

ing for the engineering of a few production hosts for the many microbial metabolites produced by different actinomycetes. The availability of production hosts of defined physiology and known genome sequence may expand the possibilities to rational approaches to strain improvement and to the biological generation of derivatives of natural products.

Experimental protocol

Bacterial strains, plasmids, and DNA manipulations. *Streptomyces lividans* ZX7 (ref. 12), *S. coelicolor* M145, ΦC31, and pIJ39 (ref. 10) were provided by professor David Hopwood; pCYPAC2 (ref. 11) was from Pieter de Jong. Manipulation of large DNA fragments and PFGE analysis followed published guidelines^{12,20}. A 2.2 kb *attP-int* cassette, engineered with *KpnI* and *BamHI* sites at the ends, was PCR-amplified from ΦC31 DNA, and ligated with the 1.8 kb *BamHI* fragment (containing *tsr*, from pIJ39) into pUCBM21. From the resulting plasmid, the *BamHI* site separating *attP-int* from *tsr* was removed by *BamHI* partial digestion and fill-in, generating plasmid pUIT4. The *attP-int-tsr* cassette was excised from pUIT4 as an *ApaI* fragment and cloned into the *NheI* site of pCYPAC2, generating pPAC-S1 and pPAC-S2.

ESAC library construction. *Streptomyces coelicolor* M145 was grown in YEME medium (ref. 10) containing 0.5% glycine for 40 h at 30°C. The pelleted mycelium was washed with 10.3% sucrose, embedded in 0.75% low-melting-point agarose, and lysed by treatment with 1 mg ml⁻¹ lysozyme, 1 mg ml⁻¹ proteinase K in 0.1% SDS for 40 h at 50°C. Chromosomal DNA was partially digested with *Sau3AI* for 20 min and resolved by PFGE. The DNA fraction (~100 kb) was excised, digested with gelase, and ligated to *BamHI*-digested pPAC-S2 (prepared as described²) in a 50 μl final volume, using an ~10:1 molar ratio of vector to insert. The ligation mixture was drop-dialyzed, and a few microliters were used to electroporate *E. coli* DH10B cells.

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Custom fluorescent-nucleotide synthesis as an alternative method for nucleic acid labeling

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The variety of potentially useful dyes or haptens available for fluorescent nucleic acid hybridization assays is far greater than what can be obtained from commercial sources^{1,2}. Since this diversity could be useful in many laboratory applications, we have developed a simple and inexpensive procedure for preparing nonpurified labeled nucleotides^{3–5}, for use in common nucleic acid labeling reactions, such as PCR and nick translation. The modified nucleotides were synthesized by coupling allylamine-dUTP to the succinimidyl-ester derivatives of the fluorescent dyes or haptens such as biotin or digoxigenin, which require fluorescently labeled proteins for detection. This method allows custom preparation of most common fluorescent nucleotides and rapid testing of new ones, while reducing the cost of procedures such as multiplex fluorescent in situ hybridization (M-FISH) by 100–200 fold.

We tested the following fluorescent dyes for their efficacy in the custom labeling reaction: amino-methyl coumarin (AMCA); diethyl aminomethyl coumarin (DEAC); Cascade Blue; fluorescein isothiocyanate (FITC); Oregon Green 488; Alexa 488; the rhodamine derivatives Carboxy-rhodamine 6G (R6G), tetramethyl rhodamine (TAMRA), and Texas Red; Cy3; Cy3.5; Cy5 and Cy5.5. The haptens we tested were biotin; digoxigenin; and 2,4-dinitrophenyl (DNP). DNA probes were labeled with the custom-made nucleotides by PCR or nick translation. Both reactions required the addition of bovine serum albumin (BSA) while PCR labeling also required additional magnesium. As the added BSA impeded proper resuspension of the DNA in hybridization buffer, it generally was removed by phenol extraction or by proteinase K digestion.

Of the sixteen dyes and haptens we tested, eleven could be successfully incorporated in DNA labeling reactions by PCR or nick translation. Oregon Green yielded only weak hybridization signals,

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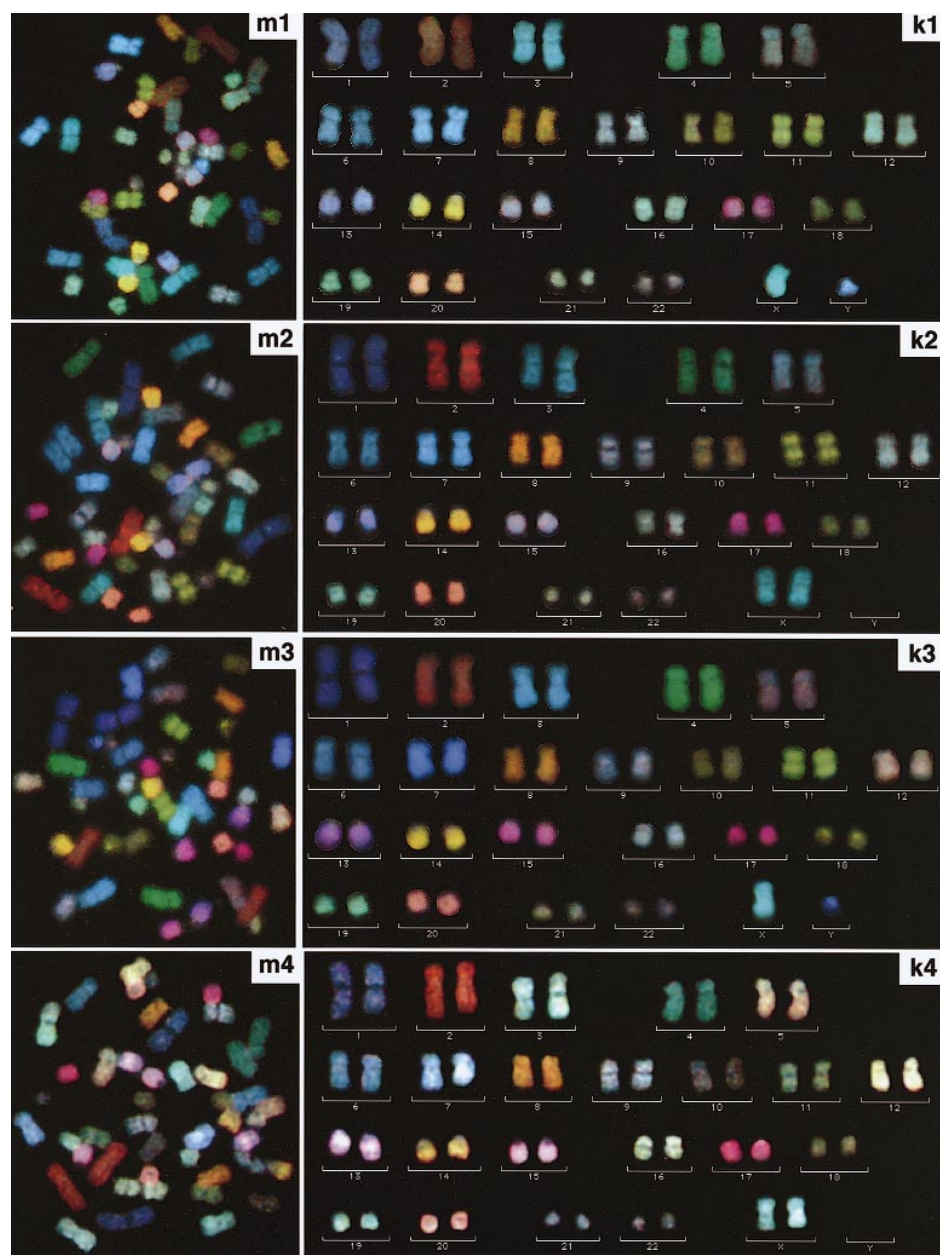


Figure 1. m1-k1 and m2-k2. Comparison of metaphases and karyotypes of M-FISH analyses with commercial labeled nucleotides (m1-k1) and custom-synthesized labeled nucleotides (m2-k2). The amount of each probe used is shown in Table 1. Probes were labeled with biotin (detected with avidin-Cy3.5), digoxigenin (detected with Cy5.5-labeled sheep anti-DIG), FITC, Cy3, and Cy5. Both approaches yielded robust hybridizations, with fluorescence signals that could be analyzed by the M-FISH software. m3-k3 show results of M-FISH using custom-synthesized, fluorescent-labeled nucleotides. The fluorophores used were: DEAC, FITC, R6G, Texas Red and Cy5 (Table 1). m4-k4 show results of M-FISH using dUTP custom-labeled with five different "haptenes" (biotin, digoxigenin, DNP, FITC, rhodamine derivatives), each detected with a fluorescently-labeled antibody.

while AMCA, Cascade Blue, Cy3.5 and Cy5.5 yielded no signals, possibly because the lack of a spacer between the dye and the allylamine-dUTP and/or the electrical charge of the dye molecule interfered with hybridization. To prepare probes for M-FISH, we used random-priming PCR to amplify chromosome-specific DNA fragments obtained by flow cytometry or microdissection of chromosomes (these probes are termed chromosome painting probes). To prepare unique probes such as plasmids, cosmids, PACs (P1 artificial chromosomes), or YACs (yeast artificial chromosomes), we used nick translation.

To compare the performance of the custom-prepared nucleotides to that of commercially available nucleotides, we conducted parallel M-FISH⁶⁻⁸ analyses (Fig. 1, m1-k1 and m2-k2). We used the same

amount of FITC, biotin and Cy5 labeled probes in each reaction, but for the custom synthesized dyes, we increased the amount of digoxigenin and Cy3 labeled probes by 30% and 60%, respectively. This was to compensate for DIG-labeled DNA losses by phenol extraction and sub-optimal deoxynucleotide coupling reaction from a poorly reactive batch of Cy3 dye. This reduced the efficiency of DNA labeling by custom-made Cy3-dUTP, and underscores the importance of storage conditions and expiration dates for the activated dyes.

The hybridization results in Fig. 1 show that the commercially- and custom-prepared nucleotides gave comparable results in M-FISH. However the commercial nucleotides required shorter exposure times, indicating that they labeled the DNA with a higher efficiency, with more labeled nucleotide being incorporated into the DNA. This was expected, since in a given volume of nucleotide solution, the commercial source contained 100% labeled dUTP whereas the custom-made source contained only 50-60% labeled dUTP. Therefore, achieving similar labeling efficiencies would require that 2/3 of dTTP in a reaction be replaced by custom-modified nucleotides, instead of 1/3 for commercial nucleotides.

We also investigated whether in M-FISH experiments, the fluorescent signal could be improved by using haptenes, which are detected by a corresponding haptene-specific fluorescent antibody or haptene binding protein. As each protein molecule carries between 2-4 fluorescent dye molecules, we expected to obtain stronger FISH signals (Fig. 1 m3-k3 and m4-k4). We therefore performed parallel M-FISH analyses using fluorescent-labeled nucleotides (DEAC, FITC, R6G, Texas Red, Cy5), or haptene-labeled nucleotides (digoxigenin, biotin, DNP, TAMRA and FITC). For the latter, the fluorophores were used as haptens and were detected with specific antibodies. The detection scheme included the following: avidin-AMCA* for biotin detection; mouse-antidigoxigenin and horse

antimouse Cy5.5 for digoxigenin detection; rat antiDNP and donkey antirat Cy5 for DNP detection; goat antiFITC and donkey antigoat FITC* for FITC detection; and rabbit antirhodamine and donkey antirabbit Cy3 for rhodamine detection. Some of these antibodies were purchased in the labeled form (designated by asterisk) whereas the others were custom-labeled in our laboratory using standard dye-protein conjugation reactions.

M-FISH results with the five "haptenes" mentioned above showed that, as expected, the labeled antibodies increased the fluorescent signal intensity, reducing the exposure time by 2-5 fold (Table 1). However, they also decreased the signal to noise ratio (S/N) and thus were not always beneficial, possibly as the result of

Table 1. Signal-to-noise ratios (S/N), exposure times, and their relationship with the amount of labeled probe used in M-FISH analysis^a.

| Dye/haptene | Chromosomes (S/N ratios) | | | | | Vol (μl) | Exp (s) |
|--------------|--------------------------|----------|-----------|-----------|-----------|----------|---------|
| | 1 | 7 | 9 | 17 | 22 | | |
| FITC-m1 | 3.2 | 5.9 | 2.8 | 3.8 | 2.3 | 133 | 0.4 |
| FITC-m2 | 2.5 | 4.4 | 2.0 | 3.7 | 1.9 | 133 | 1.0 |
| FITC-m3 | 2.8 | 4.5 | 1.8 | 3.1 | 1.4 | 100 | 0.6 |
| FITC/FITC-m4 | 2.3 | 3.9 | 1.9 | 2.9 | 1.4 | 100 | 0.2 |
| FITC-m5 | 2.0 | 2.6 | 1.4 | 2.7 | 1.4 | 60 | 3.0 |
| | 3 | 7 | 9 | 5 | 12 | | |
| Cy3-m1 | 4.8 | 4.3 | 2.6 | 1.9 | 3.5 | 166 | 0.3 |
| Cy3-m2 | 3.9 | 3.7 | 2.2 | 2.3 | 3.7 | 266 | 2.5 |
| R6G-m3 | 6.7 | 4.1 | 2.7 | 2.7 | 3.9 | 100 | 0.4 |
| Rhod/Cy3-m4 | 4.9 | 2.8 | 2.4 | 2.6 | 3.5 | 100 | 0.07 |
| Cy3-m5 | 2.3 | 3.9 | 1.8 | 1.9 | 3.2 | 100 | 3.0 |
| | 4 | 7 | 18 | 11 | 12 | | |
| BIO/Cy3.5-m1 | 2.4 | 2.4 | 1.6 | 2 | 2.2 | 133 | 0.1 |
| BIO/Cy3.5-m2 | 2.3 | 2.3 | 2.1 | 1.9 | 2.4 | 133 | 0.1 |
| TxR-m3 | 3.1 | 1.4 | 2.3 | 2.1 | 2.3 | 100 | 0.4 |
| BIO/AMCA-m4 | 2.2 | 1.9 | 1.5 | 1.8 | 2.6 | 100 | 0.4 |
| BIO/Cy3.5-m5 | 2.5 | 2.8 | 1.7 | 1.8 | 2.6 | 50 | 0.35 |
| | 10 | 8 | 9 | 11 | 22 | | |
| Cy5-m1 | 2.3 | 3.0 | 2.3 | 3.0 | 1.8 | 250 | 0.6 |
| Cy5-m2 | 2.2 | 3.8 | 1.8 | 2.8 | 1.6 | 250 | 1.2 |
| Cy5-m3 | 1.8 | 2.9 | 2.1 | 3.1 | 1.6 | 200 | 1.6 |
| DNP/Cy5-m4 | 1.3 | 2.3 | 2.4 | 1.6 | 1.1 | 100 | 1.2 |
| Cy5-m5 | 1.6 | 3.5 | 1.6 | 1.9 | 1.5 | 100 | 3.5 |
| | 2 | 8 | 18 | 17 | 12 | | |
| DIG/Cy5.5-m1 | 1.5 | 2.2 | 1.5 | 2.7 | 2.3 | 100 | 2.5 |
| DIG/Cy5.5-m2 | 3.5 | 2.9 | 2.1 | 3.2 | 2.7 | 133 | 5.0 |
| DEAC-m3 | 2.5 | 1.9 | 2.0 | 3.6 | 3.4 | 100 | 0.5 |
| DIG/Cy5.5-m4 | 2.5 | 2.3 | 1.8 | 3.5 | 3.8 | 100 | 3.0 |
| DIG/Cy5.5-m5 | 4.1 | 3.6 | 2.6 | 3.9 | 3.4 | 50 | 5.0 |

^aBold numbers indicate the chromosomes for which S/N ratios were calculated (Adobe Photoshop). Chromosomes were chosen so as to include both strongly (e.g., 7, 8, 12, 17) and weakly labeling (e.g., 5, 10, 18, 22) chromosomes. Exp, Exposure time in seconds. m1 through m5 are the metaphase(s) visualized, m1, m2, m3, and m4 being illustrated in Fig. 1. Chromosomes were labeled with commercial nucleotides in m1 and m5 and custom nucleotides in m2. Only custom-made fluorescent- or haptene-nucleotides were used for m3 and m4, respectively. In m4, FITC and rhodamine were used as haptens, and were detected with antibodies labeled with FITC and Cy3, respectively. BIO (biotin); Rhod (rhodamine derivatives); DIG (digoxigenin).

increased fluorescent detection of the non-specifically hybridized DNA probe fragments (Table 1). The use of labeled antibodies for detection yielded more punctate signals (Figure 1, m4-k4), as compared to signals of chromosomes stained with fluorescent-labeled DNA probes (Figure 1, m3-k3).

For any particular chromosome, the labeling intensity depends on several factors: concentration of the hybridization probe and how evenly it hybridizes to its target chromosome; the amount of labeled nucleotide(s) incorporated into the probe; the dye/haptene used and the overall amount of DNA in the hybridization (the latter includes the labeled DNA probe and the competitor DNA). For example, increasing the amount of labeled library DNA by two-fold in M-FISH experiments resulted in improved signal intensities for the fluorescent-labeled libraries (FITC, Cy3, Cy5) (e.g. compare m1 and m5 in Table 1). In contrast, the same two-fold increase for haptene-labeled DNA libraries resulted in unchanged (BIOTIN/Cy3.5) or decreased (digoxigenin/Cy5.5) signal quality (e.g. compare m3 and m4 in Table 1).

Choosing the appropriate fluors and haptens for FISH will depend on the sensitivity of imaging equipment, probe type (unique probes or whole chromosome painting probes), and the cost of each dye or haptene. Fluorescence filters appropriate for all dyes used in this study were obtained from Chroma Technologies, and their suit-

Table 2. Filters and corresponding dyes

| No. | Filter name | Name | Dye Absorption | Emission |
|-----|--------------------------|--------------|----------------|----------|
| 1 | Narrow band DAPI (31013) | DAPI | 350 | 456 |
| | | AMCA | 353 | 442 |
| | | Cascade Blue | 396 | 410 |
| 2 | Aqua-v2 (31036v2) | DEAC | 432 | 472 |
| | | FITC | 491 | 515 |
| 3 | MF101/Spectrum Green | A488 | 493 | 517 |
| | | OG | 495 | 521 |
| | | Cy3 | 550 | 570 |
| | | R6G | 524 | 552 |
| | | TAMRA | 547 | 573 |
| 4 | MF102/Spectrum Orange | TAMRA | 547 | 573 |
| | | Cy3.5 | 581 | 596 |
| | | Texas Red | 583 | 603 |
| 5 | Cy3.5 | Cy5 | 649 | 670 |
| | | Cy5.5 | 675 | 694 |
| 6 | Cy5 | Cy5 | 649 | 670 |
| 7 | Cy5.5 | Cy5.5 | 675 | 694 |
| 8 | Cy7 | Cy7 | 743 | 767 |

ability for different fluorescent dye combinations is summarized in Table 2. In our hands, the most convenient nucleotide combinations for M-FISH consisted of DEAC, FITC, R6G, Texas Red, and biotin (the latter detected with Avidin-Cy5). We found that the rhodamine derivatives R6G and Texas Red could successfully be used to replace Cy3 and Cy3.5. Moreover, avidin-Cy5 could be used to detect biotin labeled probes at less cost than Cy5-dUTP, and it yielded strong signals with very good signal to noise ratio (not shown). Although custom-made AMCA-dUTP yielded no FISH signals, commercial AMCA-5-dUTP could be incorporated into the DNA, probably because this version contained a spacer between the dye and the nucleotide. DEAC-dUTP yielded M-FISH signals with a good signal to noise ratio, that could be easily differentiated from DAPI or FITC signals. R6G gave the highest signal to noise ratio of all the nucleotides tested and is also cheaper than Cy3. Custom-made Texas Red-dUTP yielded a good signal to noise ratio and is much cheaper than its cyanine equivalent (Cy3 or Cy3.5). Cy5-dUTP could be easily used to label DNA in enzymatic reactions.

In contrast, custom-made Oregon Green-, Alexa488- and commercial Alexa 488-dUTP yielded poor hybridization signals, with lower intensity and reduced signal to noise ratio, when compared with FITC. TAMRA-dUTP worked well in labeling reactions but has the disadvantage that it can be detected by both the MF102 and the Cy3.5 filters. Cy3.5- and Cy5.5- dUTP were not incorporated into DNA by polymerases, but these two cyanine dyes can be used in fluorescent applications when conjugated to antibodies (unpublished data). We did not attempt to synthesize Cy7-dUTP, but found that Cy7-labeled antibodies could be used to detect various haptens (unpublished data). Good replacements for the far-red or infrared cyanine dyes are currently unavailable.

The main advantage of the custom nucleotide synthesis is the significant cost reduction over commercial sources, as a result of eliminating the nucleotide purification step. With the current reagent costs of \$400 for 10 mg allylamine-dUTP, \$800 for 5-10 mg cyanine dyes and \$100-200 for 5-25 mg of the other dyes, when using nucleotides labeled by this procedure, the total price of one M-FISH assay is less than \$1 (\$2 when using cyanine dyes), as compared to \$230/assay when using commercially available probes (e.g. Vysis Inc, Downers Grove, IL).

Experimental protocol

Nucleotide labeling procedure. Storage conditions and expiration dates for the activated dyes should be observed for best reproducibility of results. For labeling, succinimidyl-ester derivatives of the dyes and haptens were dissolved in DMSO, at 10 mM (AMCA, Oregon Green, TAMRA, Cy3.5, Cy5.5), 20 mM (DEAC, Cy3, Cy5) and 40 mM (FITC, R6G, Texas Red, biotin, digoxigenin, DNP), based on their apparent solubilities. The acetylazide derivative of Cascade Blue (40 mM) and the Alexa 488 succinimidyl-ester were dissolved in water. All cyanine dyes were purchased from Amersham Pharmacia

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Biotech (Piscataway, NJ) whereas all other dyes and haptens were purchased from Molecular Probes (Eugene, OR). The Alexa 488 succinimidyl-ester is not available commercially as a reactive dye, and small dye aliquots could only be obtained from a protein labeling kit (Cat. A-10235; Molecular Probes). As Molecular Probes considered the amount of Alexa 488 in an aliquot to be proprietary information, several dye aliquots were dissolved in various volumes of water (5-50 μ l) and used to prepare Alexa 488-dUTP. FISH tests showed that the best results were obtained when each Alexa 488 dye aliquot was dissolved in 15 μ l water and used as if it were equivalent to 20 mM in chemical coupling reactions. Commercially available 5-(3-aminoallyl)-2'-deoxyuridine 5' triphosphate (Sigma, St. Louis, MO) was dissolved in 0.2 M bicarbonate buffer (\sim pH 8.3) at 20 mM concentration.

Chemical coupling reactions were carried out at 1:1 molar concentration (dye: dUTP) in 80-100 mM bicarbonate buffer, adding the allylamine-dUTP first, and the reactive dye last. Chemical coupling with dye stocks at 10mM concentrations included one volume each of allylamine-dUTP (20 mM), water, 0.2M bicarbonate buffer and two volumes reactive dye (80 mM bicarbonate, 40% DMSO final). Coupling reactions with dye stocks at 20mM, included one volume each of allylamine-dUTP (20 mM), water, 0.2M bicarbonate buffer and dye (100 mM bicarbonate, 25% DMSO final). Coupling reactions with dye stocks at 40 mM included one volume each of allylamine-dUTP (20 mM), 0.2M bicarbonate buffer, 1.5 volumes water, and 0.5 volumes of reactive dye (100 mM bicarbonate, 14% DMSO final). For Texas Red, digoxigenin and DNP, one additional volume of DMSO was also needed in the reaction to keep all ingredients in a soluble form (final concentrations were 80mM bicarbonate, 30% DMSO). After 3-4 hours incubation at room temperature, glycine (pH 8.0, 20 mM final concentration) was added to stop the reaction, along with Tris-HCl, pH 7.75 (20 mM final concentration) to stabilize the nucleotides, and water to adjust the final nucleotide concentration to 1 mM. Previous data indicated that roughly 50% of the dUTP was labeled in such reactions. Nucleotides could be used immediately or stored at -20° C. Reactions carried out at a 2:1 molar excess of dye achieved higher dUTP labeling (80-90%) and are more cost-effective for the cheaper fluorescein, rhodamine and coumarine derivatives. Because of the higher costs of the cyanine dyes and to maintain uniformity, we carried out the reactions at 1:1 molar ratios. Solutions were stable for at least 1 year when stored at -20° C.

Enzymatic DNA labeling. A standard PCR reaction included: 0.1-0.5 ng/ μ l DNA template, 1x buffer (50 mM KCl, 10 mM Tris, pH 8.4, 1.5-2 mM $MgCl_2$), 200 μ M each nucleotide, 2 μ M primer and 2U Taq polymerase/25 μ l. A standard nick translation reaction included: DNA (20-30 ng/ μ l), 1x buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.05 mg/ml BSA), 10 mM β -mercaptoethanol, 50 μ M each nucleotide, 0.34 μ g/ml DNase, 0.25 U *Escherichia coli* polymerase I/ μ l reaction. When using commercially-labeled dUTP, the dTTP in the reactions was reduced to about 2/3 (130 μ M in PCR and 35 μ M in nick translation), the rest (1/3) being dUTP. When using custom-made nucleotides, the dTTP in the reactions was reduced to about 1/3 (70 μ M in PCR and 17 μ M in nick translation), and the following volumes of 1 mM custom-made nucleotide solutions were added to each reaction (in 100 μ l PCR or nick translation): 2 μ l (20 μ M) DEAC, Cascade Blue, Cy3.5, Cy5.5; 3 μ l (30 μ M) R6G, TAMRA, Texas Red; 5 μ l (50 μ M) Oregon Green, Alexa 488, Cy3, Cy5; and 6-7 μ l (60-70 μ M) AMCA, FITC, biotin, digoxigenin, DNP. Replacing so much dTTP was possible because in the custom-synthesized nucleotides 50% allylamine-dUTP is non-conjugated, resulting in a variable dye:dTTP ratio, based on the dye used. Nevertheless, labeling results were good, indicating that a precise dTTP/dUTP analog ratio did not appear to be necessary. With custom labeled nucleotides PCR reaction required additional BSA (Sigma) at 0.4 mg/ml and additional magnesium (up to 4-5 mM). Various Tris, glycine and ethanolamine concentrations did not influence efficiency of DNA labeling. Nick translation worked as usual, but seemed somewhat improved by an increased BSA concentration (up to 0.2 mg/ml).

For M-FISH studies, the NIH chromosome painting probes, which were obtained by microdissection and kindly provided by Dr. J. Trent, were labeled by degenerate oligonucleotide priming PCR, using a previously described degenerate primer⁹. 3-4 μ l PCR template (40-80 ng DNA) was re-amplified and simultaneously labeled in 100 μ l PCR reaction. The commercial labeled nucleotides

used were: biotin-11-dUTP or biotin-16-dUTP (Enzo Diagnostics, Farmingdale, NY or Boehringer Mannheim, Indianapolis, IN), FITC-12-dUTP, digoxigenin-11-dUTP (Boehringer Mannheim), Cy3-dUTP and Cy5-dUTP (Amersham Pharmacia Biotech) and AMCA-5-dUTP (Molecular Probes).

The chromosome labeling algorithm was previously described⁷⁻⁸. Usually, for one M-FISH slide, about 100 μ l each of the five labeled chromosome pools were mixed together (Table 1). To adjust the labeled DNA to an average length of 200-300 bp, the PCR products (DNA smear on agarose gels) were subjected to a partial DNase digestion, for 10-15 minutes at room temperature. We used a 10x DNase digestion solution, obtained by mixing 400 μ l water, 4 μ l 1M $MgCl_2$ and 1-2 μ l 3 mg/ml DNase stock solution (final DNase concentration in the reaction was 1.5 μ g/ml). Reaction was stopped by heating 2-3 minutes at 94° C.

Post-labeling DNA purification (BSA removal). The added BSA impeded proper resuspension of the labeled DNA in hybridization buffer, but only when volumes of labeled DNA probe greater than 50 μ l had to be precipitated and used. To remove the BSA, our preferred procedure was a 5 minutes phenol extraction: 1/2 volume phenol and 1/2 volume chloroform:isoamylalcohol (24:1) were added to the labeled DNA, followed by vortexing, 3-4 times, at 20-30 seconds interval, and 3 minutes centrifugation at full speed in a microfuge. The upper phase was collected, precipitated and resuspended in 12 μ l hybridization buffer (50% formamide/10% dextran sulfate/2XSSC [300 mM NaCl, 30 mM sodium citrate]). Phenol extractions led to approximately 50% losses for DNA labeled with digoxigenin and rhodamine derivatives (especially Texas Red). Such DNA could still be used for FISH, but signals were less intense (e.g. compare Fig.1 m1-k1 with m2-k2). For these dyes, alternative protein removal procedures were used. Spin columns (10 μ g DNA capacity) (Quiagen, Valencia, CA) were used to remove BSA from 100-200 μ l PCR reactions, with only slight DNA losses. Alternatively, 2 μ l proteinase K (2 mg/ml) was added to every 100 μ l PCR labeling reaction and incubated 30 minutes at room temperature or 45° C. Reaction was stopped by adding the irreversible protease inhibitor Pefabloc SC (Boehringer Mannheim), at a final concentration of 2-4 mM. No DNA losses occurred, and the DNA could be easily resuspended in hybridization buffer after precipitation.

Antibody detection of haptens. We used the following hapten specific antibodies: Avidin-AMCA (Accurate Chemical, Westbury, NY) ; mouse-antidigoxigenin (Sigma, St. Louis, MO) and horse antimouse (Vector Laboratories, Burlingame, CA); rat antiDNP and donkey antirat (Accurate Chemical); rabbit antirhodamine (Accurate Chemical), donkey antirabbit (Accurate Chemical), goat antiFITC (Accurate Chemical) and donkey antigoat FITC (Accurate Chemical). Avidin-Cy5 was purchased from Accurate Chemical (Westbury, NY). All antibodies were stored as 1 mg/ml stock solutions and were used at 1:100 dilutions in 4xSSC, 5-10 minutes at 37° C.

Slide preparation, hybridization and analysis. Slides were prepared and hybridized as described previously⁸. Slides were visualized using an AX-70 fluorescence microscope (Olympus America, Melville, NY) equipped with a cooled CCD camera (Quantix, Roper Scientific, Tuscon, AZ). Karyotyping was performed with the PowerGene M-FISH software (Perceptive Scientific Instruments, League City, TX).

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