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Genetic Analysis by Chromosome Sorting and Painting: Phylogenetic and Diagnostic Applications

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Key Words

Fluorescence-activated chromosome sorting
Chromosome painting
Fluorescence in situ hybridisation (FISH)
Karyotype evolution
Spectral karyotyping

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Abstract

Chromosome sorting from fluid suspensions of metaphase chromosomes using a fluorescence-activated cell sorter has been used for a number of years to produce chromosome-specific genomic libraries and other reagents for chromosome mapping. Improved techniques for fluorescence in situ hybridisation and the amplification and labelling of sorted chromosomes using degenerate oligonucleotide-primed PCR have led to the widespread use of chromosome painting both for the resolution of complex chromosome aberrations and for the study of karyotype evolution by cross-species reciprocal chromosome painting. The chromosomes of a large number of different species have been sorted and used to make chromosome-specific paints and already new data challenging results of earlier phylogenetic studies have been obtained. Sorted chromosomes provide the resource for multicolour chromosome analysis of all chromosomes simultaneously. Such reagents are now available for all human and mouse chromosomes and are proving particularly useful in the analysis of cancer chromosomes.
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Introduction

The most significant advances in human cytogenetics in the past 40 years can be attributed almost entirely to technical innovation. It is widely appreciated that improved tissue culture methods led to the determination of the correct chromosome number in our species in 1956 and shortly thereafter to the discovery of the common

aneuploid syndromes. Phytohaemagglutinin-stimulated lymphocyte cultures [1] introduced in 1961 allowed the establishment of chromosome diagnostic laboratories worldwide and 10 years later chromosome banding [2] permitted the detection of many previously unknown chromosomal syndromes resulting from structural rearrangements. 1969 was a particularly important year because it saw the introduction of molecular cytogenetics in the form of in situ hybridisation (ISH) of radioactive DNA probes to routine air-dried preparations of chromosomes on microscope slides [3, 4]. The most important application of ISH was in chromosome mapping, first for repetitive DNA sequences [5] and later, using recombi-

Based on the Baschirotto Award Lecture presented at the 28th Annual Meeting of the European Society of Human Genetics, London, April 11th, 1996.

nant techniques to clone specific DNA probes for single-copy sequences including genes [6–8].

The poor resolution of ISH with radioisotopic probes requiring autoradiography and the statistical analysis of grain counts soon led to its replacement by fluorescence ISH (FISH) and other non-isotopic methods using enzyme-linked probe detection systems [9]. The FISH method [10] has been highly successful and is used extensively at the time of writing, not only for the diagnosis of chromosome aberrations but also as the method of choice for mapping genes to chromosomes. The main technical innovations responsible for the success of FISH have been the modification of DNA probes using biotin [11] digoxigenin [12] and, more recently, nucleotides directly labelled with fluorescein-isothiocyanate (e.g. FITC-11-dUTP) and other fluorochromes. The larger DNA probes used in FISH applications contain interspersed repetitive sequences that may cause non-specific background signals and satisfactory techniques have been designed to suppress background using unlabelled Cot-1 DNA [13] or more simply, a pre-annealing step before ISH [14]. As FISH can be used to detect DNA probes labelled with several different colours simultaneously, the method has been applied to ordering cloned sequences along the length of the chromosome [15].

Further technical innovations have extended the use of FISH from the analysis of probes mapped to metaphase chromosomes, where differentially labelled probes can be distinguished only if they are at least 1–2 Mb apart, to the analysis of the chromosomes in interphase nuclei where the limit of resolution is about 50 kb [16]. Even greater resolution of approximately 1 kb is possible in histone-depleted DNA fibres [17, 18], and this method has been used to study the exon-intron structure of large genes [19]. A review of these and other recent applications of FISH has been provided by van Ommen et al. [20] in 1995.

Both the classical and molecular methods of cytogenetics briefly described above use conventional microscopy with preparations of chromosomes variously treated and fixed on microscope slides. A quite different, although no less important technical innovation in the examination of chromosomes was introduced by Gray et al. [21] in 1975, namely, chromosome sorting and measurement by the technique of flow cytometry. In this method, a fluid suspension of chromosomes is analysed in a fluorescence-activated cell sorter. The resolution proved to be remarkable and it is evident that only the expense of the instrumentation has prevented its wider exploitation. Chromosome sorting has been used for the analysis of chromosomal polymorphisms [22], for optimising chromosome

specific DNA libraries [23], for the measurement of the DNA content of chromosomes [24], for the detection of chromosome abnormalities [25–27], for gene mapping using chromosomes sorted onto filters for hybridisation [28–30], for reverse chromosome painting [31–33], and for evolutionary studies using cross-species chromosome painting [34, 35]. These studies demonstrate that quite small deletions and duplications of 2 Mb and over can be resolved by flow karyotyping [26, 29, 36].

One of the most useful of the early applications of chromosome sorting has been in the construction of chromosome-specific DNA libraries. These were made by inserting fragments of the sorted chromosomes into plasmid or bacteriophage vectors and cloning the inserts in bacterial hosts. A library of the human X chromosome was the first to be made using sorted chromosomes [35] and since then, DNA libraries have been made for all human chromosomes [36]. It was quickly appreciated that these libraries could be used with great efficiency in FISH experiments to identify specific chromosomes. When the cloned DNA fragments were suitably labelled and hybridised to chromosome preparations, complementary sequences along the length of the chromosome were 'painted' by the DNA library. Libraries used as chromosome paints thus became valuable for the analysis of chromosome aberrations [39, 40].

Chromosome paints prepared from chromosome libraries and from small numbers of flow-sorted chromosomes amplified and labelled by the polymerase chain reaction (PCR) are now widely used for the identification of interchromosomal rearrangements. However, they are not able to detect the breakpoints in intrachromosomal rearrangements and have to be applied on a 'trial and error' basis in the analysis of complex rearrangements such as the marker chromosomes commonly found in cancer cytogenetics. Such complex aberrations can be resolved by sorting and preparing a paint probe from the aberrant chromosome by PCR amplification and labelling. 'Reverse' painting the aberrant chromosome probe onto normal chromosomes enables the composition of the abnormal chromosome to be determined from the hybridisation pattern produced [31]. Amplification and labelling of flow-sorted chromosomes have been achieved using the degenerate oligonucleotide-primed PCR reaction (DOP-PCR) introduced by Telenius et al. [41] or, alternatively, using Alu-PCR primers [32, 33]. Probes made by the latter method tend to produce an R-banded pattern of hybridisation and so the DOP-PCR method is generally preferred for the production of chromosome-specific paint probes. The method is sufficiently sensitive and

robust to allow a similar analysis from very small numbers of chromosomes microdissected from slide preparations [42].

It is the purpose of this review to outline the current techniques of chromosome sorting and painting and describe their applications in the diagnosis of chromosomal aberrations and in the study of karyotype evolution. The importance of chromosome sorting in the construction of paint probes for these applications has recently been heightened by the developments in digital fluorescence microscopy, including spectral karyotyping [43], in which chromosome-specific probes for all chromosomes can be visualised simultaneously by multiple fluorochromes, used either individually or in combination.

Materials and Methods

A fluid suspension of chromosomes is prepared from colchicised peripheral lymphocyte cultures, lymphoblastoid cell lines, or from monolayer cultures in which the dividing cells have been shaken off after colchicine treatment for at least 6 h. The cells are resuspended in 75 mM KCl for 10 min and transferred to a buffer containing polyamines and Triton-X 100 [44]. Chromosomes are released into suspension by 12 s rapid vortexing, stained in Chromomycin A3 (final concentration 40 µg/ml) and Hoechst 33258 (final concentration 2 µg/ml) in the presence of magnesium sulphate (final concentration 2 mM), and left for 2 h at 4°C. Fifteen minutes prior to flow sorting, sodium citrate (final concentration 10 mM) and sodium sulphate (final concentration 25 mM) are added to the sample. It is important to avoid excessive disruption of the mitotic cells as this leads to chromosome fragmentation, indicated by an unacceptable level of debris at the lower end of the flow karyotype. (Cultures which contain a high proportion of cells in anaphase or telophase may also lead to reduced resolution due to contamination from the inclusion of anaphase chromosomes).

Chromosomes are sorted using a commercial fluorescence-activated cell sorter equipped with two 5-watt argon ion lasers. One laser is tuned to emit 300 mW in the UV (351–364 nm) to excite Hoechst fluorescence, and the second laser is tuned to emit 300 mW at 458 nm to excite Chromomycin fluorescence. The chromosome suspension, surrounded by sheath fluid, passes through the two laser beams sequentially to permit the fluorescence signal emitted from each chromosome to be collected separately and stored in the computer. As the chromosomes pass through the two lasers, the fluid stream breaks into a series of droplets, some of which will contain a single chromosome. Sorting is achieved by applying an electrical charge to the droplets containing the chromosome of interest so that they can be deflected into a container as they pass between two high-voltage plates. Highly pure samples of two chromosomes can be collected by the instrument simultaneously. The fluorescence measurements from each chromosome are accumulated in large numbers in the computer and used to construct a flow karyotype (fig. 1a) which reveals discrete clusters of signals, each cluster representing one or more chromosome types arranged in order according to overall size and base pair ratio. A-T-rich chromosomes sort above a diagonal line drawn through the middle of the chromosome clusters, while G-C-

rich chromosomes sort below this line. The accumulation of signals can be observed on a video screen and the chromosome of interest selected for sorting by a simple gating procedure. As chromosomes pass through the laser beams at a rate of 200 per second it only takes a few minutes to collect the 300–500 chromosomes required for PCR mapping and for the production of chromosome-specific paint probes. Larger samples of approximately 10,000 chromosomes are required for filter hybridisation and even greater numbers of over one million must be collected for preparing chromosome-specific libraries.

Chromosome-specific paint probes are made from 300–500 sorted chromosomes by DNA amplification using DOP-PCR as described by Telenius et al [41]. After a primary round of DNA amplification using the 6 MW primer (5' CCGACT CGA GNN NNN NAT GTGG 3') and unlabelled deoxynucleotide triphosphates, a secondary round of amplification is made, this time incorporating a labelled nucleotide such as biotin-11-dUTP. The biotinylated probe is hybridised to denatured chromosome preparations using standard FISH methods and can be detected using fluorochrome-conjugated streptavidin or anti-biotin antibodies. Alternatively, the nucleotides can be labelled directly with a variety of fluorescent dyes including fluorescein isothiocyanate (FITC) and the more recent Cy-dyes (Cy3, Cy5), which avoid the need for the additional detection step.

For many painting applications, two-colour FISH using, for example, propidium iodide as a chromosome stain and an FITC-labelled chromosome-specific paint probe may suffice with standard fluorescence microscopy. However, there is an increasing need for multicolour FISH in which several DNA probes are used, each labelled with a different fluorochrome or with different combinations of fluorochromes. For these applications a digital fluorescence microscope capable of detecting fluorescent signals of low luminosity is essential, coupled with image processing systems. These depend on the addition of a sensitive monochromatic, cooled charged coupled device camera with an appropriate series of excitation and emission filters. A grey-scale image of the fluorescence emitted by each fluorochrome is acquired sequentially and merged to produce a composite picture in which each DNA probe is assigned a separate false colour. As the various grey-scale images must be aligned accurately in the merged image, a system to prevent registration errors has been found useful. This depends on the use of triple dichroic band-pass filters in the epifluorescence optical path with individual excitation filters mounted on a computer-controlled motorised filter wheel in front of the lamphouse; filter changes can then be made without the risk of moving the stage or objectives. The image-processing systems used in digital fluorescence microscopy allow the fluorescence images to be digitised and acquired by a personal computer and archived onto optical disc or computer tape. The stored image is then available for colour adjustment, image enhancement, image reversal and a number of other procedures which assist in chromosome measurement and analysis. Spatial filters and contrast adjustment are particularly useful in enhancing the weak chromosome-banding patterns obtained by DAPI counterstaining. The recent advent of spectral imaging, which enables the measurement of definitive emission spectra simultaneously from multiple spectrally overlapping DNA probes [43], is a major development which has added a new dimension to molecular cytogenetic analysis based on chromosome sorting and painting.

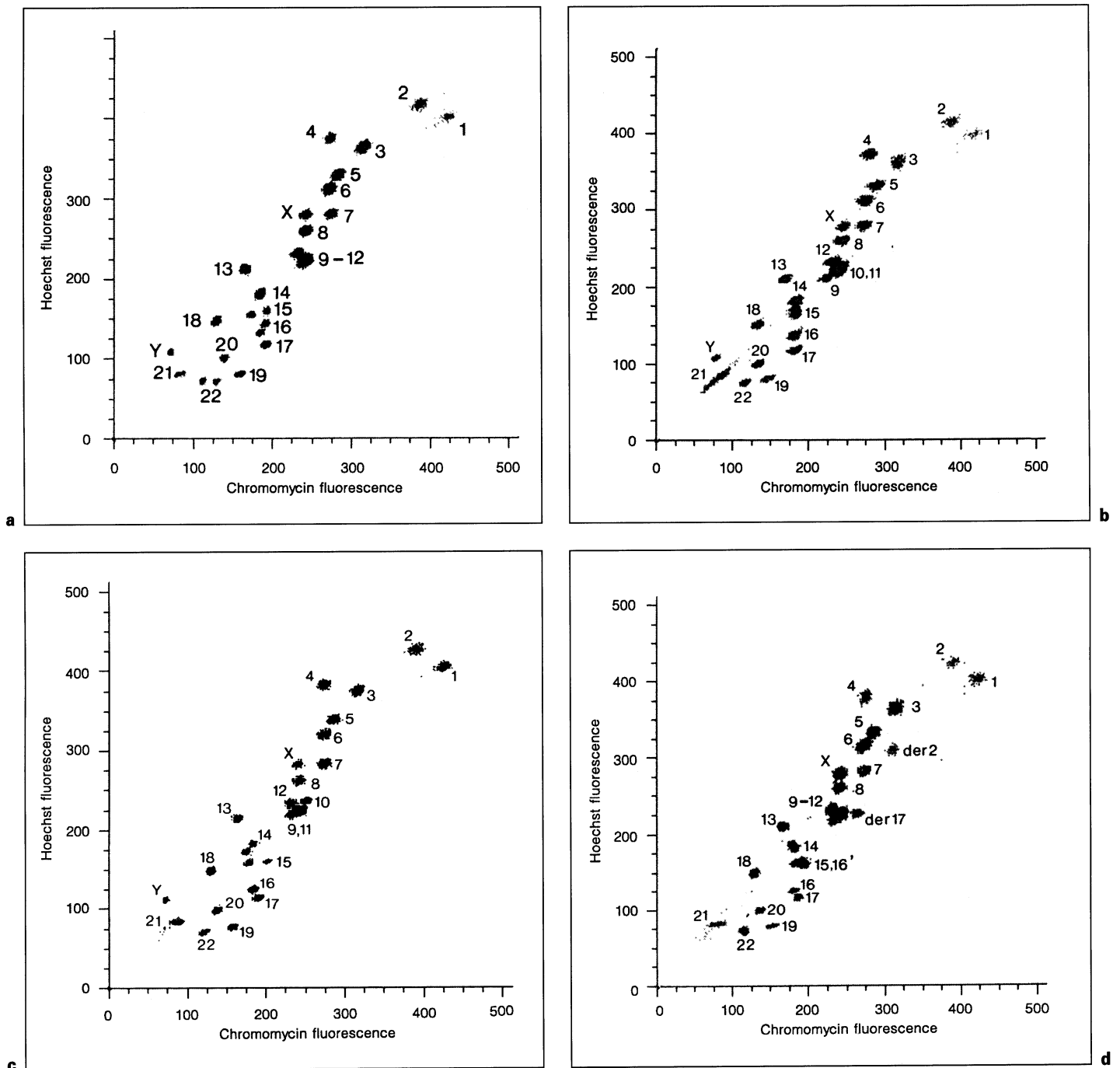


Fig. 1. Bivariate flow karyotypes produced from chromosome suspensions stained by Hoechst 33258 and chromomycin A3 and analysed in a fluorescence activated cell sorter. **a** Flow karyotype from a normal male cell line with only chromosomes 9–12 unresolved by size and base pair composition. Note that chromosomes 15, 16 and 22 are resolved into their separate homologues. **b** Flow karyotype of cell line from a normal male heterozygous for deletion of part of the centromeric heterochromatin of chromosome 9. This has been used to sort the variant chromosome 9 from other members of the 9–12 group. Chromosome 15 is resolved into separate homologues. **c** Flow karyotype from an individual heterozygous for an intrachromosomal duplication of chromosome 10. This small change has permitted the sorting of chromosomes 10 and 12 from 9 and 11. Chromosomes 14 and 15 resolve into separate homologues. **d** Flow karyotype of female heterozygous for a t(2;17)(q31;q25) reciprocal translocation; the two derivative translocations are marked. The two chromosomes 16 are heteromorphic. Normal chromosomes 2 and 17 are represented by only one homologue.

Chromosomal Polymorphism and the Production of Chromosome-Specific Libraries

The well-known variation in amounts of non-transcribed highly repetitive, DNA sequences in the human genome is reflected in the variation observed in the flow karyotypes of different individuals [22]. The most striking differences can be followed easily through a pedigree, using flow karyotyping of individual family members [25]. The most common of the chromosomal heteromorphisms involve chromosomes 1, 9, 16, the five pairs of acrocentric chromosomes, numbers 13, 14, 15, 21, 22 and the Y chromosome. Most of the variation involves the satellite DNAs which are commonly clustered at the centromeric regions of autosomes and in the distal long arm of the Y chromosome. Often, variation between individual members of a pair of homologous chromosomes is sufficient to allow them to be sorted individually. Several examples are shown in figure 1a-c.

Chromosomal heteromorphisms have been exploited to make chromosome-specific DNA libraries free from contamination with other chromosomes [23]. This has been particularly important in constructing libraries and paint probes of chromosome 9, which commonly sorts together with chromosomes 10, 11 and 12 (fig. 1a). The use of cells from an individual heterozygous for a deletion involving only the centromeric heterochromatin avoids the problem of contamination (fig. 1b). A similar strategy has been used to sort uncontaminated samples of chromosomes 10 and 12. Figure 1c shows a flow karyotype from an individual heterozygous for an intrachromosomal duplication of chromosome 10; this small change has been sufficient to separate chromosomes 10 and 12 from 9 and 11 and pure samples of both chromosomes 10 and 12 have been obtained from this preparation.

The first chromosome-specific library, an X-chromosome specific library, was prepared from sorted chromosomes and used to obtain polymorphic markers which helped to map the Duchenne muscular dystrophy locus to Xp21.2 by linkage studies [37]. At the time of writing, DNA libraries have been made from all human chromosomes, either by sorting directly from a tissue culture or from a man:rodent interspecific somatic cell hybrid. More recently, microdissection has been used to isolate chromosomes for chromosome-specific paint probes [42]. Microdissection has the advantage that regional paints can be made by dissecting individual chromosome arms or regions. Even smaller chromosome segments can be isolated for libraries and paints by coincidence cloning [45]; this involves sorting two chromosomes, one of which contains a region of

overlap with the other. Thus a paint probe is made from chromosome A which is then hybridised to multiple copies of chromosome B fixed on a microscope slide; the coincident region of A anneals to the coincident region of chromosome B and the slide is washed at high stringency to remove unhybridised A probe. The B chromosomes are treated with alkali and the eluted A probe is collected and amplified by PCR. The amplified products can then be used to make a region-specific library or to isolate homologous sequences from either genomic or cDNA libraries.

Gene Mapping

Chromosome-specific libraries made from sorted chromosomes have been widely used for isolating chromosome-specific probes. Sorted chromosomes can also be used as reagents in assigning cloned DNA sequences to their respective chromosome or chromosome region. One method uses a panel of chromosome dot blots composed of groups of 10,000 chromosomes of each type sorted directly onto nitrocellulose filters which are then baked [28]. The probe of interest is radiolabelled, hybridised to the panel of dot blots under appropriate conditions and then washed at high stringency. Autoradiography readily identifies the dot blot to which the probe has annealed [29]. Regional localisations can be made using a series of sorted translocation chromosomes which cover the chromosomal region of interest [30].

Partially sequenced DNA clones (i.e. with a sequence-tagged site) can be assigned to their respective chromosome locations by PCR amplification of the sequence-tagged site using specific DNA primers on a panel of sorted chromosomes. Once again, a more specific regional localisation can be achieved using sorted translocation derivatives with break points across the region.

Forward and Reverse Chromosome Painting

One of the most frequent applications of FISH is in the analysis of complex chromosome aberrations, either in diagnostic cytogenetics or in the analysis of cancer chromosomes. The chromosomal origin of de novo aberrations which cannot be resolved by conventional chromosome banding, can be identified by chromosome painting. Usually, the application of commercial chromosome specific paint probes (forward chromosome painting) is adequate, particularly if there is a clue as to which chromosomes are most likely to be involved. For example, a de

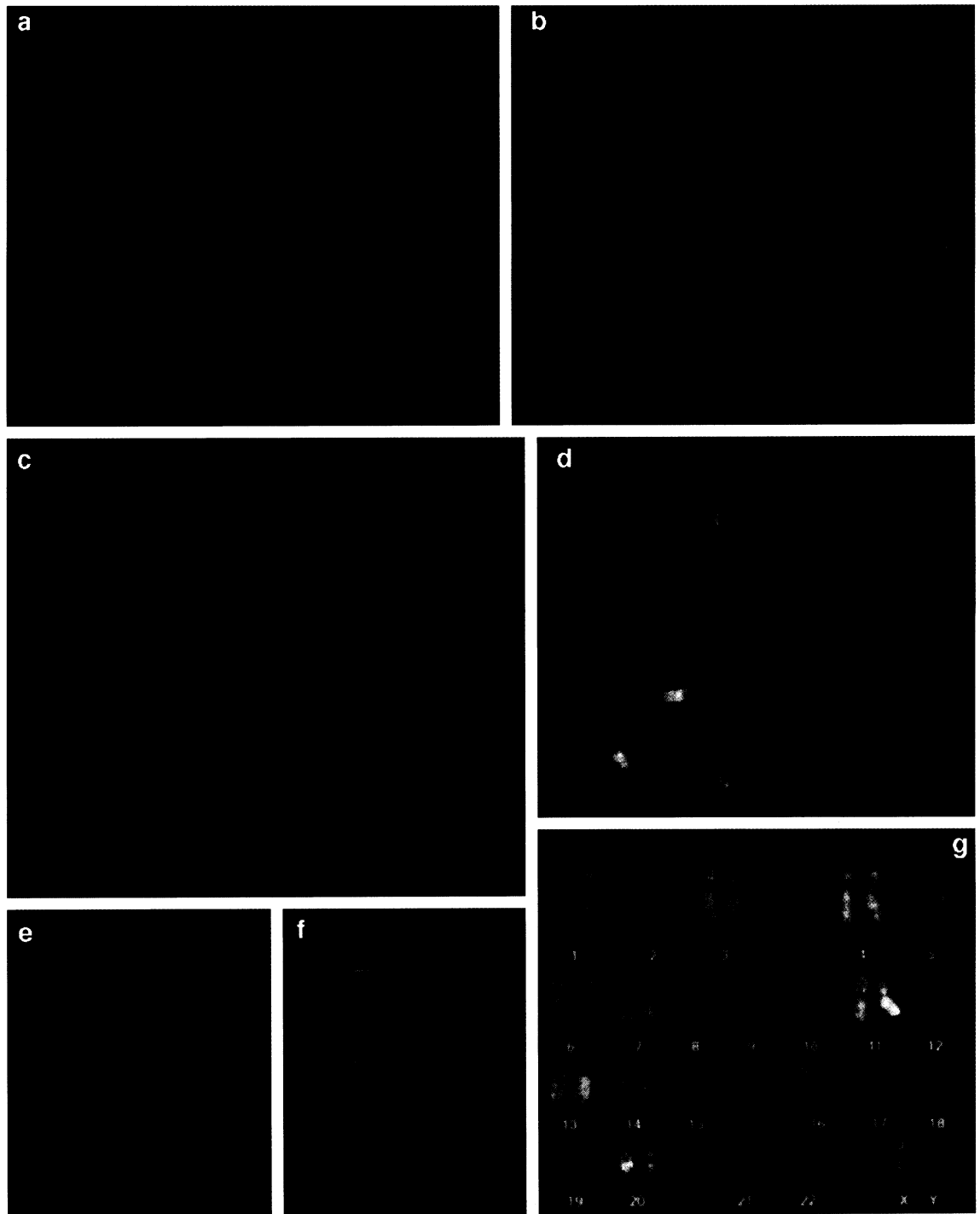


Fig. 2. **a** Dual-colour reverse chromosome painting of the two derivative translocations 2 and 17 shown in the flow karyotype in figure 1d. The paint probe from the chromosome 2 derivative is detected by FITC (green) and that from the chromosome 17 derivative is detected by Cy3 (red). When these paints are hybridised to *normal* chromosomes 2 and 17, they reveal the breakpoints and extent of the exchange associated with the translocation. **b** Cross-species chromosome painting. Mouse chromosome 11 paint hybrid-

ised to a normal human metaphase reveals regions of homology on human chromosome 17p and 17q, 5p14–15, 2p15–21, and 7q21–31, with weaker signals on chromosome 22q11 and some other chromosomes. The areas of homology are consistent with comparative genetic mapping data. **c** Cross-species chromosome painting: mouse chromosome 11 paint hybridised to Chinese hamster metaphase, reveals areas of homology on hamster chromosomes 1p and 7q only. **d** Cross-species chromosome painting. Human chromosome specific paint

novo chromosomal duplication is much more likely to be intrachromosomal than the result of an exchange with another chromosome. Therefore, the paint probe of the corresponding chromosome should always be used first. If the duplication remains unpainted, then a succession of probes or probe mixtures can be tried until the correct one is identified. Alternatively, if chromosome sorting is available, the abnormal chromosome can be sorted, labelled by DOP-PCR and used as a paint probe (reverse chromosome painting) onto a normal metaphase cell [31]. For example, the flow karyotype of an individual heterozygous for a reciprocal translocation between the long arm of chromosome 2 (breakpoint at 2q31) and the distal end on the long arm of chromosome 17 (breakpoint at 17q25) is shown in figure 1d. The two translocation derivatives of chromosomes 2 and 17 are the approximate size of chromosomes 6 and 12, respectively. In order to determine the size of the fragment from chromosome 17 involved in the translocation, approximately 500 copies of the two derivative chromosomes were sorted into separate tubes, amplified by DOP-PCR and labelled with biotin and Cy3 fluorochrome, respectively. The labelled paint probes were then hybridised to normal metaphases and the derivative chromosome 2 paint detected with streptavidin coupled with FITC while the derivative chromosome 17 paint was visualised directly by the Cy3 label. The normal metaphase in figure 2a shows the chromosomes 2 and 17 painted in both red and green indicating the particular composition of the two derivative chromosomes; only a small part of the distal end of chromosome 17 is involved in the derivative chromosome 2 (green) whereas nearly half of the long arm of chromosome 2 is involved in the derivative chromosome 17 (red).

Cross-Species Reciprocal Chromosome Painting

Chromosome-specific paints derived from sorted chromosomes have been made from a number of different species in recent years. A list of species sorted in the Molecu-

lar Cytogenetics Research Laboratory, Cambridge, is shown in table 1. The chromosome paints are being used in comparative mapping experiments, and in the case of the mouse, for assistance with chromosome analysis and radiation dosimetry experiments. In view of the similarity between many of the chromosomes in some species, e.g. the mouse [46], dog [47] pig [48] and sheep [49], the availability of sorted chromosomes and chromosome-specific paint probes has greatly assisted routine cytogenetic studies and has been helpful in resolving mapping problems.

Flow karyotypes in the mouse made from short-term lymphocyte cultures (from spleen) are of remarkably high resolution [46] but because several chromosomes are of similar DNA content and base-pair ratio, a number are difficult to sort apart. We have therefore used a judicious selection of several different inbred mouse strains, each homozygous for a number of centric chromosome polymorphisms, which has allowed the construction of a complete panel of 21 sorted chromosomes (including X and Y) and chromosome-specific paint probes (fig. 3, table 2). Each mouse strain has a characteristic flow karyotype which is reproducible within the strain (unpublished data). The chromosomal heteromorphisms are similar to the human heteromorphisms previously mentioned and reflect variable amounts of satellite DNA at the centromeres. Like the human, the Y chromosome is highly variable being largest in the BALB/c strain of *Mus musculus* (fig. 3c) and smallest in the *Mus spretus* sub-species (fig. 3f). The X chromosome of *M. spretus* is also smaller than in the other strains.

In view of the conservation of genes between species, chromosome-specific paint probes from one species are able to identify syntenic blocks of homology in other species. We have confirmed this in the mouse, where the genetic map is sufficiently advanced to allow detailed comparison with the human map, by hybridising mouse paints to human chromosomes. Figure 2b shows that a chromosome paint probe made from mouse chromosome 11 hybridises to at least 5 specific regions of human chromosomes for chromosome 1 (red) chromosome 2 (green) and chromosome 6 (yellow) hybridised to a metaphase from *Hylobates hoolock*. Chromosomes 1 and 2 are each homologous to regions on 5 different chromosomes and chromosome 6 is homologous to parts of two chromosomes. **e** Cross-species chromosome painting. Human chromosome 1 paint hybridised to Indian muntjac metaphase (male) reveals areas of homology on muntjac chromosomes 1 and 3 (see fig. 4). **f** Cross-species chromosome painting. Six chromosome-specific paint probes from Chinese muntjac chromosomes have been hybrid-

ised to Indian muntjac chromosome 3. The order of these probes from centromere (top) are: chromosome 18 (green), 6 (red), 5 (green), 9 (red), 16 (green) and 21 (red). Note that, with the exception of chromosome 5 (part of which is homologous to a region on Indian muntjac chromosome 1), segments homologous to complete Chinese muntjac chromosomes are arranged in tandem and that fragments of centric heterochromatin (revealed by the red C5 probe) are detectable at the points of fusion. **g** Multicolour spectral karyotype of normal male metaphase.

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Table 1. Species used for chromosome sorting, with diploid number

Human (<i>Homo sapiens</i>) 46	Bat (<i>Tadarida brasiliensis</i>) 48
Chimpanzee (<i>Pan troglodytes</i>) 48	Goose (<i>Anser anser</i>) 78
Pygmy chimpanzee (<i>P. paniscus</i>) 48	Quail (<i>Coturnix coturnix</i>)
Gorilla (<i>Gorilla gorilla</i>) 48	Cat (<i>Felis catus</i>) 38
Orangutan (<i>Pongo pygmaeus</i>) 48	Giant panda (<i>Ailuropoda melanoleuca</i>) 38
White-handed gibbon (<i>Hylobates lar</i>) 44	Mouse (<i>Mus musculus domesticus</i> and <i>Mus musculus castaneus</i>) 40
White-browed gibbon (<i>H. hooleck</i>) 38	Short-tailed mouse (<i>Mus spretus</i>) 40
White-cheeked gibbon (<i>H. leucogenys</i>) 52	Chinese hamster (<i>Cricetulus griseus</i>) 22
Siamang gibbon (<i>H. syndactylus</i>) 52	Rat (<i>Rattus norvegicus</i>) 42
Slow loris (<i>Nycticebus intermedius</i>) 50	Domestic sheep (<i>Ovis aries</i>) 54
Rhesus monkey (<i>Macaca mulatta</i>) 42	Domestic yak (<i>Bos grunniens</i>) 60
Pig-tailed macaque (<i>M. nemestrina</i>) 42	Reindeer (<i>Rangifer tarandus caribou</i>) 70
Silvered leaf monkey (<i>Presbytis cristata</i>) 44	Red deer (<i>Cervus elaphus</i>) 68
Vervet monkey (<i>Cercopithecus aethiops</i>) 60	Brown brocket deer (<i>Mazama gouazoubira</i>) 70
Snub-nosed monkey (<i>Rhinopithecus bieti</i>) 44	Tufted deer (<i>Elaphodus cephalophus</i>) 46/48
Marmoset (<i>Callithrix jacchus</i>) 46	Indian muntjac (<i>Muntiacus muntjak vaginalis</i>) 6, 7
Black lemur (<i>Eulemur macaco</i>) 44	Chinese muntjac (<i>M. reevesi</i>) 46
Brown lemur (<i>Eulemur fulvus</i>) 60	Black muntjac (<i>M. crinifrons</i>) 8, 9
Tree shrew (<i>Tupaia belangeri</i>) 60	Tammar wallaby (<i>Macropus eugenii</i>) 14
	Brush-tailed possum (<i>Trichosurus vulpecula</i>) 18
	Long-nosed potoroo (<i>Potorus tridactylus</i>) 11

Table 2. Chromosomes which sort independently (+) in different mouse inbred strains

Chr. No.	PL/J	BALB/c	Spretus	129Sv	C3H/He	C57BL/10	101
1	-	-	+	-	+	+	-
2	-	-	+	-	+	+	-
3	+	+	+	+	+	+	+
4	+	+	-	+	-	+	-
5	+	+	-	+	-	+	-
6	+	+	-	+	+	+	+
7	+	+	+	+	+	+	+
8	+	+	+	-	+	-	-
9	+	-	-	+	+	-	+
10	+	-	-	-	-	-	-
11	+	+	-	-	+	+	-
12	+	-	+	-	-	-	-
13	-	-	-	-	+	+	-
14	-	-	+	+	+	-	+
15	-	+	+	+	+	+	+
16	-	+	+	+	+	-	+
17	+	+	+	+	+	-	+
18	-	+	+	+	+	+	+
19	+	+	+	+	+	+	+
X	+	+	+	+	+	+	+
Y	+	+	+	+	+	+	+

mosomes 17, 5p, 2p, 22q, and 7q as expected from previously known man:mouse homologies. Hybridisation of mouse chromosome 11 paint to the chromosomes of the Chinese hamster reveals two much larger syntenic blocks (fig. 2c). As expected, fewer rearrangements have occurred during the evolutionary divergence of the two rodent species than have occurred between the divergence of mouse and man.

Having established that cross-species ISH is a reliable indicator of DNA conservation, we are in the process of establishing comparative maps of the species listed in table 1. The success of the method relies on the well-known extensive divergence of repetitive DNA between even closely related species. To ensure that only conserved expressed sequences are involved in cross-species hybridisation, it is helpful to allow the chromosome-specific probe to pre-anneal with itself for up to 1 h before applying it to the denatured chromosome preparations [14]. This effectively removes non-specific background hybridisation due to repetitive DNA dispersed throughout the chromosomes. An example of the construction of a comparative map between the Indian muntjac and human is shown in figure 4: in this example the sites of homology of the entire human chromosome complement can be identified within the three pairs of Indian muntjac chromosomes [50]. Figure 2e shows the hybridisation of human chromosome 1-specific paint to the three major

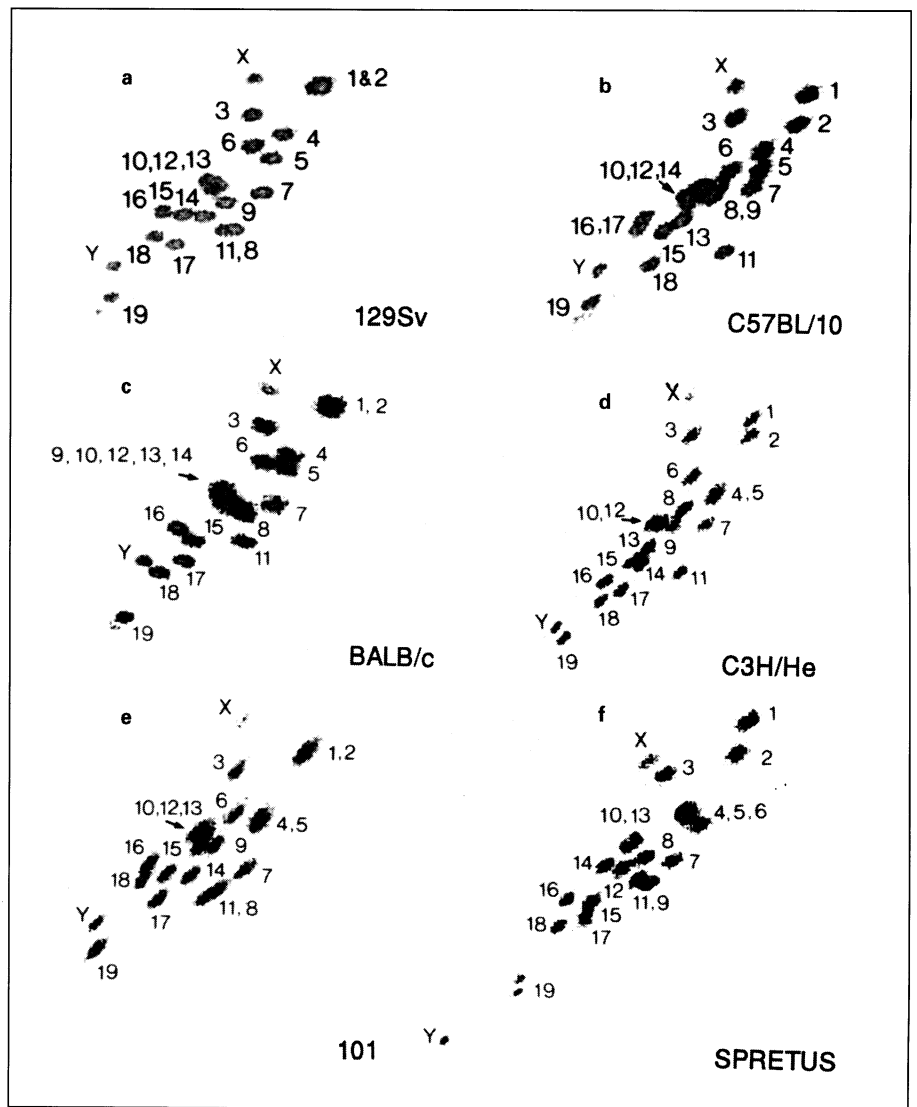


Fig. 3. Flow karyotypes of 5 inbred mouse strains and one subspecies (*M. spretus*). Various centric heteromorphisms allow each individual mouse chromosome to be sorted from a panel of inbred strains. Note variation in size of Y chromosome and also the small X chromosome in *M. spretus*.

sites on Indian muntjac chromosomes 1 and 3. By the reciprocal procedure of painting Indian muntjac chromosome-specific paints to human metaphases (not shown), it is possible to identify the intrachromosomal derivation of each separate hybridisation site when a specific chromosome paint hybridises to more than one chromosome.

Reciprocal chromosome painting is a convenient method for the rapid construction of a preliminary genetic map of any species, based on the human genetic map (fig. 4). It can provide the foundation on which the construction of more detailed physical and genetic maps is based. It may simplify the identification of disease genes in animals and, at the same time, lead to the localisation of genes important for quantitative and behavioural traits in man.

Reciprocal chromosome painting has important applications in the study of karyotype evolution and phylogeny, particularly of distantly related species. It complements the use of chromosome banding and gene mapping with somatic cell hybrids which have given important insights into the genome evolution of primates, rodents and other animals. Linkage groups have been found to be conserved intact in species which have diverged many million years ago. However, these techniques give only an imprecise picture of the chromosomal rearrangements which have occurred during evolution, and banding patterns can be interpreted with confidence only in closely related species [51]. Chromosome painting demonstrates the major patterns of homology which, in almost all mam-

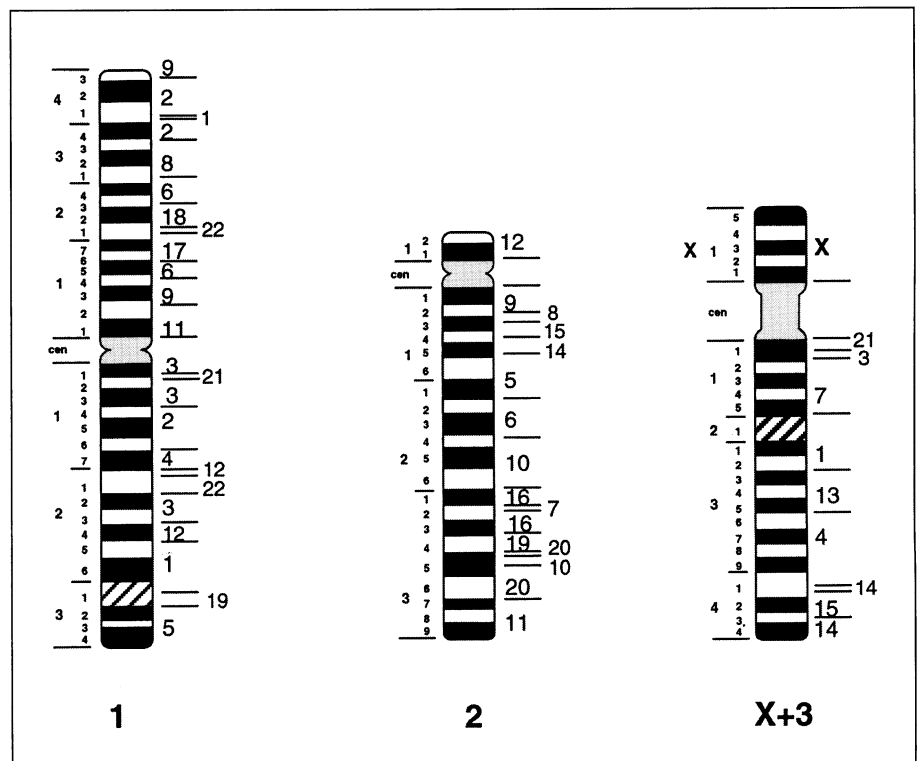


Fig. 4. Comparative idiogram showing the homology of all human chromosomes to the three pairs of chromosomes of the Indian muntjac as revealed by cross-species chromosome painting using human chromosome-specific paints hybridised to muntjac chromosomes.

malian species, are evident in comparatively large blocks of synteny easily identified in the fluorescence microscope [52]. For example, while the great apes and humans show almost complete homology, with the well-known exception of the fusion of the ancestral chimpanzee chromosomes 12 and 13 to form what has become human chromosome 2, and the rearrangement between ancestral human 5 and 17 to produce chromosomes 4 and 19 in the gorilla, the lesser apes show that numerous rearrangements have occurred since the existence of their common ancestor with the great apes. Thus, the white-cheeked gibbon (*Hylobates concolor*) shows over 31 translocations when painted with chromosome-specific human paint probes [53]. Figure 2e shows a metaphase from the white-browed gibbon (*Hylobates hoolock*) hybridised with human chromosome-specific paints for chromosomes 1 (red), 2 (green) and 6 (yellow); chromosome 1 is homologous to parts of 5 pairs of gibbon chromosomes, chromosome 2 to 5 different pairs and chromosome 6 to parts of two pairs of gibbon chromosomes.

Assumptions about cross-species homology from G-banding and other non-molecular techniques can be misleading. An early conclusion that nucleolus-organising chromosomes were shared between Old World monkeys

and lesser apes (gibbons) was disproved by chromosome painting, while at the same time showing that regions homologous to human chromosome 22 were present in the nucleolus-organising chromosomes of all Old World monkeys, great apes and humans [54]. Similarly, the postulated translocation between chromosomes in the orangutan homologous to human chromosomes 8 and 20 was refuted by chromosome painting [55].

Chromosome painting across mammalian species suggests that segments which have diverged in humans are linked in widely divergent species. For example, chromosomal segments homologous to human chromosomes 14 and 15 are linked on the same chromosomes in all lower monkeys and non-primate species studied to date (including Indian muntjac, fig. 4). Segments homologous to chromosomes 3 and 21 are similarly linked in species as different as the tree shrew, cat, pig and mouse [56]. It seems likely that linkages such as these are ancestral to all mammalian species, and that our understanding of genome evolution is likely to be illuminated by extending these chromosome-painting comparisons even to other non-mammalian vertebrates.

A striking example of chromosome fusion during evolution is shown by comparative studies in various deer

species. These suggest that the ancestral karyotype of many deer species had a diploid chromosome number of 70. The diploid number of the Indian muntjac (*Muntiacus muntjak vaginalis*) is $2n = 6,7$ and in the Chinese muntjac (*Muntiacus reevesi*) is $2n = 46$. In both cases, the reduction in chromosome number from 70 can be demonstrated by chromosome painting to be due largely to a simple process of fusion of whole chromosomes [35, 57]. Fragments of ancestral centric heterochromatin can be demonstrated between almost every syntenic block (fig. 2f).

Chromosome painting on its own has limitations in the study of chromosome aberrations and karyotype evolution for it cannot identify many types of intrachromosomal rearrangement, such as paracentric inversions and insertions, within segments homologous to a single chromosome. Moreover, it cannot identify the orientation of homologous chromosome segments within a chromosome in relation to the centromere. Subregional specific paints, and specific cosmid and YAC DNA clones are often used in conjunction with chromosome paints to solve these problems [58, 59]. More recently, colour bar code probes have been used to confirm and extend our knowledge of intrachromosomal rearrangements in the great apes [unpubl. data].

Multiplex-FISH and Spectral Karyotyping

For a number of years it has been possible to use several DNA probes simultaneously in multicolour FISH, and this has considerably extended the applications of molecular cytogenetics. Up to 7 different chromosome-specific paint probes can be used together in digital fluorescence microscopy by labelling each with a different combination of 3 fluorochromes [60]. The technique has now been extended by Speicher et al. [61] using different combinations of 5 fluorochromes to label each of the human chromosomes (or chromosome arms) in a 27-colour FISH termed 'combinatorial multicolor FISH' (M-FISH). The DNA probes are made from amplified, microdissected chromosomes and all fluorochromes were excited with a 75 watt xenon lamp. Both excitation and emission spectra were carefully selected using appropriate filter sets each with waveband widths in the range of 5–15 nm. The emission from each dye is analysed separately and the various images merged to provide the final image. The labelling procedure provides a specific spectral signature for each probe which the computer program translates into distinct false colours displayed on the computer monitor.

The method has high resolving power and its utility has been well demonstrated in the analysis of constitutional and cancer chromosome aberrations.

An alternative method for multicolour FISH has independently and simultaneously been developed by Schröck et al [43]. The method once again uses 5 different fluorochromes in different combinations to provide a distinctive label for each of the 24 human chromosome paints produced from flow-sorted chromosomes (fig. 2g). Fluorescence is excited by a xenon lamp and analysed through a triple filter set which allows all dyes to be excited and measured simultaneously with the SD-200 spectral bio-imaging system (Applied Spectral Imaging Ltd., Migdale Haemek, Israel) incorporating a Fourier transform spectrometer. This system acquires a conventional fluorescence image through the microscope, at the same time measuring the visible and near-infrared emitted light spectrum for each pixel in the image. The definitive spectrum which identifies the combination of dyes in each DNA probe is translated into a distinctive false colour which can be viewed on the computer monitor. A complete measurement of each metaphase takes about 50 s with a 15-nm spectral resolution; the computer then builds a spectral image of the metaphase which is available for visualisation, image processing and analysis. The system has been tested for its utility in the diagnosis of both constitutional and cancer chromosome aberrations and in cross-species reciprocal chromosome painting. Multicolour chromosome specific paints are available for all mouse chromosomes [62] as well as for human chromosomes and are to be expected for other species as well. The resolving power is excellent and there is no question that spectral karyotyping will have an important place in molecular cytogenetics where it can be expected to advance our knowledge of cancer cytogenetics and karyotype evolution.

Acknowledgements

The author is grateful for helpful discussions with Johannes Wienberg and colleagues in the human molecular cytogenetics laboratory and, in particular, to Fengtang Yang for the hybridisations illustrated here and to Patricia O'Brien for flow sorting all the species listed in table 1. The work of the laboratory is supported by an MRC Programme Grant (MAF-S).

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