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Simultaneous visualization of seven different DNA probes by *in situ* hybridization using combinatorial fluorescence and digital imaging microscopy

(PCR-probe labeling/CCD camera/gene mapping/centromere repeats/cytogenetics)

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ABSTRACT **Combinatorial labeling of probes (i.e., with two or more different reporters) increases the number of target sequences that can be detected simultaneously by fluorescence *in situ* hybridization. We have used an epifluorescence microscope equipped with a digital imaging camera and computer software for pseudocoloring and merging images to distinguish up to seven different probes using only three fluorochromes. Chromosome-specific centromere repeat clones and chromosome-specific "composite" probe sets were generated by PCR in which different mixtures of modified nucleotides, including fluorescein-conjugated dUTP, were incorporated. Cosmid clones were labeled similarly by nick-translation. The technique has been used to delineate the centromeres of seven different human chromosomes, on both 4′,6-diamidino-2-phenylindole-stained metaphase spreads and interphase nuclei, to map six cosmid clones in a single hybridization experiment and to detect chromosome translocations by chromosome painting. Multiparameter hybridization analysis should facilitate molecular cytogenetics, probe-based pathogen diagnosis, and gene mapping studies.**

A variety of methods for labeling and detecting DNA probes by nonradioactive *in situ* hybridization has become available over the past few years (1, 2). This enables one to visualize more than one target sequence in a single hybridization experiment. Haptenated probes [e.g., labeled with biotin (Bio), digoxigenin (dig), or dinitrophenol (DNP)] have been most frequently detected by direct or indirect immunofluorescence, leading to the acronym FISH (fluorescence *in situ* hybridization). Three sets of distinguishable fluorophores, emitting in the green (fluorescein), red (rhodamine or Texas Red), and blue (AMCA or Cascade Blue) have been used for FISH to date. Three separate chromosomal DNA sequences have been delineated simultaneously by combining appropriate fluorophores with three differentially labeled probes (3). Nederlof and collaborators (4) recently extended the number of simultaneously detectable targets to four by double haptenization of one probe molecule by using a combination of chemical and enzymatic labeling procedures.

In principle, combinatorial labeling of probes with two or more different reporters can markedly increase the number of distinguishable targets relative to the number of available fluorophore detectors. For example, with three haptens (e.g., Bio, dig, and DNP) and three fluorophores (e.g., fluorescein, rhodamine, and Cascade Blue), a total of seven probes should be resolvable. Probes 1, 2, and 3 would be visualized as a pure fluorophore, while probes 4–7 would appear as fluorophore mixtures as follows: probe 4, fluorescein + rhodamine; probe 5, fluorescein + Cascade Blue; probe 6, rhodamine + Cascade Blue; probe 7, fluorescein + rhodamine + Cascade Blue. With four labeling and fluorescent detection systems, the number of different targets could be increased to 15, again assuming that only equimolar mixtures of reporters in all pairwise and triple-labeling combinations are used. Using a conventional epifluorescence microscope and photomicroscopy, these theoretical possibilities would be difficult to achieve in practice, mainly because multiple exposures of color film cannot adequately display and resolve images from combinatorially labeled probes. This restriction should be overcome with digital imaging camera systems, such as silicon intensified tube or charge coupled device (CCD) cameras, which allow one to collect separate gray scale images of each fluorophore; these can be subsequently pseudocolored, aligned, and merged with the appropriate computer software. Since multiparameter hybridization methods could prove to be extremely useful for addressing a broad spectrum of clinical and biological problems, we have extended the combinatorial labeling strategy of Nederlof *et al.* (4) by using a cooled CCD camera-based digital imaging microscope. Here we report simple protocols, including a single-step PCR method for combinatorial probe labeling, and document the feasibility of visualizing up to seven probes simultaneously.

MATERIALS AND METHODS

Human metaphase chromosomes were prepared by standard procedures. Prior to *in situ* hybridization, slides were washed in 1× phosphate-buffered saline (5 min; room temperature) and dehydrated through an ethanol series (70%, 90%, and 100%; 5 min each). Slides were stored at −70°C with Drierite powder.

DNA Probes. The following chromosome-specific α satellite DNA clones were used: pBS10.7AE0.6 (A.B., unpublished data), chromosome 3; p7tet (5), chromosome 7; pMR9A (6), chromosome 9; pBR12 (7), chromosome 12. pαH2 (chromosome 18) and pαH5 (chromosome 8) were cloned in our laboratory, while pRB2 (chromosome 11) was a gift of M. Rocchi (Bari, Italy). The chromosome-specific plasmid libraries (8) were a gift of J. Gray (Livermore, CA). The following cosmid and phage clones were used: cpt1, mapping to Xp21 (9); c-myc, mapping to 8q24 (10); c512, mapping to 21q22 (11); cosmid clone 26, mapping to 5q32 (unpublished data); cosSB1, mapping to 6p21 (12); cosmid K40, mapping to 11p15 (13). The cosmid clones specific for chromosome 5 (clones 26, 29, 56, 58, 92, and 121) were provided by Greg Landes (Integrated Genetics, Framingham, MA) and were previously mapped by Jennifer Lu and D.C.W. (unpublished results).

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Abbreviations: Bio, biotin; dig, digoxigenin; DNP, dinitrophenol; FISH, fluorescence *in situ* hybridization; AMCA, 7-amino-4-methylcoumarin-3-acetic acid; CCD, charge coupled device; DAPI, 4′,6-diamidino-2-phenylindole.

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DNA was prepared according to standard techniques (14).

Probe Labeling. PCRs were performed with 10 ng of alphoid DNA clones or 100 ng of chromosome-specific libraries as template. Preferential amplification of insert DNA was achieved by using primers flanking the polylinker of each plasmid vector. T3 and T7 primers were used for the pBS vector, and M13 forward and M13 reverse primers were used for pUC and pCR1000 vectors (all at a final concentration of 1 μM). PCR was carried out in 1.5 mM $MgCl_2$/10 mM Tris·HCl/50 mM KCl/0.001% gelatin/1.25 units of *Taq* polymerase (AmpliTaq; Perkin–Elmer/Cetus) in a total vol of 50 μl (10 μl when fluorescein-12-dUTP was used due to the limited amount of this reagent). The dNTP concentrations used in the PCR-labeling reactions are listed in Table 1. The highest concentration of modified nucleotides used was 75 μM. However, DNP-11-dUTP at a concentration >37.5 μM strongly reduced the amplification efficiency (data not shown). When DNP-11-dUTP was used for combinatorial labeling, the concentrations were the same as for fluorescein-12-dUTP. The modified nucleotides were obtained from Boehringer Mannheim (dig-11-dUTP, fluorescein-12-dUTP), Sigma (Bio-11-dUTP), and Novagen (Madison, WI) (DNP-11-dUTP). The thermocycling was performed with a commercially available machine (Ericomp, San Diego). After an initial denaturation at 95°C for 3 min, 32 cycles of PCR were carried out with denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 4 min (last cycle, 10 min). PCR products from the chromosome libraries were treated with DNase I to obtain an average fragment size of ≈250 base pairs (bp) and were separated from free nucleotides by Sephadex G50 spin column. Cosmid and phage clones were labeled by standard nick-translation reactions. The final concentration of the modified nucleotides and the DNA clones used in these reactions were as follows: Bio, 50 μM (cosSB2, clone 58); dig, 40 μM (K40, clone 121); DNP, 40 μM (c512, clone 92); Bio/dig, 20 μM/30 μM (cptl, clone 56); Bio/DNP, 20 μM/30 μM (c-myc, clone 29); dig/DNP, 20 μM/30 μM (clones 28 and 26).

***In Situ* Hybridization and Detection.** *Centromeric repeats.* After PCR amplification, the probes were used without further purification. The DNA solution was diluted 1:5 in 10 mM Tris·HCl/1 mM EDTA. One microliter of each probe was precipitated with 5 μg of salmon sperm DNA and 5 μg of yeast RNA and resuspended in 10 μl of 60% formamide, 2× standard saline citrate (SSC), and 5% dextran sulfate. Probe DNA was denatured at 75°C for 5 min and immediately applied to the denatured chromosome specimens; a coverslip was added and was sealed with rubber cement. The slides were denatured separately in 70% formamide/2× SSC for 2 min at 80°C and dehydrated in an ethanol series. After overnight incubation at 37°C, the coverslips were removed and the slides were washed at 45°C in 50% formamide/2× SSC three times followed by three washes at 60°C in 0.1× SSC. After a blocking step (in 4× SSC/3% bovine serum albumin for 30 min at 37°C), the biotinylated probes were detected with streptavidin conjugated to the infrared dye Ultralite 680 (Ultra Diagnostic, Seattle; final concentration, 2 μg/ml); the dig-labeled probes were detected with a rhodamine-labeled anti-dig IgG (Boehringer Mannheim). The fluorescein-12-dUTP (Boehringer Mannheim) labeled probes did not require any immunological detection step. 4′,6-Diamidino-2-phenylindole (DAPI) was used as a chromosome counterstain.

Table 1. Labeling of seven centromere probes

	dNTP, μM						
	AE.06	p7tet	pBR12	pMR9A	pRB2	pαH2	pαH5
Bio-11-dUTP	75			37.5	37.5		25
dig-11-dUTP		75		37.5		37.5	25
FITC-11-dUTP			75		37.5	37.5	25
TTP	225	225	225	225	225	225	225
dA, dC, and dGTP	300	300	300	300	300	300	300

FITC, fluorescein isothiocyanate.

Chromosome painting. The amplification products were treated with DNase I to an average size of 150–500 bp. Five microliters of the amplification reaction mixture (50 μl) was precipitated with 5 μg of salmon sperm DNA and 5 μg of yeast RNA, together with 10 μg of total human competitor DNA, and then resuspended in 10 μl of 50% formamide/2× SSC/10% dextran sulfate. The probe was denatured as described above and allowed to preanneal for 1 h at 37°C. Slides were denatured as described for the centromeric repeats. Hybridization took place overnight at 37°C. Slides were washed at 42°C in 50% formamide followed by three washes at 60°C in 0.5× SSC. The biotinylated sequences were detected with streptavidin conjugated to the infrared dye Ultralite 680; the dig-labeled sequences were detected with rhodamine-labeled anti-dig IgG (Boehringer Mannheim). DNP-labeled probes were detected with a monoclonal rat anti-DNP antibody (Novagen) and a secondary goat anti-rat antibody, conjugated to fluorescein isothiocyanate (Sigma). DAPI was used as a DNA counterstain.

Cosmid clones. Eighty nanograms of each cosmid or phage was precipitated with 20 μg of human competitor DNA and 5 μg each of yeast RNA and salmon sperm DNA. The detection of the differently labeled probe DNAs was performed as described above for the chromosome-specific libraries.

Digital Imaging. Images were obtained with a Zeiss Axioskop epifluorescence microscope coupled to a cooled CCD camera (Photometrics, Tucson, AZ; PM512). Camera control and digital image acquisition (8-bit gray scale) used an Apple Macintosh IIx computer. Fluorophores were selectively imaged with filter cubes specially prepared by Zeiss (filter 487910 for fluorescein, filter 487915 for rhodamine, and filter 487901 for DAPI) to minimize image offsets. The infrared filter (excitation 620–658 nm; dichroic, 650 nm; bandpass, 670–680 nm) is not a precision filter. Images taken with the latter filter were therefore slightly shifted. These were digitally realigned with the probe signals as reference.

Each set of three gray scale fluorescence images revealed probe signals that appeared in only one, in two, or in all three of the images (i.e., the seven combinatorial possibilities). Since the probe-positive regions were visually distinct and were relatively few in number, their combinatorial participation was readily identifiable by visual inspection of the image groups. As a step toward uniquely pseudocoloring these data regions on a combinatorial basis, the regions were isolated and segregated into seven separate (but still spatially aligned) gray scale subimages by using interactive graphics software. Data regions were blended (intensity was averaged) in those cases in which probe signals appeared in more than one of the original images.

The visual identification and manual interactive segregation of data regions was necessary due to limitations of currently available graphics software. Software to more fully automate these procedures remains to be developed.

The seven intermediate gray scale images were then separately pseudocolored, a process that converts a gray scale to a tint scale. The pseudocolored images were recombined through a simulated overlay. This is a relatively trivial process since no data overlap exists after the segregation step performed previously. The multicolored composite image was simultaneously merged with a DAPI counterstain image (also pseudocolored) using software developed in our laboratory that combines images by picking maximum signal intensity at each pixel position. This software package, termed Gene Join, can be obtained for a fee by writing to the

Office of Cooperative Research, Yale University, Suite 401, 246 Church Street, New Haven, CT 06510.

Photographs were taken with an Agfa matrix procolor slide printer using Kodak 100 HC color slide film.

RESULTS

Combinatorial Labeling of Probes by PCR. Chromosome-specific centromeric repeats and chromosome-specific DNA libraries are frequently used as probes for FISH because of their utility in revealing chromosome aneuploidy or aberrations in interphase cells and tissues as well as the identification of marker chromosomes unrecognizable by conventional banding methods (15, 16). Since clones containing such sequences generally have relatively small inserts, ranging in size from a few hundred nucleotides to a few kilobase pairs, we first chose to assess vector PCR as a general method for the combinatorial labeling of such clones. Bio, dig, DNP, and fluorescein, all conjugated to dUTP, could be efficiently incorporated during the amplification reaction, alone or in combination, resulting in selective enrichment of labeled chromosome-specific sequences. Several combinations of nucleotide analogs were tested in order to establish the appropriate concentrations necessary to give an approximately equimolar mixture of each reporter in the probe. These nucleotide concentrations are listed in Tables 1 and 2. Alphoid DNA clones specific for chromosomes 3, 7, 8, 9, 11, 12, and 18 and chromosome-specific libraries for chromosomes 1, 2, 4, 8, 14, and X were then labeled combinatorially by vector PCR. Each combination with Bio, dig, and DNP (or fluorescein-dUTP) was singularly tested by *in situ* hybridization and each combination gave comparable signal intensities (data not shown).

Simultaneous Detection of Seven Centromere Repeat Probes. The chromosome-specific alphoid DNA clones and the modified nucleotides used to label them are given in Table 1. The biotinylated probes were detected with an infrared fluorophore emitting at 680 nm (Ultralite 680) conjugated to streptavidin. The dig-labeled probes were detected with anti-dig antibodies coupled to rhodamine (630-nm emission), while the probes labeled with fluorescein-11-dUTP (580-nm emission) were detected directly. A separate gray scale image of each fluorophore was then acquired by using the CCD camera system. As shown in Fig. 1 (*A–C*), four pairs of chromosome-specific hybridization signals are seen in each image, as expected from the experimental design. Each of the source gray scale images has been pseudocolored to highlight the hybridization signals. One pair of signals appears uniquely on each of the images (see arrowheads), reflecting those probes that were labeled with only a single reporter. Two other signal pairs appear on two images each, while the third appears on all three images (see arrows). Thus, each probe could be selectively identified by the fluorophore image combination on which the hybridization signal was detected. The gray scale signal regions from the images were segregated, pseudocolored, and merged with computer software as described. Fig. 1*D* shows this merged image. Each of the seven centromere probes are seen as distinct colors on the DAPI (blue) counterstained metaphase chromosomes. The probes could also be clearly distinguished after hybridization to fixed human lymphocyte nuclei. Fig. 1*E* shows a merged image of an interphase nucleus hybridized with a mixture of the seven centromere probes.

Table 2. Labeling of chromosome-specific libraries

	dNTP, μM					
	pBS2	pBS14	pBS1	pBS4	pBSX	pBS8
Bio-11-dUTP	75			37.5	37.5	
dig-11-dUTP		75		37.5		37.5
DNP-11-dUTP			37.5		37.5	37.5
TTP	225	225	262.5	225	225	225
dA, dC, and dGTP	300	300	300	300	300	300

Simultaneous Painting of Six Chromosomes and Detection of a Chromosomal Translocation. Chromosome painting is a powerful and general approach to study chromosomal abnormalities. Here the probes are a complex composite of sequences cloned in plasmid or phage vectors with flow-sorted chromosomes used as the starting DNA source (17, 18). To demonstrate that combinatorial labeling also could be used for whole chromosome analysis, the libraries for chromosomes 1, 2, and 4 were singly labeled with Bio, dig, and DNP, while the libraries for chromosomes 8, 14, and X were labeled combinatorially (see Table 2). Each probe set decorated a single chromosome pair when analyzed by FISH, with signal intensities on each fluorophore channel being of similar intensity (data not shown). The merged image (Fig. 1*F*) highlights the six target chromosomes in different pseudocolors while the remaining chromosomes exhibit the blue DAPI counterstain.

The PCR-generated libraries can also be used for detection of chromosomal translocations as exemplified in Fig. 1*G*. Metaphase spreads were obtained from lymphocytes of a healthy female donor whose karyotype (Giemsa banding) was shown to be 46,XX,t(2;14)(q37;q22). The metaphase spreads were investigated in order to clarify the origin of an identical translocation detected in the fetus of the donor. Using PCR-generated libraries for chromosome 2 (Bio) and chromosome 14 (dig), the reciprocal character of the translocation could be clearly demonstrated (see arrowheads).

Combinatorial Labeling and Gene Mapping. The feasibility of mapping multiple genes simultaneously by using the combinatorial labeling paradigm is demonstrated by the data presented in Fig. 1 (*H–J*). Six different cosmid and phage clones, previously mapped to chromosomes 5, 6, 8, 11, 21, and X in independent experiments, were cohybridized and separate gray scale fluorescence images were collected and processed as described above. The merged image on a DAPI-counterstained metaphase spread is shown in Fig. 1*H*. The chromosomal location of each clone, as measured by both fractional length measurements (13) and *Alu*–PCR hybridization banding (19), was identical to that obtained before (data not shown). Six cosmid clones with known locations on chromosome 5 were also hybridized simultaneously. Fig. 1*I* shows the distribution of these clones on both chromosome 5 homologs in a metaphase spread, while Fig. 1*J* demonstrates that the relative order of the clones (i.e., the pattern of colors) is maintained in the interphase nuclei of a T lymphocyte. It should be noted that many of the signals appear as doublets, reflecting the fact that these sequences have already undergone DNA replication in this nucleus. Conversion of a probe signal from a singlet to a doublet can be used to monitor the replication timing of DNA segments during S phase.

DISCUSSION

In this paper, we report a procedure that permits analysis of up to seven probes simultaneously. Combinatorially labeled probes can be produced rapidly and reproducibly by either nick-translation or PCR amplification. However, the latter approach is particularly attractive for labeling clones with relatively small inserts (≈6 kilobases or less) since vector-derived PCR primers permit selective amplification of insert DNA sequences with high efficiency. For example, with the alphoid DNA clones, a typical 50-μl amplification reaction mixture yields sufficient labeled probe for ≈250 *in situ* hybridizations. Not surprisingly, the yield for the chromosome library clone pool is lower; nevertheless, 100 ng of template gave enough amplification products to hybridize 10 slides. Reamplification of the primary PCR product pool could also be done without any detectable loss of probe complexity (data not shown). In contrast, using nick-translated plasmid libraries, 200 ng of DNA was required per

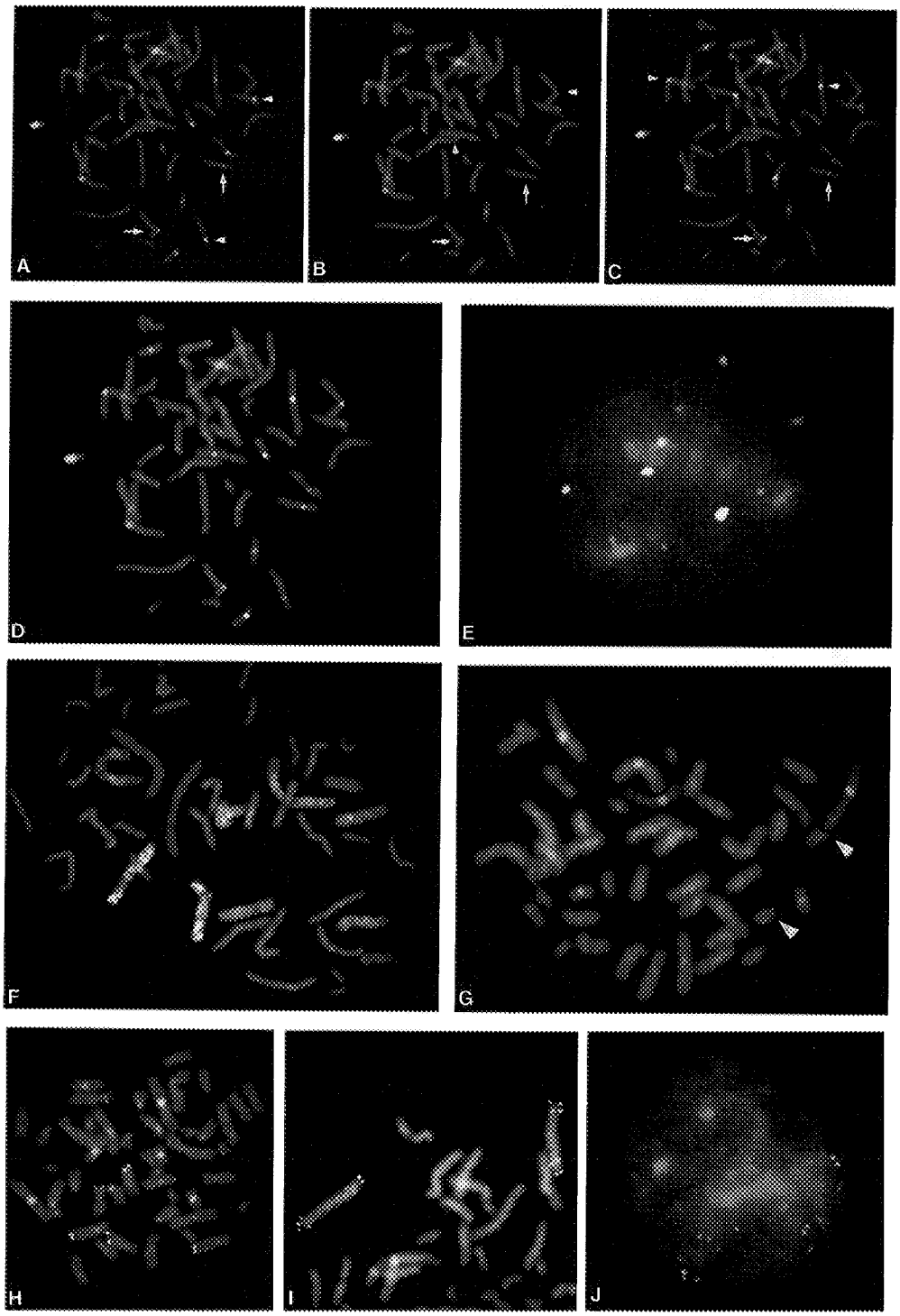

FIG. 1. (*A–C*) Hybridization signals from centromeric repeat probes on metaphase chromosomes from a normal male. The labeling combinations used are given in Table 1. The images were taken separately with the appropriate filters and pseudocolored. (*A*) Image taken with the fluorescein filter, displaying the fluorescein-12-dUTP-labeled probes for the centromeres of chromosomes 8, 11, 12, and 18. Arrowheads indicate the centromere for chromosome 12, which was singly labeled with fluorescein-dUTP. Arrows show the centromere of chromosome 8, which was labeled with a triple combination. (*B*) Detection of the dig-labeled probes with the rhodamine-specific filter. Centromeres of chromosomes 7 (arrowheads), 8 (arrows), 9, and 18 reveal hybridization signals. (*C*) Using the infrared filter combination, the biotinylated probes that were detected with streptavidin conjugated to the infrared dye Ultralite 680 are shown. Chromosomes 3 (arrowheads), 8 (arrows), 9, and 11 were detected. (*D* and *E*) Independently acquired gray scale images were merged and pseudocolored, resulting in seven differentially colored centromeric sequences on metaphase chromosomes (*D*) and in an interphase nucleus (*E*). DAPI was used as a DNA counterstain. (*F*) Example of combinatorial labeling of chromosome-specific libraries with PCR. The libraries for chromosomes 1, 2, 4, 8, 14, and X were labeled singly or combinatorially (see Table 2) and pseudocolored in green, violet, white, red, and orange, respectively. (*G*) PCR-labeled chromosome-specific libraries were used for detection of a t(2;14) translocation. The library for chromosome 2 was labeled with biotin and detected with avidin fluorescein; the chromosome 14 library was labeled with dig and detected with anti-dig rhodamine. Both translocation chromosomes are clearly visible (arrowheads). (*H*) Single gene probes for six different chromosomes were hybridized simultaneously. Chromosomes 5, 6, 8, 11, 21, and X show hybridization signals in yellow, violet, white, orange, green, and red, respectively. Combinatorial labeling of cosmid and phage clones is described in *Materials and Methods*. (*I*) Combinatorial labeling of six cosmid clones specific for chromosome 5. The differentially pseudocolored probes label six loci on this chromosome simultaneously. (*J*) Hybridization of the chromosome 5-specific probes to an interphase nucleus. The order of the cosmid clones is maintained.

slide. The negligible amount of labeled vector sequences in the PCR products also reduces the potential for vector sequence cross-hybridization, a problem that was described by Nederlof *et al.* (3).

The digital imaging capabilities of the cooled CCD camera and the computer software for pseudocoloring and merging signals from combinatorially labeled probes will play an important role in extending the number of simultaneously detectable probes beyond the seven reported here. The CCD camera is sensitive to light over a broad spectral range. Infrared dyes, such as Ultralite 680, which are not visible by eye, can be imaged quite readily by the CCD camera. A series of fluorophores emitting in the 650- to 900-nm range, have recently been reported (20); this should increase the number of different fluorophores that can be used combinatorially for probe identification. Furthermore, the infrared dyes, such as

Ultralite 680, offer certain advantages over the blue fluorophores, 7-amino-4-methycoumarin-3-acetic acid (AMCA) or Cascade Blue: (*i*) sample autofluorescence is minimal at the longer wavelength, (*ii*) DAPI counterstaining of metaphase chromosomes and interphase nuclei is possible (the emission of DAPI, AMCA, and Cascade Blue overlap), and (*iii*) the observed bleed-through of rhodamine signals with the DAPI filter when imaging AMCA fluorescence is more severe than the bleed-through of rhodamine signals using the infrared filter.

Digital imaging of combinatorially labeled probes also circumvents a universally thorny problem in multicolor analysis—that of precise image registration. When filter cubes are moved to collect the fluorescence emission of a single fluorophore, optical imperfections or mechanical motion may cause image displacement relative to each other; these registration offsets can be as large as 1 μm. This is extremely problematic when spatial relationships between signals are critical, such as in gene mapping. However, when multiple probes, combinatorially labeled, are cohybridized, signals from these probes appear on two or more of the separate fluorophore images, thus providing internal reference points for image registration. Provided that one hybridization signal set is directly tied to the complete image of a metaphase spread or interphase nucleus—i.e., by using a dual band-pass filter (21)—all images can be aligned irrespective of the number of separate images to be merged.

Fluorescence *in situ* hybridization is becoming an increasingly powerful experimental tool, both for basic research (16, 22, 23) and for clinical applications (9, 10, 15, 24). The ability to visualize multiple probes simultaneously should streamline the screening of specimens for chromosomal aneuploidies and/or chromosomal rearrangements. This is of particular importance in cases in which clinical samples are limited in number. In addition, by incorporating one or more appropriate reference clones (e.g., centromere repeats or unique sequence genes) in the experimental protocol, the assessment of gene dosage (loss of heterozygosity, aneuploidy, and mosaics) or defining boundaries of chromosomal deletions should be more definitive and require less statistical analysis. The generation of physical mapping data, using either metaphase (13) or interphase mapping strategies (25, 26), should be facilitated with combinatorial fluorescence as would studies focused on understanding the intranuclear topography of genes and chromosomes. It should be stressed that the assessment of the chromosomal map positions of several combinatorially labeled clones does not necessarily require the pseudocoloring and merging procedures. Displaying the signals separately as gray scale images, as shown in Fig. 1 (*A–C*), allows the physical ordering of probes, since combinatorially labeled clones appear in several gray scale images and can thus be identified. Manual segregation of the images is time-consuming, which in its present format reduces the rate at which clones can be mapped. This limitation will be eliminated as soon as software becomes available to fully automate this step; this software is presently being developed. The use of commercially available nucleotide analogs conjugated to fluorescein is of particular value for clinical applications since it circumvents time-consuming and sometimes troublesome immunological steps required to visualize haptenized probes. In addition, this results in an improved signal/noise ratio, which could enhance overall detection sensitivity, especially if a cooled CCD camera were used for imaging. It can be expected that other nucleotides with additional conjugated fluorophores will be available soon, which should both simplify and expand the combinatorial labeling strategy for multicolor hybridization assays even more.

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