THE NEW CYTOGENETICS: BLURRING THE BOUNDARIES WITH MOLECULAR BIOLOGY

Michael R. Speicher and Nigel P. Carter‡*

Exciting advances in fluorescence *in situ* hybridization and array-based techniques are changing the nature of cytogenetics, in both basic research and molecular diagnostics. Cytogenetic analysis now extends beyond the simple description of the chromosomal status of a genome and allows the study of fundamental biological questions, such as the nature of inherited syndromes, the genomic changes that are involved in tumorigenesis and the three-dimensional organization of the human genome. The high resolution that is achieved by these techniques, particularly by microarray technologies such as array comparative genomic hybridization, is blurring the traditional distinction between cytogenetics and molecular biology.

BANDING
A method that uses chemical treatments to produce differentially stained regions on chromosomes.

**Institut für Humangenetik, Technische Universität München, Trogerstrasse 32, D-81675 München and GSF-Nationales Forschungszentrum für Umwelt und Gesundheit, Ingolstädter Landstrasse 1, D-85764 Neuherberg, Germany.*
‡The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, CB10 1SA, United Kingdom.
Correspondence to M.R.S.
e-mail: speicher@humangenetik.med.tu-muenchen.de
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Traditionally, the term cytogenetics has referred to studies of the cellular aspects of heredity, especially the description of chromosome structure and the identification of genomic aberrations that cause disease. Cytogenetics has been used for many years for various applications, from clinical diagnostics to basic genomic research. Conventional chromosome analysis, which is based on BANDING and was developed in the 1970s (BOX 1), is still widely used. However, over the past 25 years molecular cytogenetic techniques of increasingly higher resolution have been developed.

Molecular cytogenetics is usually based on fluorescence *in situ* hybridization (FISH) (FIG. 1). In this technique a labelled DNA probe is hybridized to cytological targets such as metaphase chromosomes, interphase nuclei, extended chromatin fibres or, in the most recent development, DNA microarrays. As cytogenetic methods have traditionally been based on microscopic examination of individual cells or chromosomes, it can be argued that the latest genome-scanning methods, such as microarray technologies, cannot be classed as cytogenetic methods. However, the cytogenetic use of these new technologies is designed to provide a description of chromosome structure at a resolution that exceeds that of microscopic analysis. Therefore, these technologies bridge the gap between cytogenetic and molecular approaches.

The latest cytogenetic technologies have become important components of molecular diagnostics, particularly for diagnosing congenital syndromes in which the underlying genetic defect is unknown. These methods are also widely used in cancer research for the identification of regions that harbour tumour-suppressor genes or oncogenes. Another important feature of molecular cytogenetic methods is the ability to analyse single cells, which, in light of the heterogeneity of primary tumours and metastases, is likely to provide new insights into tumour progression and invasiveness. Molecular cytogenetics is also increasingly used to study normal cell biology and genetics. For example, cytogenetic analysis using array-based technologies has highlighted the extent of large-scale copy number variation between individuals of the same species. Furthermore, cytogenetic techniques are now being used to unravel the three-dimensional organization of the genome, and even epigenetic features of higher-order chromatin structure.

Here we review recent advances in cytogenetic technologies, especially those that use array platforms, and highlight how the field is moving away

BIOTIN
A vitamin and mobile carrier of activated CO_2 that has a high affinity for avidin and is used for non-radioactive labelling.

COT-1 DNA
DNA that is mainly composed of repetitive sequences. It is produced when short fragments of denatured genomic DNA are re-annealed.

METAPHASE SPREAD
Preparations of chromosomes in dividing cells that have been artificially arrested at metaphase, when chromosomes are highly condensed and shortened, so that they are visible under a light microscope.

DEGENERATE OLIGONUCLEOTIDE-PRIMED PCR
A method for the unbiased amplification of any DNA source using partially degenerate primers.

CHROMOSOME MICRODISSECTION
A technique in which an entire chromosome or region of a chromosome (for example, a chromosome arm or a chromosome band) is isolated using a micro-manipulated glass needle or highly focused laser beam. The sample is then transferred to a tube for subsequent amplification and probe labelling.

FLUOROCHROMES
Non-radioactive labels that can emit fluorescence after excitation by light. Also known as fluorophores.

NICK TRANSLATION
A widely used method for DNA-probe labelling. Nick translation uses a combination of DNase I to nick double-stranded probe DNA, and the polymerase and endonuclease activity of DNA polymerase I to proceed along the target strand from the nicks, incorporating labelled nucleotides.

RANDOM-PRIMED LABELLING
A method for labelling single-stranded probe DNA that uses a mixture of random short oligonucleotides to prime the incorporation of labelled nucleotides using polymerase.

HAPTEN
A small molecule that has binding affinity for a protein receptor.

Box 1 | A brief history of cytogenetics

Early uses of cytogenetics

The first images of human chromosomes date back to the nineteenth century[107–109]. However, it took several decades to establish 46 as the correct chromosome number in humans[110,111]. When this was finally achieved in 1956, the availability of reliable methods for obtaining chromosome preparations paved the way for the identification of numerical aberrations in patients with Down syndrome[112] in 1958, and Klinefelter syndrome[113] and Turner syndrome[114] in 1959. In tumour cytogenetics, one of the most exciting findings was the identification in 1960 of a minute chromosome, later named the Philadelphia chromosome, which was regularly found in the peripheral blood of patients with chronic myeloid leukaemia[115].

Chromosome banding

More detailed chromosome analyses had to await the development of chromosome banding in the late 1960s. Using fluorochromes that were coupled to an alkylating agent (such as quinacrine mustard), a highly characteristic fluorescence pattern for each chromosome could be obtained and a complete human karyotype identified[116]. In the 1970s, Giemsa banding rapidly displaced quinacrine banding. The improvement in the resolution of chromosome analyses by banding revealed in 1973, 13 years after its initial discovery, that the Philadelphia chromosome was not the result of a deletion in chromosome 22, but of a translocation between chromosomes 9 and 22 (REF. 117).

Molecular cytogenetics

The first application of molecular techniques to chromosome cytology was based on the observation that complementary nucleotide sequences could anneal or hybridize to each other to form more stable complexes than non-complementary sequences. The first *in situ* hybridization analysis was reported in 1969 by Joe Gall and Mary Lou Pardue, who used DNA–RNA hybridization to localize the genes that encode ribosomal RNA[118].

Advances in detection

Early *in situ* hybridizations depended on radioactive detection, followed in 1977 by the introduction of fluorescently labelled antibodies that recognized specific DNA–RNA hybrids[119]. A more straightforward approach used the chemical coupling of a fluorochrome to an RNA probe for rapid and direct visualization, known as fluorescent *in situ* hybridization[120]. The coupling of a fluorochrome to a DNA or RNA probe is often referred to as 'direct labelling'. By contrast, 'indirect labelling' refers to the enzymatic or immunological detection of tags that have been incorporated into a probe. The syntheses of modified nucleotide derivatives that contain a BIOTIN label, which could be incorporated by polymerases into probes, was instrumental in the development of indirect labelling techniques[121]. A pre-incubation of a denatured complex probe with excess unlabelled total human DNA[122] or later, COT-1 DNA, allowed the specific staining of entire chromosomes[123–125].

from traditional methods towards molecular genetic approaches. We discuss advances in FISH, the effect of arrays on cytogenetics, and how such technologies have aided both diagnostics and basic research. Finally, we discuss how molecular cytogenetics is being used to understand the architecture of the genome.

Advances in FISH-based techniques

An important aim in the development of cytogenetic methods has been to increase the resolution at which chromosome rearrangements can be identified. This has been achieved by advances that involve the two crucial elements of cytogenetic analysis — the target and the probe. Target resolution has improved from the ability to study whole chromosomes in METAPHASE SPREADS (at a resolution of ~5 Mb), through the analysis of interphase nuclei (50 kb–2 Mb) to the level of chromatin strands (5 kb–500 kb) using fibre FISH. As discussed below, the use of DNA microarrays now provides high resolution down to the single-nucleotide level. Probe development has also advanced, with more effective labelling strategies and a wider range of clones and sequences available for probe generation (FIG. 2).

Advances in metaphase spread analysis. Following the sequencing of the human genome[1], large-insert clones that have been mapped and sequenced, and can be used as probes, are now readily available for almost any genomic region[2]. Probes can be selected easily using internet-browsers such as Ensembl Cytoview, NCBI Map-Viewer or the UCSC genome browser. Chromosome-painting probes, which stain entire chromosomes or chromosome regions, are also widely available, mainly owing to the development of universal PCR approaches that allow the amplification of large DNA regions. Perhaps most commonly used for this purpose is DEGENERATE OLIGONUCLEOTIDE-PRIMED PCR (DOP-PCR)[3]. It facilitates the production of chromosome paints from small numbers of flow-sorted chromosomes[4] and of sub-regional painting probes by CHROMOSOME MICRODISSECTION[5]. DOP-PCR has also been used for reverse chromosome painting[6], in which probes are generated from flow-sorted rearranged chromosomes and analysed by hybridization onto normal metaphase spreads. This process reveals the content and breakpoints of the rearranged chromosome.

Another important development, not only for the analysis of metaphase chromosomes but also for that of other cytogenetic targets, is the increase in the number of differentially labelled probes that can be hybridized and imaged. The discrimination of many more targets than the number of available, spectrally resolvable FLUOROCHROMES can be achieved using combinatorial labelling[7] or ratio labelling[8]. Combinatorial labelling uses the calculation of a Boolean spectral signature for each probe — each probe is identified by its

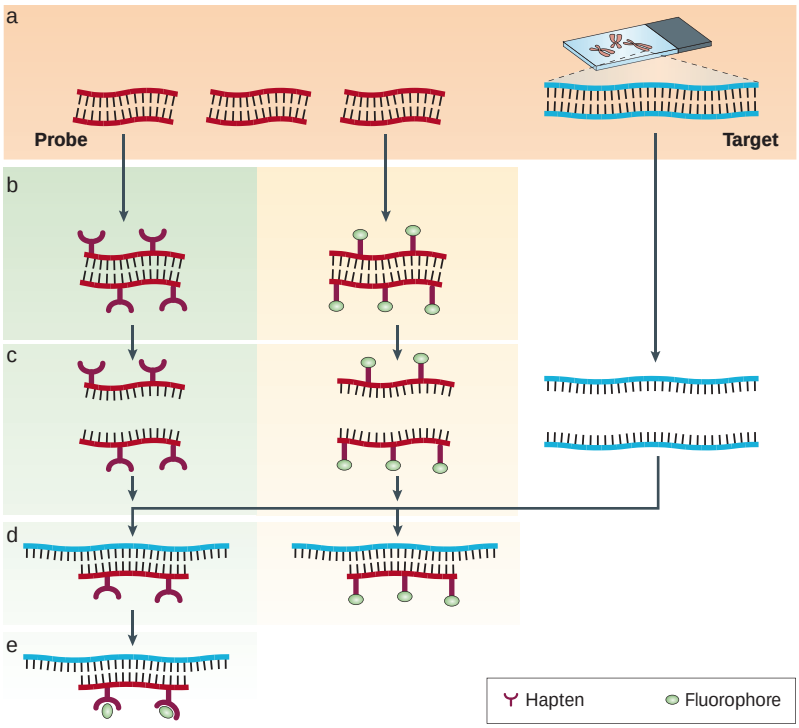


Figure 1| **Principles of fluorescence *in situ* hybridization. a** | The basic elements of fluorescence *in situ* hybridization are a DNA probe and a target sequence. **b** | Before hybridization, the DNA probe is labelled by various means such as NICK TRANSLATION, RANDOM-PRIMED LABELLING and PCR. Two labelling strategies are commonly used — indirect labelling (left panel) and direct labelling (right panel). For indirect labelling, probes are labelled with modified nucleotides that contain a HAPTEN, whereas direct labelling uses the incorporation of nucleotides that have been directly modified to contain a fluorophore. **c** | The labelled probe and the target DNA are denatured to yield ssDNA. **d** | They are then combined, which allows the annealing of complementary DNA sequences. **e** | If the probe has been labelled indirectly, an extra step is required for visualization of the non-fluorescent hapten that uses an enzymatic or immunological detection system. Whereas FISH is faster with directly labelled probes, indirect labelling offers the advantage of signal amplification by using several layers of antibodies, and might therefore produce a signal that is brighter compared with background levels. Finally, the signals are evaluated by fluorescence microscopy (not shown).

unique combination of absences and presences of each fluorochrome. In ratio labelling, different probes can be labelled with the same fluorochrome combinations, but are distinguished by the different proportions of the fluorochromes used.

These labelling strategies allow the simultaneous visualization of all 24 human chromosomes, each in a different colour, in a single hybridization. Specific technologies that use these approaches include MULTIPLEX-FISH (M-FISH)[9], SPECTRAL KARYOTYPING (SKY)[10] and COMBINED BINARY RATIO LABELLING (COBRA)[11], and have a wide range of uses. These include the characterization of structural interchromosomal aberrations and complex chromosomal rearrangements, which are often observed in tumour cells, and the analysis of marker chromosomes in prenatal and postnatal diagnostics[12]. Although the detection sensitivity for small interchromosomal rearrangements (involving <3 Mb of sequence) is poor using these multicolour approaches, resolution can be significantly improved by increasing the number of fluorochromes that are used for probe labelling[13].

More specific multicolour FISH assays have also been developed, for example to screen particular regions of the genome, such as those near telomeres[14,15]. Furthermore, identifying intrachromosomal rearrangements is facilitated by multicolour banding technologies, such as CROSS-SPECIES COLOUR SEGMENTATION[16], or by the use of overlapping microdissection libraries that are differentially labelled[17]. The latter method produces reproducible and unique patterns of fluorescence ratios along chromosomes, which can be transformed into a PSEUDOCOLOUR BANDING PATTERN using appropriate imaging software.

In addition to probe technology, there has also been considerable improvement in both the hardware and software that are used for the analysis of FISH images. COOLED CHARGE-COUPLED-DEVICE (CCD) cameras and fluorescence filter sets for microscopy that are more specific and efficient have improved the sensitivity and resolution of imaging, and sophisticated software facilitates the acquisition and processing of images.

Comparative genomic hybridization on chromosomes. The preparation of high-quality metaphase spreads, especially from solid tumours, is often difficult. As a consequence, leukaemias, from which metaphase chromosomes are readily obtained, are more thoroughly investigated than solid tumours, although the latter represent most malignant diseases[18]. To overcome this problem, comparative genomic hybridization (CGH) was developed (FIG. 2f)[19,20].

In CGH, DNA is extracted directly from the test sample and a normal reference sample. The two DNA samples are differentially labelled — for example, with the test labelled in green and the reference in red. The combined probes are then applied to target metaphase chromosomes and compete for complementary hybridization sites. Therefore, if a region is amplified in the test sample the corresponding region on the metaphase chromosome becomes predominantly green. Conversely, if a region is deleted in the test sample the corresponding region becomes red. The ratios of test to reference fluorescence along the chromosomes are quantified using digital image analysis. Gains and amplifications in the test DNA are identified as chromosomal regions with increased fluorescence ratios, whereas losses and deletions result in a reduced ratio (FIG. 2f). One of the main advantages of CGH is its use as a discovery tool, as it requires no *a priori* knowledge of the chromosome imbalance that is involved.

The scope of CGH has been extended to include the analysis of small amounts of DNA that have been obtained from small subregions of a specimen, such as microdissected tumour samples, by carrying out unbiased PCR amplification that reflects the copy number differences of the original genome[21–23]. CGH has even been applied to the analysis of single cells[24–26], and such approaches have been used for prenatal diagnosis[27,28] and analyses of MINIMAL RESIDUAL DISEASE[29,30]. Single-cell CGH results can be verified by the sequential application of interphase FISH followed by single-cell CGH of the same cell[31].

MULTIPLEX-FISH
Painting of the entire chromosome complement such that each chromosome is labelled with a different combination of fluorophores. Images are collected with a fluorescence microscope that has filter sets for each fluorochrome, and a combinatorial labelling algorithm allows separation and identification of all chromosomes, which are visualized in characteristic pseudocolours.

SPECTRAL KARYOTYPING
Similar to M-FISH, except that an interferometer is used for fluorochrome discrimination and imaging.

For rearrangements that do not involve genomic imbalances, such as balanced chromosome translocations and inversions, the use of CGH is limited. In addition, whole-genome copy number changes (ploidy changes) cannot be detected. Furthermore, CGH provides no information about the structural arrangements of chromosome segments that are involved in gains and losses. Despite these limitations, CGH has become one of the most widely used cytogenetic techniques in both basic research and molecular diagnostics. It has also altered our view of cancer biology, revealing that tumours of the same type have similar patterns of regional gain and loss, and that the frequency of copy number changes increases with tumour progression.

Interphase cytogenetics. In interphase cytogenetics, chromosomes or chromosomal subregions are visualized in the nucleus, which allows the analysis of the genome of individual cells in a more 'natural' context than in metaphase spreads, and enables the study of three-dimensional genome organization (see below). Furthermore, as chromatin in the interphase nucleus is almost a tenth as condensed as in metaphase chromosomes, interphase cytogenetics has allowed the ordering of probes over shorter distances (50 kb–2 Mb), compared with ~5 Mb using metaphase spreads[32,33]. With careful selection of probes even structural rearrangements, such as translocations and inversions, can be visualized in interphase nuclei[34,35].

As for CGH, interphase analysis is useful in diagnostic applications for which metaphase spreads cannot be obtained. For example, interphase analysis with FISH probes is used to detect amplification of *HER2* (v-erb-b2 erythroblastic leukaemia viral oncogene homologue 2) in human breast cancer tissue sections[36], which identifies patients who might benefit from TRASTUZUMAB (herceptin) treatment[36]. Interphase FISH on tissue sections also allows the analysis of primary tumours, which might avoid possible artefacts being introduced by *in vitro* cell-culture systems or in the generation of animal models. For example, this advantage of interphase FISH allowed the observation to be made that TELOMERE CRISIS occurs at an early stage in the development of breast cancer[37].

Interphase analysis is also useful when cells are rare, such as in minimal residual disease or micrometastasis. Disseminated cells in patients with early-stage cancers occur at low frequencies, making it almost impossible to isolate live cells for culture and subsequent preparation of metaphase spreads[38]. Studies of disseminated cells by interphase analysis have revealed a high degree of cellular heterogeneity even at early stages of tumour progression[39]. Interphase FISH with centromere probes also allows the rapid screening of large numbers of cells to identify chromosomal instability in tumour cell nuclei[40].

COMBINED BINARY RATIO LABELLING
A multicolour karyotyping system that uses a combination of combinatorial and ratio labelling for probe discrimination.

CROSS-SPECIES COLOUR SEGMENTATION
A FISH-based multicolour banding technology that uses flow-sorted gibbon chromosome paints, which generate a cross-species banding pattern when hybridized to human metaphase spreads.

PSEUDOCOLOUR BANDING PATTERN
A fluorescence multicolour banding pattern along chromosomes that is generated by hybridization of multiple differentially labelled region-specific probes.

COOLED CHARGE-COUPLED DEVICE
A highly sensitive area imager that is widely used for capturing FISH images. Cooling, which reduces random noise during long exposures, is often not required as modern fluorochromes with microscope optics usually require only short exposure times.

MINIMAL RESIDUAL DISEASE
The low numbers of tumour cells that remain after therapy, which are often below the detection limits of classical morphological methods.

TRASTUZUMAB
A monoclonal antibody that targets cancer cells that overexpress HER2, which is found on the surface of some cancer cells.

TELOMERE CRISIS
The erosion of the telomeres so that chromosome ends are no longer protected, resulting in unstable chromosomes.

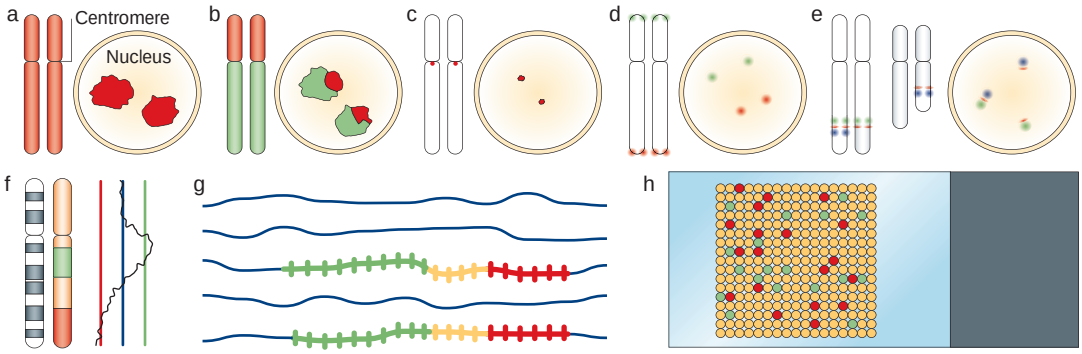


Figure 2 | **Fluorescence *in situ* hybridization probes for different applications. a** | Painting probes stain entire chromosomes. In a normal diploid interphase nucleus, two chromosome territories are typically visible. **b** | Regional painting probes can be generated by chromosome microdissection. Differentially labelled probes that are specific for each of the arms of a particular chromosome are shown. **c** | Centromeric-repeat probes are available for almost all human chromosomes. Because of their ease of use and high signal intensities, these probes are popular for counting chromosome copy numbers in interphase nuclei. **d** | Large-insert clones are available for most genomic regions. Subtelomeric probes, which are often used to screen for cryptic translocations that are not usually visible in conventional chromosome-banding analyses, are shown in this example. **e** | Special probe sets can be designed to facilitate diagnosis of known structural rearrangements. In this example, the probe set includes a breakpoint-spanning probe (red) and two breakpoint-flanking probes (green and blue). Probe sets of this type allow structural rearrangements to be detected even in interphase nuclei. **f** | Genomic DNA is used as the probe in comparative genomic hybridization (CGH) to establish copy number. An analysis of chromosome 8 is shown as an example. Simultaneous visualization of both test DNA (green region) and normal reference DNA (red region) fluorochromes shows balanced regions in orange (equal amounts of green and red fluorochromes). In regions of gain in copy number, green fluorescence dominates, whereas regions of loss appear red. Quantification is carried out using software that calculates a ratio profile for the two colours. The standard representation of the ratio profile consists of a centre line (black), which reflects a balanced ratio of green and red fluorescence. The green line represents the upper threshold for significant gain, the red line the lower threshold for significant loss. **g** | For high-resolution analysis, DNA fibres can be used as the target for probe hybridization. The simultaneous hybridization of two different probes is shown, labelled green and red. The yellow colour indicates an overlap between these two probes. **h** | Microarrays can be used as targets for hybridization to provide resolutions down to the single-nucleotide level. A BAC array is shown, to which test DNA and reference DNA are hybridized. Individual clones show different colours after hybridization depending on whether the corresponding DNA in the test sample is lost (red on the array), gained (green on the array) or neither (yellow on the array), in a similar way to conventional CGH.

Fibre FISH. The highest resolution target for FISH analysis on microscope slides is provided by the preparation of released chromatin fibres (FIG. 2g). These consist of chromatin from which proteins such as histones are removed, allowing it to unfold and extend. This method, known as fibre FISH, provides a resolution of 1–500 kb.

Several alternative techniques for the release of chromatin for fibre FISH have been developed. The earliest protocols used an alkaline lysis buffer[41] or high-salt treatment in an SDS-containing lysis buffer[42,43] to generate fibres. These approaches produce fibres of varying length and compaction so that the size of a probe that is hybridized to fibres might not closely correlate with its signal length, making these preparations unsuitable for quantitative analyses. For quantification, highly uniform, evenly stretched DNA fibres are required, such as those that are provided by MOLECULAR COMBING[44]. An example of the benefits of the increased resolution of fibre FISH is in the direct visualization of X-chromosome deletions in patients with Duchenne muscular dystrophy, which allows accurate sizing of the variable deletions for correlation with disease phenotype[45].

Fibre FISH is also one of the most powerful tools for mapping DNA sequences onto specific regions of the genome, because it allows accurate sizing of gaps and overlaps between probes. Overlaps between two differentially labelled probes are sized by measuring the 'length' of the signal on the DNA fibres that show mixed fluorescence (fluorescence from both probes) (FIG. 2g). By contrast, gaps are sized by measuring the length of non-hybridized regions between two hybridization signals[45]. Fibre FISH has been particularly useful for measuring the size of regions of the human genome that have been impossible to sequence, which is achieved by hybridization of the clones that flank gaps in the sequence[46].

Array-based cytogenetics

Array CGH. In array CGH, metaphase chromosomes are replaced as the target by large numbers of mapped clones that are spotted onto a standard glass slide. This has greatly increased the resolution of screening for genomic copy number gains and losses. In array CGH, the test and normal reference genomes, which are used as probes, are differentially labelled and co-hybridized to a microarray (FIG. 2h). The array is then imaged and the relative fluorescence intensities are calculated for each mapped clone, with the resulting intensity ratio reflecting the DNA copy number difference. The resolution of the analysis is restricted only by clone size and by the density of clones on the array. A further advantage is the ease with which array CGH can be automated for high-throughput applications.

The first descriptions of array CGH with large-insert clones in the late 1990s[47,48] were followed rapidly by the development of whole-genome arrays that had one clone for every megabase, which are now widely used[49,50]. The flexibility of array design has also allowed the development of specialized arrays for applications such as telomere screening[51] or for specific diseases (for example, B-cell leukaemia)[52]. In these applications, arrays are constructed with clones that are specific to the regions of interest. This improves resolution, with a more complete coverage of clones for the target region, and lowers production and hybridization costs by reducing array size. For whole-genome arrays, the density of clones on the slide has continued to increase such that arrays printed with over 30,000 overlapping clones that cover the whole human genome are now available[53,54].

DNA sequences other than large-insert genomic clones are also used for array CGH. An advantage of cDNA arrays[55] is that expression analysis can be carried out using the same platform, but they suffer from uneven coverage of the genome and lower sensitivity to small copy number changes than arrays that are produced from genomic clones of a similar size. This lack of sensitivity is probably mainly due to sequence mismatches between genomic DNA and corresponding cDNAs where intronic sequences have been spliced out during transcription. In addition to cDNAs, several groups have used genomic PCR amplicons for the construction of arrays. For example, Dhami *et al.* used small PCR amplicons that represent exons from 5 human genes that are involved in inherited genetic disorders for the identification of exonic gains and losses[56]. The highest resolution for array CGH is now provided by oligonucleotide arrays, which are produced either by spotting oligonucleotides onto a slide[57,58] or by direct synthesis onto glass or silicon substrates[59–62] that contain as many as 500,000 elements.

SNP arrays are high-density oligonucleotide-based arrays that can identify both LOSS OF HETEROZYGOSITY (LOH) at individual nucleotides and copy number alterations[61,62]. Arrays of up to ~100,000 SNPs[63,64] were originally designed for genotyping purposes to identify regions of the genome that are associated with particular phenotypes. SNP array probes comprise 25-mer oligonucleotides, and each SNP is represented by both a sense and an antisense strand. The probe intensities that correspond to the two possible alleles of the SNP reveal which of the three expected genotypes (for example, *AA*, *BB* or *AB*) is present.

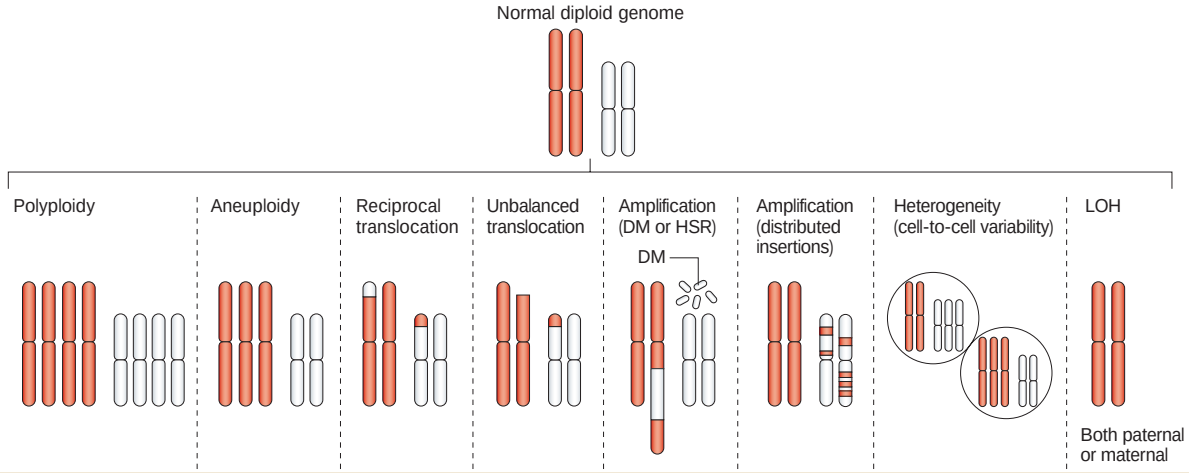
These probe intensities can also be used to estimate DNA copy numbers[61]. In contrast to the CGH approaches, in SNP arrays only one genomic sample is hybridized to the array, so copy number changes are identified by comparison with independent control hybridizations. Using this approach, Raghavan *et al.* identified large regions of UNIPARENTAL DISOMY in patients with leukaemia who had an apparently normal karyotype[65]. These findings have considerable effect on our understanding of the genomic changes in leukaemia and it is likely that SNP arrays will become an important method in the analysis of this and other malignant diseases.

Instead of using genomic DNA, some oligonucleotide arrays use 'representations' of the genome, which are generated by restriction-enzyme digestion and subsequent PCR amplification. This reduces probe complexity, as it can be optimized for fragments of a

MOLECULAR COMBING
High molecular-weight DNA in solution is stretched at the meniscus as a glass slide is removed from the solution at a constant rate, generating fields of evenly stretched DNA fibres that have a parallel orientation.

LOSS OF HETEROZYGOSITY
A loss of one of the alleles at a given locus as a result of a genomic change, such as mitotic deletion, gene conversion or chromosome missegregration.

UNIPARENTAL DISOMY
A condition in which an individual or embryo carries two chromosomes that are inherited from the same parent.



Technique	Detection							
Banding	+	+	+	+	+	–	+	–
M-FISH/SKY	+	+	+	+	+	+	+	–
(Array) CGH	–	+	–	+	+	+	(+)*	–
SNP-array	–	+	–	+	+	+	(+)*	+
Digital karyotyping	–	+	–	+	+	+	–	+
Interphase‡	+	+	+	+	+	+	+	–

Figure 3 | **Comparison of cytogenetic techniques for identifying chromosomal abnormalities.** Various chromosomal aberrations that might be present in clinical samples are shown, with the ability of different cytogenetic techniques to detect them summarized below. A '+' indicates that an approach is suited for identifying the chromosomal rearrangement, a '–' indicates that the aberration would be missed. In the case of heterogeneity, both array comparative genomic hybridization (CGH) and conventional CGH could detect the abnormality, but only if numerous small subpopulations are analysed. We distinguish between amplifications in which the amplified region is easily visible in banding analysis as DOUBLE MINUTE (DM) chromosomes or homogeneously stained regions (HSR), and amplifications where the amplified region is scattered throughout the genome as small insertions (distributed insertions), making identification using banding analysis impossible. M-FISH, multiplex fluorescence *in situ* hybridization; SKY, spectral karyotyping. *Indicates that several experiments are needed. ‡The detection rate of interphase cytogenetics depends on probe selection. Modified, with permission, from *Nature Genetics* REF. 126. © (2003) Macmillan Magazines Ltd.

CONSTITUTIONAL REARRANGEMENTS
Chromosomal rearrangements that are present in an individual at birth.

TILING CLONE ARRAY
A high-resolution array consisting of multiple overlapping clones.

FLOW SORTING
After staining with base-pair-specific fluorochromes, cells or chromosomes are sorted according to their DNA content and base pair ratio using a flow cytometer.

DOUBLE MINUTE
An acentric, extra-chromosomally amplified chromatin, which usually contains a particular chromosomal segment or gene. Double minutes occur frequently in cancer cells.

ACROCENTRIC CHROMOSOME
A chromosome with a near-terminal centromere so that one arm is very short. The short arms of acrocentric chromosomes consist mainly of repetitive DNA sequences.

specified size range (usually small restriction fragments), which improves the signal-to-noise ratio. However, hybridization to these arrays is still inherently noisy owing to the small size of the targets. This requires the calculation of an average signal over several loci, followed by statistical analysis for the confident identification of small gains and losses.

Array CGH methods have been most widely used for the analysis of gains and losses in tumours, but are increasingly being used for the analysis of patients with CONSTITUTIONAL REARRANGEMENTS. For example, Vissers *et al.* used array CGH at a resolution of 1 Mb to identify a deletion of ~5 Mb at 8q13 in a patient with CHARGE (coloboma, heart, choanal atresia, retardation, genital and ear) syndrome[66]. Using a TILING CLONE ARRAY of chromosome 8, this group was then able to refine the deletion interval in a second patient to 2.3 Mb, which allowed the subsequent identification of the gene that is affected in most patients with CHARGE[66].

As well as its use in diagnostics, array CGH has provided important insights into aspects of normal genomic variation. Initial analyses of DNA from about 120 normal individuals using array CGH, in combination with other methods, has uncovered a previously under-appreciated level of variation in the human genome. This involves variation in the copy numbers of genomic regions that are as large as hundreds of kilobases in size[67–69]. Many of these copy number variations are common in gene-rich regions of the genome, and are associated with segmental duplication. Similar variations have also been observed in other organisms, such as mice and yeast. These copy number variations are likely to influence gene expression, and might be associated with phenotypic variation and susceptibility to disease.

Array painting: structural aberrations at high resolution. Array CGH is of little use for studying chromosomal abnormalities that do not involve copy number changes, such as inversions or balanced translocations. For the latter, array painting, a modification of the array CGH method, has been developed[70]. Array painting uses FLOW SORTING to separate the abnormal chromosomes that result from these rearrangements from the rest of the genome on the basis of their altered sizes and base pair ratios, after which they are hybridized to an array[70].

In the case of balanced translocations, differential labelling of the two derivatives results in the sequences that are proximal to the breakpoint being labelled in

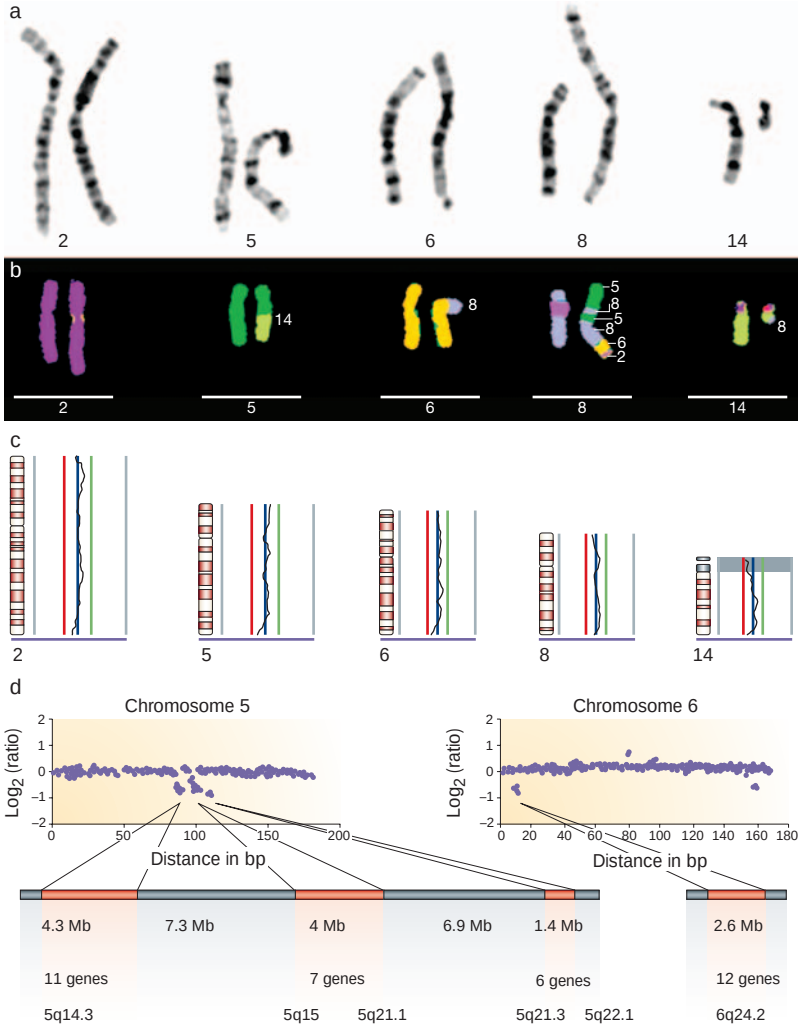


Figure 4 | **Combining cytogenetic approaches to understand a complex chromosomal rearrangement.** Analyses were carried out for a child with dysmorphic features and mental retardation. **a** | Banding analysis reveals aberrant banding patterns for several chromosomes. In particular, the significant size differences within chromosome pairs 8 and 14 indicate the presence of a complex chromosomal rearrangement. **b** | Multiplex fluorescence *in situ* hybridization identifies interchromosomal rearrangements that involve chromosomes 2, 5, 6, 8 and 14. For each chromosome, where regions have been translocated from other areas of the genome, the number of the chromosome that this came from is indicated. Note that the colour change on the left-hand chromosome 8 is caused by an overlapping chromosome. The p-arms of ACROCENTRIC CHROMOSOMES consist of repetitive sequences, which are not easily evaluated. Automated classification algorithms often assign a random classification colour, as is visible for the p-arm regions of both copies of chromosome 14. **c** | Conventional comparative genomic hybridization (CGH) does not identify any imbalances in this case. The profiles for the ratio of fluorescence from the normal reference genome (detected by red fluorescence) and the genome of the patient (detected by green fluorescence) are on the black line, and do not exceed the thresholds for over- or underrepresentation of particular chromosomal regions (indicated by the red and green lines, respectively). However, this could be due to the low resolution of chromosome CGH, which is estimated to be ~10 Mb for deletions. **d** | In array CGH, deletions are identified by a decreased intensity ratio at particular positions along the chromosome. Array CGH identifies 4 deletions on chromosomes 5 and 6, which are the chromosomes that are involved in the complex rearrangement in this patient. The deletions range in size from 1.4 Mb to 4.3 Mb.

one colour, whereas sequences that are distal to the breakpoint are labelled in another. When the two derivatives are hybridized to the array, the ratio of the two colours on the chromosomes that are involved in the rearrangement switches from high to low (or *vice versa*) at the breakpoint. If a clone on the array spans the breakpoint it can report an intermediate value. Array painting can also identify other rearrangements within the derivative chromosomes, such as inversions at the breakpoint, deletions[70], and translocations or insertions of extra material from other chromosomes[71].

The combined use of array CGH for the identification of copy number changes across the whole genome and array painting for analysis of the translocated derivatives provides a comprehensive analysis of genomic rearrangements in patients who have apparently balanced translocations. These methods have uncovered a surprisingly high frequency of complexity in these cases; as a result, traditional cytogenetic methods that rely on microscopic analysis can no longer be considered to be adequate for determining whether a translocation is balanced[71].

Study designs for diagnostic cytogenetics

Selecting the correct strategy in molecular cytogenetics. Matching the cytogenetic assay to the biological question is important, as summarized in FIG. 3. From the discussion above, it is clear that different chromosomal abnormalities require different cytogenetic methods. In addition, there are fundamental differences between monitoring the state and the rate of chromosomal aberrations and rearrangements[72]. The state refers to the existence of one or several mutations, whereas the rate refers to the frequency with which these are formed. The state is not a measure of the rate of chromosome rearrangement or genomic instability. The detection of multiple numerical and structural abnormalities in a metaphase spread from a patient or tumour (identification of the state) might suggest high genomic instability, but if identical abnormalities are found in other metaphase spreads from the same patient or tumour, the rearrangements are clearly stable, with a low rate of change. The rate can be best assessed by single-cell approaches, as it refers to the variability between cells. The assessment of the rate is especially important for analyses of cell populations in which the genome can undergo frequent changes, and is of particular importance in tumour genetics and in monitoring minimal residual disease.

Combining techniques. Cytogenetic methods can be combined to provide a comprehensive analysis of complex chromosomal rearrangements. An example of this is shown in FIG. 4, which shows the case of a child with mental retardation and dysmorphic features. Banding analysis revealed a complex chromosomal rearrangement, but could not identify the origin of extra material on chromosome 8 or correctly assign other structural chromosomal rearrangements. M-FISH identified a rearrangement involving chromosomes 2, 5, 6, 8 and 14, but this did not provide information about genomic imbalance. In this case, conventional CGH failed to identify any copy number changes. However, the enhanced resolution of array CGH clearly identified four genomic deletions that were below the resolution limits of standard CGH, and allowed direct mapping of the deletion breakpoints onto the human reference sequence so that

the number of genes involved could be determined using publicly available genome browsers. Therefore, combining traditional and molecular cytogenetic techniques provides a powerful strategy for modern diagnostics.

Cytogenetic approaches to genome architecture

Chromatin structure and function. Chromatin organization and the epigenetic modification of chromatin-associated proteins have important effects on fundamental processes, such as transcription, recombination, replication and DNA repair. Cytogenetic methods, particularly when combined with chromatin fractionation, are proving to be useful tools in the study of chromatin structure and function. For example, Gilbert *et al.* separated compact and 'open' chromatin structures using sucrose gradients and analysed the distribution of these chromatin states through hybridization of the enriched DNA onto metaphase chromosomes and through array CGH[73]. This revealed that regions of open chromatin are correlated with high gene density, but not necessarily with high gene expression.

Perhaps the most powerful approaches to studying chromatin structure and function use the fractionation and enrichment of DNA through chromatin immunoprecipitation (ChIP). Chromatin that is associated with DNA–protein interactions or modifications is specifically enriched by precipitation with an antibody that is directed against the protein of interest. The enriched DNA sequences can then be mapped and quantified, often using DNA microarrays, which provide the high resolution required. This combined methodology has been termed ChIP on chip. For example, ChIP that is directed against phosphorylated H2AX, a marker of double strand breaks, was used to show that DNA damage that is a consequence of telomere shortening is associated with cellular senescence[74].

For most ChIP on chip studies much higher resolution is required than can be achieved with large-insert clone arrays as the chromatin modifications and DNA–protein interactions that are associated with gene regulation occur over small regions. Therefore, high-resolution arrays that are targeted to small or specific regions of the genome have been used for this purpose. For example, Kondo *et al.* used antibodies against acetylated H3 histone (a marker of active chromatin) and H3 histone that is methylated at lysine 9 (a marker of silenced chromatin) to carry out ChIP in a cancer cell line[75]. The precipitated chromatin was hybridized to a human CpG ISLAND microarray, allowing the identification of novel targets of gene silencing in cancer[75].

Replication of the human genome proceeds as an ordered process throughout S phase of the cell cycle, and the timing of replication correlates with gene activity, chromatin structure and nuclear position[76–78]. Cytogenetic methods that include REPLICATION BANDING, fractionation of S-phase nuclei followed by semi-quantitative PCR[79,80], and counting FISH signals in S-phase nuclei[81] have been used to measure replication timing. Using these techniques, experiments in mice showed that, for each pair of autosomes, alleles on either the maternal or paternal copy — selected at random in each cell — are replicated earlier than those on the homologous chromosome. Therefore, the non-equivalence of chromosome pairs is not limited to sex chromosomes, but is a fundamental property of all mouse chromosomes[82].

Despite these successes, the applications that are described above are not easily applied to the whole genome. Microarrays have recently been used to overcome this limitation[83–86]. For example, as a direct measure of replication timing, Woodfine *et al.* used array CGH to compare copy numbers of sequences throughout the genome in S phase and G1 phase of asynchronously dividing human cells[85]. They were able to confirm the correlation of replication timing with gene density, GC content, *Alu* repeat frequency and the probability of gene expression across the whole genome. They were also able to study correlations at an even higher resolution for chromosomes 6 and 22 (REFS 85,86).

Studying the genome in three dimensions. In three-dimensional FISH (3D-FISH), nuclei are fixed so that the spatial relationships between CHROMOSOME TERRITORIES are maintained. Sophisticated three-dimensional image-acquisition technology is used to collect a series of images throughout different sections of the nucleus, which allows a detailed three-dimensional reconstruction. 3D-FISH has greatly benefited from improvements in imaging technologies that use confocal lasers or sophisticated DECONVOLUTION ALGORITHMS (reviewed in REF. 87). Interphase FISH, both on fixed nuclei and in living cells, allows the study of the functional organization of the genome and the dynamic interplay between the genome and its regulatory factors[87,88]. However, these technologies currently lack the resolution and speed that is required for the clinical diagnosis of structural rearrangements and the instrumentation that is required is expensive.

Studies of chromosomes within their natural tissue context using 3D-FISH have allowed the study of higher-order chromatin architecture[87–89]. The nucleus is thought to have a compartmentalized structure, consisting of chromosome territories separated by an interchromatin compartment. Combining the latest multicolour technologies with three-dimensional imaging tools and reconstructions, it has become possible to visualize all 46 chromosome territories in different colours in intact human cell nuclei, which is an important achievement[90] (FIG. 5). In most tissues, the radial arrangement of chromosome territories has been shown to correlate with gene density[91,92]. However, in fibroblasts the distribution of chromosome territories correlates more closely with chromosome size, indicating that there are tissue-specific differences in three-dimensional genome organization[90,93]. Three-dimensional studies are also providing increasing evidence that chromatin location within the nucleus is an important constraint on gene activity. Recent 3D-FISH studies indicated that portions of different chromosomes interact with each other, which implies that related genes are brought together in the nucleus to coordinate their expression[94].

CpG ISLANDS
Sequences of 200 bp or more that have high GC content and a high frequency of CpG dinucleotides. CpG islands are found upstream of many mammalian genes.

REPLICATION BANDING
Chromosome banding using differences in staining between early and late replicating regions of the genome after timed incorporation of bromodeoxyuridine (BrdU), a nucleoside that substitutes for thymidine in DNA.

CHROMOSOME TERRITORIES
Compartments within a cell nucleus that are occupied by a chromosome.

DECONVOLUTION ALGORITHMS
Computational techniques for removing out-of-focus haze from stacks of optical sections, so restoring sharpness and clarity to an image.

KARYOTYPING
A process in which metaphase chromosomes are ordered and numbered according to morphology, size, arm-length ratio and banding pattern.

DIGITAL KARYOTYPING
A technique that provides quantitative analysis of DNA copy number by isolation and enumeration of short sequence tags from specific genomic loci.

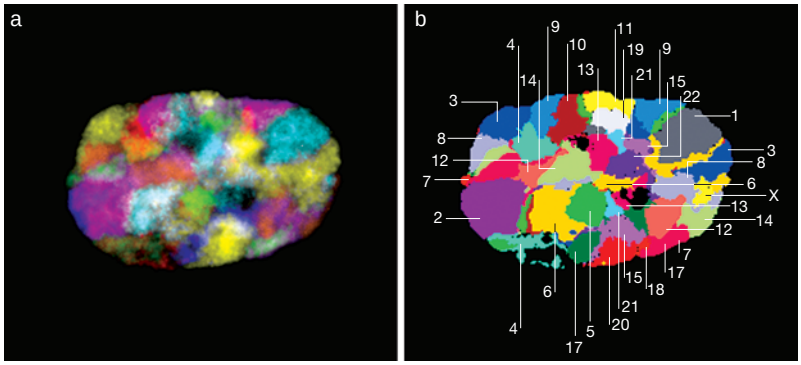


Figure 5 | **Studying genome organization using three-dimensional fluorescence *in situ* hybridization.** All the chromosome territories that make up the human genome can be visualized simultaneously in intact interphase nuclei, each in a different colour. **a** | A red, green and blue image of the 24 labelled chromosomes (1–22, X and Y) was produced from deconvoluted mid-plane nuclear sections from a three-dimensional stack by superposition of the 7 colour channels. **b** | As in 24-colour KARYOTYPING, each chromosome can be identified by using a combination labelling scheme in which each chromosome is labelled with a different set of fluorochromes. In this way, each chromosome territory can be automatically classified using appropriate software, which assigns the corresponding chromosome number to a territory. If a stack of these images is collected throughout the nucleus, a simultaneous three-dimensional reconstruction of all chromosome territories is possible. Some of the dark regions represent unstained nucleoli. For further details see REF. 90.

PADLOCK PROBE
A probe with two target-complementary segments, which on hybridization are brought close to each other so that they can be covalently linked, resulting in a circularized probe.

ROLLING-CIRCLE AMPLIFICATION
A method for the general amplification of DNA by DNA polymerase, which replicates circularized oligonucleotide probes with either linear or geometrical kinetics under isothermal conditions.

HYPERBRANCHED STRAND-DISPLACEMENT AMPLIFICATION
Isothermal amplification of genomic DNA that is driven by strand-displacing polymerases, such as phage φ29, for random-primed amplification of human genomic DNA.

Four-dimensional technologies: living-cell cytogenetics. The development of new *in vivo* fluorescent labelling techniques has allowed the visualization of DNA and proteins in living cells (reviewed in REFS 89,95,96). Chromosomal regions and nuclear architecture can be visualized by the incorporation of fluorescent nucleotides into DNA during replication in S phase. Further growth and division of labelled cells results in the segregation of labelled and non-labelled chromatids during subsequent mitoses, which allows genomic organization to be followed in subsequent generations. At present the analysis of living cells is limited to gross features, such as the location of recently replicated DNA or protein aggregates. Therefore, increases in resolution are needed in the future to study living cells in more detail.

Conclusions and future perspectives

Modern cytogenetics is blurring the distinction between cytological and molecular analysis. It is now straightforward to combine methods that range from identifying gross structural changes in the genome down to single-nucleotide differences. In addition to SNP arrays, other approaches such as DIGITAL KARYOTYPING[97] and the use of PADLOCK PROBES[98] provide resolution down to the base pair level. These high-resolution technologies are increasingly allowing us to understand the processes that are involved in normal genomic function and disease. Furthermore, the future analyses of minute amounts of DNA might benefit from amplification protocols, such as ROLLING-CIRCLE AMPLIFICATION[99,100] and HYPERBRANCHED STRAND-DISPLACEMENT AMPLIFICATION[101], which are suited for a high-throughput approach. The resulting technologies are becoming established for use in basic research, but only time will tell what effect they will have on routine clinical cytogenetics.

Today it is rarely adequate to use a single cytogenetic method for detailed analysis of a genomic disease or cellular structure and function. For example, in tumour genetics integrated genomic and epigenomic analysis enables the identification of biallelic gene inactivation in tumours, as inactivation can occur both genetically and epigenetically[102]. The inclusion of genome-wide analyses of DNA methylation and histone acetylation in addition to copy number changes will therefore become an integral part of future cancer research. Furthermore, the integration of array-based genetic maps with gene expression signatures offers the potential to rapidly identify new genes that are involved in tumorigenesis[103]. In addition, the increasing number of observations that report the regulatory influence of chromosome rearrangements on genes as much as 1 Mb away demonstrates the importance of epigenetic mechanisms in disease processes[104,105]. In this respect, analyses of higher-order chromatin structure by 3D-FISH will be of particular importance in the future. New studies should also address distinct three-dimensional organizations in different tissue types, as higher-order chromatin arrangements are likely to have fundamental implications for development and cell differentiation. Therefore, future developments will include more advanced and sophisticated hardware and software platforms to carry out multicolour 3D-FISH analyses in a high-throughput format.

In a recent study, Tuzun *et al.* used an *in silico* sequence-comparison method for identifying not only copy number polymorphism but also other structural variants such as inversions[106]. As current advances in sequencing become more widely available, with increased speed and decreased cost, it is likely that sequence-based approaches for the analysis of chromosome rearrangements will become more important and widely used. Ultimately, these approaches might replace many current cytogenetic methods. In conclusion, we predict that high-resolution methods for the analysis of changes in the architecture of the genome that are the fundamental causes of inherited disease and cancer will become increasingly available to cytogeneticists.

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Competing interests statement

The authors declare no competing financial interests.

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