–42) and Y (MGY1 or cos alpha Y, No22-B12), labeled by Cy3-dCTP or FluorX-dCTP. An example of dual-color FISH for the simultaneous detection of sex chromosomes X (in green) and Y (in orange) in shown in Fig. 1 c. The same group of interphase nuclei with trisomy 21 co-hybridized with the chromosome-1-specific alphoid probe (Cy3 dye) and probe cos13-r34, specific to NORs of all acrocentric chromosomes (FluorX dye) (Fig. 1 d, e). In addition, tri-color hybridization signals could be seen corresponding to many NORs of all acrocentrics (in green, FluorX only), several alphoid DNA regions in chromosomes 13 and 21 (in red, Cy3 only), and five yel-

Table 1 Chromosomal localization of DNA probes labeled with Cy3 and FluorX dyes

Chromosomes		Probe name	Optimal hybridization time	
Major site[a]	Minor sites[b]		Cy3	FluorX
1 cen	5, 7, 19	p2hs-5	10–30 min	4 h
1 cen	3, 6, 7, 10	alphaRI-12		
2 cen	18, 20	gen-s-2	15 min	4 h
3 cen	1, 5, 6, 7,	pHSO5	2 min	30 min
4 cen	9, 18, 20	pYAM 11–39	10 min	1 h
5 cen/19cen	1, 3, 7, 16	alphaRI-14	15 min	4 h
6 cen	1, 7, 19	alphRI-12	10 min	2 h
7 cen	1, 5, 19	alphaRI-5	10 min	2 h
8 cen	2, 9, 18, 20	gen-s -8	15 min	4 h
10 cen	1, 3, 5, 16, 19	pYAM 11–19 pBR 1365	2–3 min	30 min
11 cen	1, 17, X	pHS5 3	2 min	30 min
13 cen/ 21 cen	2, 9, 14, 15 20,22	alphaR6 cosmid-gen-s-13, 21	4–5 min	45–60 min
14 cen/ 22 cen	2, 8, 9, 13, 15 17, 18, 20, 22	gen-s-14, 22	2–3 min	30 min
15 cen	Acrocentrics	gen-s-15 class	15–20 min	5–6 h
17 cen	1, 11, X	pYAM 7-29	2 min	30 min
18 cen	2, 4, 8, 9, 20	pYAM 3–86, 4–22 pYAM9–60 pBRHS 13	3–5 min	45–60 min
20 cen	2, 4, 9, 18, 21, 22	gen-s-20	15–20 min	5–6 h
Y cen	All centromeres	gen-s-Y-alpha	30–60 min	8–10 h
X cen	1, 11, 17	pYAM 10–40	2–3 min	30 min
Centromeres of all chromosomes[c]		gen-s-Y-alpha	10 min	4 h
1qh, 9qh, 15p, 16gh, Yq	All other chromosomes (centromeres)	Cot1 DNA (Gibco, BRL)	2 min	10–30 min
NORs	All acrocentric chromosomes	cos13-r34	30–60 min	8–10 h

[a] These chromosomal regions are clearly seen at high (50% formamide, 2 × SSC at 42–43° C or 0.2 × SSC, 70° C) and medium (50% formamide, 2 × SSC at 37–39° C or 0.2 × SSC, 50–60° C) stringency conditions of post-hybridization washing of slides

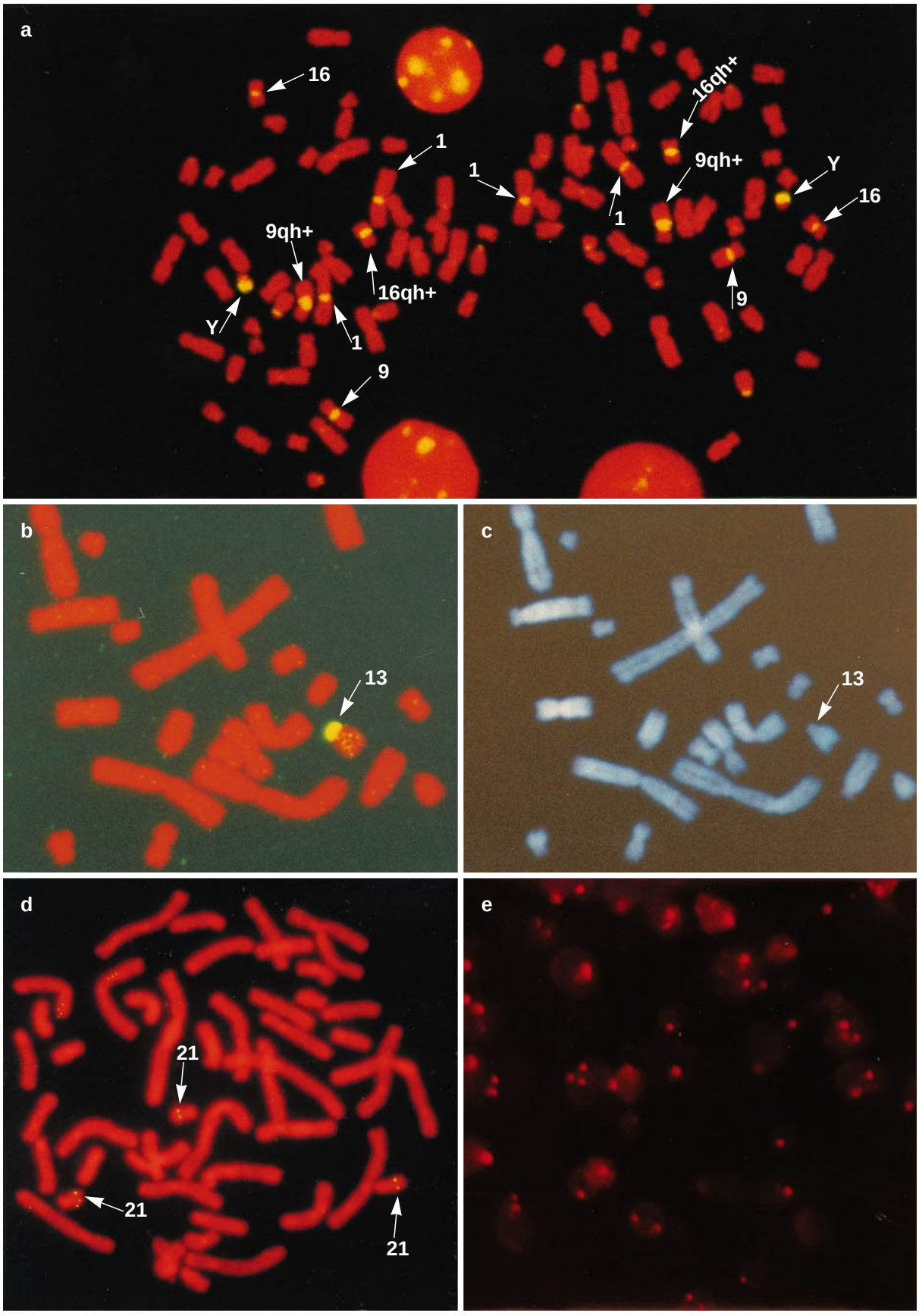
[b] These chromosomal regions are clearly seen at medium stringency conditions of posthybridization washing of slides, but are not detectable under high stringency

[c] Hybridization and washing of slides at room temperature

low signals in chromosomes 13 and 21 (green plus red), composed of closely situated and overlapping areas of hybridization signals for alphoid and ribosomal DNAs (Fig. 1 e). These results underline the potentials of Cy3 and FluorX labels for the production of tri- or multicolor FISH.

Application of digital imaging with a CCD camera can be extremely helpful for the analysis of both interphase and metaphase chromosomes hybridized with Cy3, Cy5 and FluorX dyes (Fig. 1 f, g). Nevertheless, clear accurate signals can be seen using a conventional fluorescence mi-

Fig. 2 a–e Examples of FISH with Cy3- and FluorX-labeled DNA probes. **a** Human Cot1 DNA labeled by FluorX-dCTP and hybridized to human metaphase chromosomes for 10 min at room temperature and washed in 0.2 × SSC at 70° C for 1 min. The chromosomes were counterstained by propidiun iodide *(red)*. The regions corresponding to highly repetitive "classical" satellite DNA in chromosomes 1, 9, 16, all acrocentric chromosomes, and Y are more intensely labeled. The homologs of chromosomes 9 and 16 are heteromorphic regarding the amount of repetitive pericentromeric DNA (chromosomes 9qh+ and 16qh+ contain more Fluor X-labeled hybridized probe). **b** Ribosomal DNA probes (cos13-r34), labeled by FluorX-dCTP and hybridized for 30 min to metaphase chromosomes of a somatic cell hybrid line (Chinese hamster x human), containing only chromosome 13 as the human material (counterstained by propidium iodide). Chromosome 13 contains an intense hybridization signal at the p-arm, and minor signals in the q-arm because of cross-hybridization of interspersed human-specific Alu and L1 sequences present in this cosmid clone. **c** The same metaphase chromosomes as in **b**, counterstained by DAPI, producing Q-banding for chromosome identification. **d** Chromosome-21-specific cosmid probe cos-21-02, labeled by FluorX-dCTP and hybridized for 2 h to metaphase chromosomes of a patient with trisomy 21. Each chromatid of the three chromosomes 21 contains a clear hybridization signal at region 21q22. **e** Sperm nuclei of a patient with unmatured ("big" cells) and matured ("small" cells) spermatozoa, hybridized for 2 min at 42° C with chromosome-18-specific alphoid probe pBRHS13, labeled by Cy3-dCTP, visualized and photographed through the coverslip without washing of the slide. The sample of ejaculate was washed in phosphate-buffered saline, resuspended and washed in fixative (methanol : acetic acid, 3 : 1) three times for 15 min, placed on slides and air-dried. Before hybridization, the sperm preparations were treated for 4–8 min in 2 *M* NaOH, 2 × SSC at room temperature, passed through an ethanol series and air-dried. The DNA probe (40 ng in 10 µl hybridization mixture of 50% formamide, 2 × SSC, 10% dextran sulfate) was applied to the denaturated sperm nuclei and incubated for 10 min at 70° C for probe denaturation. Hybridization was carried out at 42° C for 2 min. After microscopic analysis and photographic documentation of the signals, the slides were rinsed in 0.2 × SSC, 70° C for 5 s, mounted in antifade solution with 200 ng/ml DAPI and stored at 4° C ▶


a
16
1
1
16qh+
9qh+
Y
16
9qh+
16qh+
Y
1
9
1
9
b
13
c
13
d
21
21
21
e

croscope with repetitive or site-specific DNA probes (Fig. 2). As an example, Fig. 2 a shows the hybridization of fluorescein-labeled Cot1 human DNA, enriched by highly repetitive sequences that mark pericentromeric regions of all human chromosomes with intense signals in regions of variable C-heterochromatin of chromosomes 1, 9, 15, 16, and Y, corresponding to highly repetitive "classical" satellite DNA sequences. Moderately repeated sequences, such as ribosomal DNA, can be detected in a one-step FISH protocol, using FluorX or Cy3 dyes (Fig. 2 b, c). Hybridization of the site-specific cosmid probe (chromosome 21q22), labeled with FluorX, to metaphase chromosomes of a patient with Down syndrome leads to the detection of trisomy 21 by the one-step FISH experiment (Fig. 2 d).

For multicolor FISH, we have used chromosome-specific alphoid probes, labeled by different fluorophores. As an example, Fig. 1 f shows multicolor FISH in interphase nuclei after hybridization with three different alphoid probes, labeled by Cy3-dCTP for chromosome 3, Cy5-dCTP for chromosome 8, and FluorX-dCTP for chromosome X. The signals are pseudocolored in purple for chromosome 3, in white for chromosome 8, in yellow for chromosome X, and in blue for interphase nuclei, by using digital imaging microscopy.

Discussion

The results of this study indicate that novel, recently available, fluorophorated nucleotides (Cy3, Cy5, and FluorX-dCTP) may be applied as additional tools in human molecular cytogenetics. We have tested a number of chromosome-specific alpha-satellite DNA probes that are commonly used in many basic and clinical applications, involving high resolution uni- and multicolor FISH. All of these direct fluorophores can be used for the accurate identification and enumeration of chromosomes in normal and aberrant human cells.

We routinely use these probes, the chromosome-21-specific cosmid, and yeast artificial chromosome (YAC) probes for the detection of the most frequent aneuploidies in newborns, involving autosomes 13, 18, 21, and both sex chromosomes, in pre- and postnatal clinical studies (Soloviev et al. 1995; Yurov et al. 1995 a, b; Vorsanova et al. 1995). Using standard and rapid FISH protocols, we can obtain satisfactory results with conventional microscopy without the application of digital imaging devices, such as the CCD camera, and appropriate computer and software, which are ultimately necessary for multicolor FISH with the simultaneous visualization of several targets (Ried et al. 1992 a, b; Divane et al. 1994). Directly labeled chromosome-enumeration probes allow us to perform one-color FISH and to rehybridize the slides several times with different probes, if necessary; it also provides an alternative approach to multicolor FISH protocols. Therefore, directly labeled probes and the rapid FISH protocol can be recommended for clinical cytogenetic laboratories lacking digital imaging systems.

The above battery of alphoid probes has been applied to aneuploidy detection in uncultured tumor cells, prepared from frozen samples of colorectal cancer, spermatozoa, and buccal smears, characterized by condensed chromatin, by using thermal or strong alkaline denaturation procedures without special pretreatment for the disruption of the nuclear envelope cytoplasm and chromatin decondensation. Directly labeled chromosome-enumerated alphoid probes produce clearly detectable signals, whereas biotin-labeled probes fail to be detected under the same conditions (e.g., Fig. 1 c). We propose that FISH protocols with the application of the secondary detection systems (FITC-avidin and biotin-anti-avidin) are not effective because of the low accessibility of targets to macromolecular reagents. Directly labeled DNA probes, cut to 200–1000 bp by DNAaseI during the nick-translation reaction, are characterized by increased penetration to the targets through the membranes and cytoplasm of the cell.

It should be noted that each fluorophore tested can be used for specific applications. FluorX-labeled probes usually have a decreased sensitivity in comparison with Cy3, biotin, or hapten-labeled alphoid probes, used in control experiments. However, the strength and brightness of the hybridization signals of FluorX-labeled alphoid probes are adequate for the detection of target chromosomes in interphase or metaphase cells, even by conventional microscopy, as we have demonstrated using repetitive alphoid or ribosomal DNA probes. Moreover, we have successfully used FluorX-labeled cosmid probes, ranging from 20 to 40 kb in length, for interphase cytogenetic detection of chromosome 21 in uncultured and cultured human amniocytes (Soloviev et al. 1995).

The Cy5 dye fluoresces in the far-red region of the spectrum; therefore, this fluorophore is invisible by eye in conventional microscopy. However, Cy5 dye is an ideal additional dye for multicolor FISH using imaging equipment. Cy3 dye has fluorescent characteristics similar to tetramethylrhodamine and can be used with many existing rhodamine or Texas Red filter sets for producing orange-red signal. FluorX- and Cy3-labeled alphoid probes are optimal tools to produce dual- or tri-color FISH by conventional microscopy, as we have demonstrated in the case of the simultaneous visualization of chromosomes X and Y, or the detection of several different chromosomes in interphase nuclei by means of digital imaging (Fig. 1).

High resolution FISH detection of nucleic acids in artificially extended chromatin (Heng et al. 1992), DNA halo preparations (Wiegant et al. 1992) or in directly stretched DNA fibers (Parra and Windle 1993) have been used for genome mapping studies and for the structural analysis of alphoid sequences. All of these studies have utilized biotin- or digoxogenin-labeled probes with secondary detection reagents and digital imaging systems. We have shown that these studies may be performed by directly fluorophorated probes with high resolution using decondensed chromatin and stretched DNA fibers, even by conventional microscopy (Marçais et al., in preparation). DNA fiber "fluorography" may be applied for rapid size measurements of cosmid or YAC clones by in situ hybridiza-

tion (Y. B. Yurov, unpublished) as an alternative approach to pulse field electrophoresis and to the arrangement of cosmid clones during chromosome mapping in addition to the DNA halo technique (Wiegant et al. 1992).

Alternative molecular-cytogenetic techniques, called PRINS (primed in situ labeling) and cycling PRINS, have been developed for rapid chromosome detection (Koch et al. 1989; Gosden and Hanratty 1993). PRINS is considerably faster than classical FISH, and the whole process with one pair of primers with denaturation, annealing, extension, and detection can be completed within 2 h. Gosden and Lawson (1995) have introduced a new protocol, called instant PRINS, in which the entire procedure can be completed in 15–20 min or even less than 5 min for highly repeated sequences. Is it possible to perform FISH faster and more simply than PRINS?

Recently, a new FISH procedure has been described that allows the in situ detection of nucleic acids in a time compatible with the PRINS technique (Haar et al. 1994). The FISH protocol, developed by C. Cremer's group is based on the principle of the avoidance of denaturing chemical agents, such as formamide, and the application of fluorescein-11-dUTP labeled probes. It has been demonstrated that the hybridization time for highly repeated "classical" satellite DNA probes can be limited to 15–30 min. The intensity of hybridization with directly fluoresceinated probes is, in general, lower than that for digoxigenen-labeled probes, when using the same technique, and needs computer-aimed quantification by digital image analysis. The potential of this method for middle and low repetitive probes, such as chromosome-specific alphoid DNA, and the possibility of using the common hybridization mixture with formamide have not been determined.

An alternative rapid FISH, based on micro-wave activation of hybridization and immunochemical detection steps in the standard FISH protocol, has recently been described (Soloviev et al. 1994). We have positively tested this method using our panel of alphoid DNA probes, specific to different human chromosomes. All steps of this method are identical to the classical FISH protocol with biotin- or digoxigenenin-labeled probes and following application of secondary detection reagents as FITC-avidin. Micro-wave activation dramatically reduces the hybridization time to 5 min, and the immunochemical detection to 7 s. The whole FISH procedure using chromosome-enumeration alphoid DNA probes needs, therefore, less than 15 min, with no significant loss in the intensity of signals in comparison with overnight hybridization (Soloviev et al. 1994).

In our present work, we have developed a new rapid and highly sensitive FISH protocol, utilizing directly Cy3-fluorophorated alphoid and site-specific DNA probes. We have tested different types of cytological preparations, including metaphase and interphase lymphocytes, uncultured and cultured amniocytes, frozen cancer cells, spermatozoa, and buccal epithelium smears, with positive results. The use of Cy3-labeled probes allows us (1) to reduce the hybridization time to 2–10 min at room temperature because of the extremely bright fluorescence of the Cy3 dye (the hybridization can be continued for up to 24 h and terminated at any time, if necessary); (2) to visualize the process of hybridization under a coverslip during the renaturation of the probe and target DNA in situ because of the low fluorescence of Cy3 dye in solution, and, therefore, to control both the efficiency of hybridization before the termination of the experiment and the efficiency of the washing of slides by microscopic inspections; (3) to rehybridize the slide several times with other probes, if necessary: (4) to perform several steps of immunochemical detection and amplification procedures when probes labeled both by Cy3-dCTP and biotin or hapten labels are used; (5) to store the hybridized slides for up to 1 year at 4° C in antifading solution containing 80% glycerol and reanalyze (or rehybridize) the slides, if necessary. Cy3-hybridization signals can be analyzed under the microscope for up to 2 h, because of the marked photoresistance of this dye to strong light. The rapid FISH protocol with Cy3-labeled probes can be recommended for routine cytogenetic studies, such as chromosome identification in clinical and oncogenetics, especially in prenatal diagnosis.

In conclusion, Cy3 dye has a brighter and the more photostable fluorescence over the other fluorophores tested at the present time. This fluorophore can be utilized in basic and clinical cytogenetic studies, both of which require high resolution and multicolor FISH.

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