

Methods

Slide preparation and hybridization. In general, metaphases were spread with little or no cytoplasmic residue, and the slides subjected to very little ageing before hybridization. Cell suspensions were stored at -20°C in standard 3:1 methanol:acetic acid fixative. Slides were prepared 1 h before hybridization using common spreading techniques. After preparation, slides were aged 10–30 s at 94°C in ethanol (chemical ageing) and immediately subjected to 30–45-s treatment with 0.005% (w/v) pepsin, followed by several seconds rinse in PBS, pH 7.5, and an ethanol series (3 min each in 70%, 90%, 100%). Slides were denatured in 70% formamide/2 \times SSC at 75°C for 2 min, passed through another ethanol series and air-dried. The denatured DNA probe was placed on the slide, covered with a coverslip and hybridized. Alternatively, the probe cocktail was placed on the slide before denaturing, coverslipped, sealed with rubber cement, and denaturing of the chromosomal DNA and the probe DNA was done ‘simultaneously’ by placing the slide on a heated metal block at 75°C for 2 min. Both denaturing techniques produced similar results and the entire slide preparation process required 30–40 min. Hybridization was done overnight (14–20 h). The freshness of the slides made hybridization very efficient, and longer hybridization times (2–4 d) were not necessary.

Probes, dyes and antibodies. Microdissected chromosome painting probes were kindly provided as PCR products by J. Trent, P. Meltzer and M. Bitner. PCR re-amplification of each library was performed using a described primer¹ and the following cycling conditions: 45 s at 94°C , 45 s at 54°C and 4 min at 68°C , for 30 cycles. To propagate these probe libraries, for each chromosome, 3–4 μl original product was re-amplified in 100 μl PCR volume. Labelling was done using the same PCR conditions and any fluorescent- or hapten-labelled dUTP. The proportion of labelled dUTP to dTTP was 1:8 for Texas Red, 1:5 for TAMRA, 1:3 for Cy3, Cy5, BIO, DIG, and 1:2 for AMCA, FITC and DNP. Labelled dUTPs were either synthesized in our laboratory using amine-succinimide ester coupling reactions or were purchased (Boehringer, DIG, BIO, AMCA, FITC; Amersham Life Sciences, Cy3, Cy5; Molecular Probes, TAMRA, Texas Red; NEN Life Sciences, DNP; Enzo Diagnostics, BIO). Before labelling, probes were mixed together in cocktails to be labelled with the same dye/nucleotide, either as indicated in Table 1 (for ‘3+3’) or as proposed by Speicher *et al.* for Table 2 combinations (‘3+2 and 2+3’). In every PCR cocktail, each probe template was added in an amount proportional to chromosomal size, then further adjusted by testing. After PCR labelling, DNA size was brought to below 500 bp using a controlled DNase I digestion for 15 min at RT, followed by 2–3 min incubation at 95°C (stop reaction). The 10 \times DNase solution contains 10–20 ng/ μl DNase I and 20 mM MgCl_2 . In general, for one hybridization, the following amounts of labelled PCR products were used: 150 μl for AMCA, Cy3, Cy5; 75 μl for TAMRA, Texas Red, FITC, DNP; 50 μl for BIO, DIG. Probes were ethanol-precipitated and resuspended in 12 μl hybridization buffer. Antibodies were purchased (Accurate Chemical, avidin Cy3, avidin Cy5; Boehringer, Avidin FITC, sheep anti-mouse Cy3; Sigma, mouse anti-digoxin, goat anti-rabbit FITC; Vector Laboratories, avidin D, avidin AMCA, horse anti-mouse, horse anti-mouse Texas Red). Cy3.5- and Cy5.5-labelled antibodies were prepared using standard dye-protein coupling protocols (Amersham, Molecular Probes)

Probe detection and image capture. After hybridization, 3 \times 5 min posthybridization washes were done at $42\text{--}45^{\circ}\text{C}$ in 2 \times SSC/50% formamide, then in 0.2 \times SSC, followed by incubation with the primary antibody layer, which included mouse anti-digoxin and rabbit anti-DNP. For the 2+3 algorithm 4, avidin-AMCA was also added at this step. All antibodies were stored as 1 mg/ml stock solution and diluted 1:100 or 1:200 in 4 \times SSC for use. After 5–10 min incubation at 37°C , slides were rinsed 3 \times 3 min in wash solution (4 \times SSC/0.1% Tween) and mounted with antifade medium without DAPI. Images of the fluorescent labelled probes were captured using either a digital photographic camera (Olympus DP-10) attached to an Olympus AX70 fluorescent microscope or using specialized software (Vysis or PSI) controlling cooled CCD cameras (Photometrics) on Leica Aristoplan or Olympus Provia microscopes, respectively. Images of 10–15 metaphases were stored and their position coordinates recorded. The coverslips were then removed using tweezers and the slides rinsed in wash solution for 10–15 min at 42°C . Secondary antibodies were added according to the detection

algorithm (usually goat anti-rabbit FITC, sheep anti-mouse Cy3 or horse anti-mouse Texas Red, and avidin Cy3.5 or avidin Cy5). After 5–10 min incubation at 37°C and rinsing in wash solution, slides were stained with DAPI and mounted with antifade media. Images of the same metaphases were captured. The chip of the digital photographic camera tested (DP-10) allows imaging of fluorophores from DAPI to Texas Red, only. Unlike video CCD and cooled CCD cameras, the digital camera could not detect infrared emitting fluorophores (Cy5, Cy5.5). When using a digital camera, it is important to choose a brand allowing manual exposures, so that exposing times can be changed at will. With any digital or CCD camera, imaging sequence should proceed from the fluorophore with the longest wavelength toward the fluorophore with the shortest wavelength. Exposure to the higher energy blue light can decrease the signal from other fluorophores on the metaphase. For CCK, the three most common channels to use would be blue (DAPI and AMCA), green (FITC) and red (rhodamine, Cy3, TAMRA, Texas Red, Cy5.5). If more channels are desired, fluorescence filters can be purchased (Chroma) to visualize the fluorophore AQUA (Vysis) with emission between DAPI and FITC. Also, filters can be purchased to allow separate detection of orange-red dyes (Cy3) and red dyes (Cy3.5, Texas Red).

Image processing. Individual channel/fluorophore images captured with the digital photographic camera (DP-10) were stored in JPG format and transferred to a computer running Adobe Photoshop (versions 3.0, 4.0 or 5.0). As this camera records colour images, the first step was to open every image in Photoshop and convert them to greyscale images, by choosing “mode” and “greyscale”. When CCD or cooled CCD cameras were used for image capturing, the Vysis or PSI software used to control these cameras allowed raw images of every channel/fluorophore to be exported as individual greyscale image files in PICT or TIFF format.

Three greyscale JPG or PICT files at one time were simultaneously opened with Photoshop, then merged into a tri-colour image by clicking on the arrow located in the upper right corner of the “channels” window, and selecting “merge channels”, and then “RGB image”. When prompted, the colours red, green and blue can be selected for any of the three raw images opened. The software then provides a multicolour image of all three channels. Each channel can be selected individually by clicking on the channel name in the channel window, and can be processed for background subtraction, lightness and contrast, sharpening or smoothing using the commands under the top menus. Usually, two RGB files were created, one with the channels captured under the “first” image and another with the channels captured under the “second” image (Fig. 1*a,b,d,e,i,j,k,l*). Chromosome analysis can be done at this time using the charts provided (Fig. 1*g,h*). To merge these two RGB files in Photoshop and obtain a true multicolour image in which each chromosome has a different shade, the “second” RGB was further processed. Its colours were altered so as to be different from the “first” image by going to the top menu and selecting “image”, “adjust” and “hue/saturation” and then moving the “hue” arrow left or right until a desired colour combination was obtained. These changes were stored. The modified “second” RGB image was then copied and pasted on top of the “first” RGB image. In the layer/channel palette, by clicking on “layers”, the transparency of the “second” image was then adjusted and set to ~50%. The shift between the first and second images was apparent immediately and was corrected using the arrows on the keyboard, which move the pasted “second” image in the desired direction by one pixel for each click. As the two merged images have different colour shades, each chromosome pair in the final image has a different colour shade.

When CCD or cooled CCD cameras and FISH/M-FISH software are available, the channels captured for the “first” and “second” CCK images can be stored in a single file. The M-FISH software (PSI) will allow correction for the position shift and will automatically process, colourize and merge the images, and create a karyotype, similar to a standard M-FISH. The version of the Vysis FISH software tested allowed for only three channels to be captured, pseudo-coloured and merged at one time, yielding RGB images. The “first” and “second” coloured CCK images were then merged using Photoshop.