# Demystified . . .

## Comparative genomic hybridisation

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**Abstract**
**Comparative genomic hybridisation (CGH) is a technique that permits the detection of chromosomal copy number changes without the need for cell culturing. It provides a global overview of chromosomal gains and losses throughout the whole genome of a tumour. Tumour DNA is labelled with a green fluorochrome, which is subsequently mixed (1:1) with red labelled normal DNA and hybridised to normal human metaphase preparations. The green and red labelled DNA fragments compete for hybridisation to their locus of origin on the chromosomes. The green to red fluorescence ratio measured along the chromosomal axis represents loss or gain of genetic material in the tumour at that specific locus. In addition to a fluorescence microscope, the technique requires a computer with dedicated image analysis software to perform the analysis. This review aims to provide a detailed discussion of the CGH technique, and to provide a protocol with an emphasis on crucial steps.**
(*J Clin Pathol: Mol Pathol* 1999;**52**:243–251)

Comparative genomic hybridisation (CGH) is a technique that permits the detection of chromosomal copy number changes without the need for cell culturing. It gives a global overview of chromosomal gains and losses throughout the whole genome of a tumour. Thus, CGH is a relatively fast screening technique that can point at specific chromosomal regions that might play a role in the pathogenesis or progression of tumours. Guided by CGH results, more specific molecular biological techniques (such as fluorescence in situ hybridisation, loss of heterozygosity analysis, and sequencing) can be used to identify oncogenes and/or tumour suppressor genes in these regions.

Kallioniemi *et al* at the University of California, San Francisco were the first to report CGH as a new chromosome analysis technique in 1992,[1] shortly followed by du Manoir *et al*.[2] In short, tumour DNA is labelled with a green fluorochrome, mixed (1:1) with red labelled normal DNA, and hybridised to normal human metaphase preparations. The normal reference DNA and the metaphases are obtained from a healthy volunteer and do not need to be from the patient. The green and red labelled DNA fragments compete for hybridisation to their locus of origin on the chromosomes. The green to red fluorescence ratio measured along the chromosomal axis represents loss (ratio < 1) or gain (ratio > 1) of genetic material in the tumour at that specific locus (figs 1 and 2). In addition to fluorescence
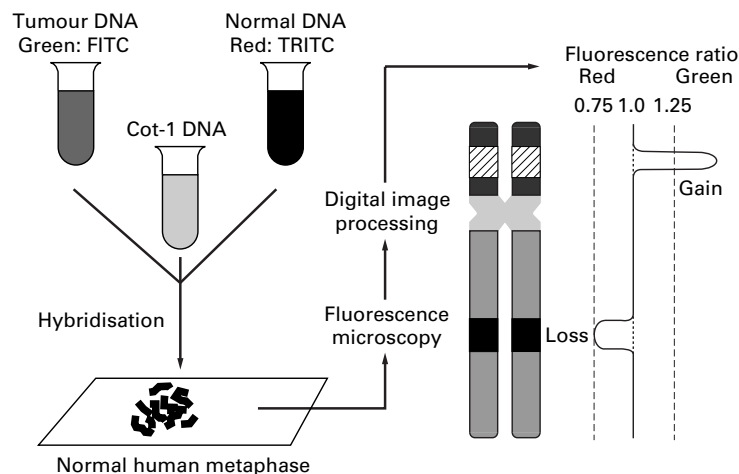


*Figure 1 Principle of comparative genomic hybridisation (CGH). Schematic overview of the CGH technique. Tumour and reference DNA are labelled with a green and red fluorochrome, respectively, and hybridised to normal metaphase spreads. Images of the fluorescent signals are captured and the green to red signal ratios are quantified digitally for each chromosomal locus along the chromosomal axis.*
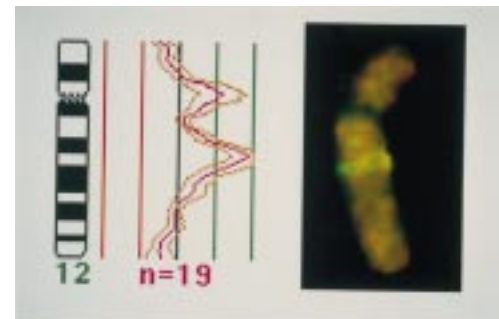


*Figure 2 High level gain on the long arm of chromosome 12. The clear green band shows the high level gain or amplification on the long arm of chromosome 12 in a carcinosarcoma of the nasopharynx (chromosomal band 12q15). In addition, the comparative genomic hybridisation (CGH) profile displays a green to red ratio >> 1. Because of suboptimal blocking of repetitive DNA sequences in the profile, another peak appeared at the site of the centromere, which was interpreted as an artefact and neglected when interpreting CGH results.[3]*
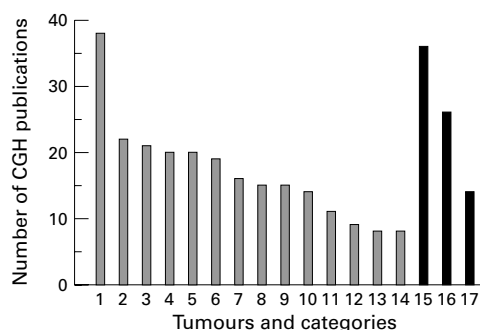
*Figure 3   Diversity of tumours analysed by comparative genomic hybridisation (CGH) and types of reports (1992–8). Categories are: (1) neurological tumours; (2) tumours of the male genital tract; (3) tumours of the urinary tract; (4) breast cancer; (5) haematological diseases; (6) tumours of the digestive tract; (7) lung cancer; (8) tumours of the female genital tract; (9) soft tissue tumours; (10) lymphomas; (11) oral tumours; (12) bone cancer; (13) neuroendocrinal tumours; (14) skin cancer; (15) technical reports; (16) reviews; (17) other types of report.*

microscopy, the technique requires dedicated computer software for the analysis of results. Initially, it appeared to be rather difficult to perform the technique in other, less specialised laboratories, and it was only after the publication of an article reviewing the method in more detail, and after the necessary software became commercially available, that CGH became more accessible.[4] Because no cell culturing is required for CGH, this technique has enabled tremendous progress in the analysis of chromosomal changes in solid tumours. Applications of CGH in cancer research include screening of tumours for genetic aberrations,[5–18] searching for genes involved in the carcinogenesis of particular subsets of cancers,[19] analysing tumours in experimental models to obtain an insight into tumour progression,[20] diagnostic classification,[19] and prognosis assessment.[21] At present, CGH is performed in a growing number of laboratories and a wide variety of tumours have been analysed (fig 3). Apart from these oncological applications, CGH analysis has also been used to study chromosomal aberrations in fetal and neonatal genomes.[22–24]

Until now, over 300 articles on CGH have been published by research groups from several countries, including reviews[25–27] and technical papers[28–45] (fig 4). This boom is caused mainly by the reduced demands on the material used for CGH. It has become possible to use archival formalin fixed and paraffin wax embedded material, whereas initially only DNA from
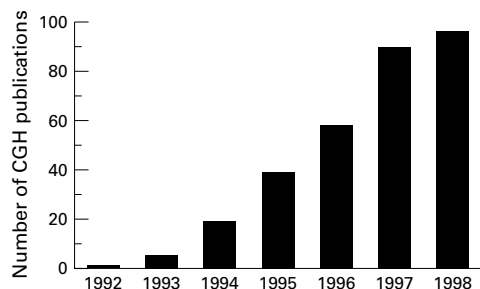


*Figure 4   Number of comparative genomic hybridisation (CGH) publications from 1992 to 1998.*

fresh and frozen tumours and material from cell lines could be used. In addition, by using techniques such as microdissection and degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR), CGH became applicable to smaller lesions. However, the high number of technical reports indicates that CGH is still viewed as a relatively difficult method. Therefore, we aim to provide a detailed discussion of the CGH technique, and to provide a protocol with an emphasis on crucial steps.

## Sensitivity and limitations

CGH is a relatively time consuming and difficult technique with a number of limitations. It cannot detect structural chromosomal aberrations without copy number changes, such as balanced chromosomal translocations, inversions, or ring chromosomes, and it does not yield information in the context of tissue architecture. CGH detects gains and losses relative to the ploidy level. It also has the disadvantage of being less sensitive than PCR based methods in detecting deletions. CGH results are not linearly distributed. Theoretically, the loss or gain of one copy of a certain chromosome in a diploid genome should result in a fluorescence ratio of 0.5 or 1.5. However, in experiments comparing male and female DNA, where the fluorescence ratio for the X chromosome should be 0.5 or 2.0, these ratios are never found in practice.

The sensitivity of CGH can be hampered by contamination of tumour material with normal cells, which will be explained in detail elsewhere in this article. Furthermore, the sensitivity of CGH depends on the level and size of the copy number changes. Based on simulation experiments, a copy number increase of 50% should be detectable if the region is 2 megabase pairs (Mb) or larger, and an amplified region (amplicon) of 250 kilobase pairs (kb) would need a 400% copy number increase.[46] The lower limit of detection is determined by the product of the excess copy number and the size of the amplified region. When the deletion is 100% (no copies present), a resolution of 1–2 Mb can be achieved.[43] Based on CGH experiments, the crucial detection size was estimated to be in the range of 10–20 Mb.[3 32 47]

## Description of the protocol and problem areas of CGH

METAPHASE SLIDE PREPARATION

Metaphase chromosomes are prepared according to standard protocols using phytohaemagglutinin stimulated peripheral blood lymphocytes from a karyotypically normal man or woman. Because women have two X chromosomes and the Y chromosome does not contain much genetic information, it is preferable to use metaphase spreads from women. High quality metaphase preparations for CGH should ideally have little cytoplasm (too much cytoplasm causes high background levels and may prevent optimal denaturation), minimal overlapping of the chromosomes (overlapping chromosomes need to be excluded from CGH analysis), and low cell density paired with a

high mitotic index. In addition, the chromosomes should be of adequate length (400–550 bands) and not contain separated chromatids. Finally, for good banding strength, chromosomes should appear dark, not shiny, when looking through a phase contrast microscope.[36]

In short, 1 ml of heparinised blood is added to 10 ml Ham F10 culture medium (Gibco BRL, Breda, Netherlands) containing fetal calf serum (10%), L-glutamine (1%), penicilin and streptomycin (1%), and phytohemagglutinin (1.5% in distilled water) and incubated at 37°C in an atmosphere of 5% $CO_2$ for 72 hours. Cells are arrested in mitosis by the addition of colchicine to a final concentration of 0.01 μg/ml, harvested, treated with hypotonic KCl (0.075 M) and fixed in 3:1 methanol/acetic acid. A crucial step in the procedure is the dropping of the fixed cell solution on to the slides. The success of this step is dependent on many factors (such as weather conditions, cell suspension quality, and laboratory conditions), but in our hands it is best to drop the cell suspension from a distance of ~ 30 cm on to ethanol cleaned slides. Usually, one drop of the cell suspension results in a sufficient number of metaphase spreads for one CGH experiment. Optimal conditions are a room temperature of 24°C and a relative humidity of 60–70%. The slides should be evaluated with a phase contrast microscope. If large amounts of residual cytoplasm are still visible on the slides, the fixation step (3:1 methanol/acetic acid) should be repeated a few times. In addition, a postfixation step—dropping 3:1 methanol/acetic acid on to a vertically positioned slide—might be helpful in removing cytoplasm. Slides are air dried ("aged") overnight at room temperature and stored at −20°C in groups of four, in the presence of either silica beads or nitrogen to keep them dry. It is important to test several batches of metaphase slides from different donors when setting up CGH experiments, because their behaviour in hybridisation can be variable (fig 5). Alternatively, fully prepared metaphase slides are commercially available (Vysis, Downers Grove, Illinois, USA; catalogue number: 30-806010). However, these slides still need to be tested before use, and the quality is not necessarily better than that of slides produced in house, as described above.

DNA ISOLATION FROM TUMOUR TISSUE
The quality of DNA is a very important issue when performing CGH. DNA extracted from fresh or frozen tissue is usually of high molecular size (not degraded) and will be of the best quality for labelling purposes. However, when using formalin fixed, paraffin wax embedded tissue, the extracted DNA will be partly degraded (300–20 000 bp) and crosslinked. The quality of DNA from paraffin wax embedded tissues depends greatly on the pH of the formalin fixative, and on the duration of fixation; thus, neutral buffered formalin (pH 7.0) and fixation for no longer that 24 hours usually preserves DNA of sufficient quality. During the labelling process, by means of nick translation (that is, cutting DNA and substituting unlabelled nucleotides with digoxigenin, biotin, or fluorochrome labelled nucleotides), DNA becomes even shorter and, in the case of formalin fixed material, the DNA fragments may become too small for optimal CGH experiments. The crosslinking of DNA caused by the fixative can hamper the function of the enzymes, DNA polymerase and DNase, resulting in impaired incorporation of modified nucleotides. This will lead to an irregular and speckled hybridisation pattern of the metaphase chromosomes. As a result, differences in staining intensities between reference and tumour DNA are less pronounced.

Both the standard phenol extraction method[19 21 28] and commercially available DNA isolation kits, based on affinity columns, can be used and optimised for paraffin wax embedded, formalin fixed tissue. For paraffin wax embedded material in general, a series of 10–20 sections of 10 μm is sufficient to obtain 4–20 μg of DNA, depending on the amount and cellularity of the tissue. To reduce the number of crosslinks between the DNA strands in formalin fixed material, an overnight incubation at 37°C with 1 M sodium thiocyanate might be helpful. A prolonged incubation period (three days) with lysis buffer (155 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM $Na_2EDTA$, pH 7.4) and high concentrations of proteinase K (final concentration 2 μg/μl, freshly added twice a day) yields a sufficient quantity of relatively good quality DNA from paraffin wax embedded material. A protocol for the extraction of DNA from formalin fixed, paraffin wax embedded tissue, using affinity columns, is given in appendix 1.

CONTAMINATION
Contamination (or dilution) of the tumour DNA with normal DNA (for example, from stromal and inflammatory cells) is an undesirable but inevitable problem when analysing tumours. The effect on CGH results will be that the green to red ratio shifts towards 1.0, so
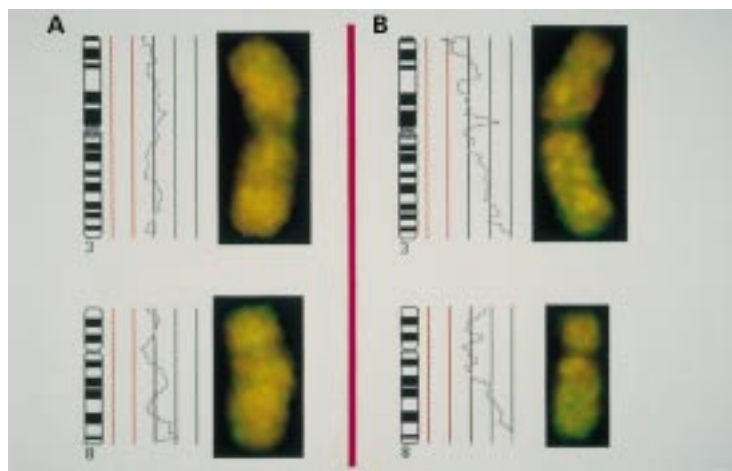


*Figure 5    Effect on comparative genomic hybridisation (CGH) results of differences in blocking efficiency between metaphases of different individuals. Metaphase slides of lymphocyte suspensions of two healthy individuals were prepared using standard procedures. On these metaphases, CGH experiments of the same tumour versus normal DNA was performed with the same amount of Cot-1 DNA. Blocking efficiency differed between chromosomes from different individuals (A v B) and insufficient blocking (individual A) caused repression of the amplitude of the green to red ratio.*
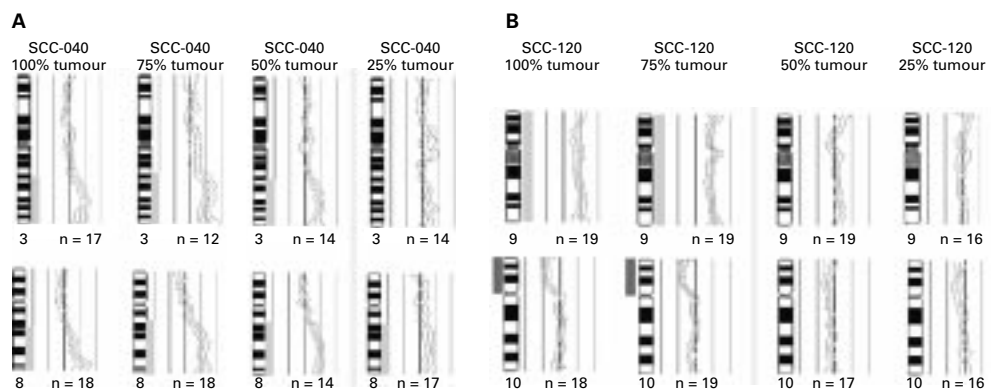
*Figure 6   Effect of contamination of tumour DNA with normal DNA on comparative genomic hybridisation (CGH) results. DNA extracted from two tumour cell lines (92-VU040T and 93-VU120T,[48] referred to hereafter as SCC-040 and SCC-120, respectively, was contaminated (mixed) with normal DNA in different concentrations and analysed by CGH. Cell line 1 (SCC-040) is a diploid oral squamous cell carcinoma and cell line 2 (SCC-120) is a triploid oral squamous cell carcinoma. Both cell lines were cytogenetically karyotyped, so most of the genetic aberrations were known. (A) The CGH profiles of certain chromosomes from the diploid tumour; (B) the CGH profiles of certain chromosomes from the triploid tumour. In the triploid tumour, a single copy gain (4:3) or loss (2:3) becomes undetectable at 50% contamination, whereas in the diploid tumour, a single copy gain (3:2) or loss (1:2) disappears at 75% contamination. The 95% confidence interval was used as the threshold.*

that if the tumour sample contains too much normal DNA, chromosomal aberrations can go undetected. In a triploid tumour, a single copy gain (4:3) or loss (2:3) becomes undetectable at 50% contamination, whereas in a diploid tumour a single copy gain (3:2) or loss (1:2) disappears at 75% contamination (fig 6). Therefore, when more than 75% of the cells are neoplastic, whole sections can be processed. In cases when the tumour content is less than 75%, tumour tissue can be microdissected from the sections by demarcating with a marker, scraping it off with a surgical blade, and collecting it in Eppendorf tubes (fig 7A and B). In addition, advanced laser microdis-



*Figure 7   Microdissection of a 10 µm tissue section. Example of a 10 µm haematoxylin stained tissue section in which most of the tumour cell rich part was demarcated (A) and scraped off with a surgical blade (B). The scraped off tissue from 10–20 such sections is collected in an Eppendorf tube for DNA extraction.*

section equipment can be used[49]; however, this is expensive and often not necessary. Inflammatory cells are particularly hard to dissect because they have usually infiltrated the tumour. Cell sorting techniques (for example, antibodies attached to magnetic beads or flow cytometric sorting) might permit the selection (and extraction) of tumour cells, or the elimination of inflammatory cells, to produce a more purified tumour DNA sample.

AMOUNT OF DNA

Approximately 0.5–1 µg of DNA is required for one CGH experiment. In the case of carcinomas this is usually not a problem. However, small samples—for example, biopsies from premalignant lesions, might not yield enough DNA. In this situation, DOP-PCR[35 50] and other techniques[51] have been used for DNA amplification. Because of the non-random way in which DOP-PCR may amplify DNA it could give unreliable results. Therefore, additional control experiments (such as a repeated CGH experiment with reverse labelled probes) need to be included in every CGH experiment. In our hands, CGH with DOP-PCR amplified normal DNA hybridised against non-amplified normal DNA yielded CGH profiles with fluorescence ratios smaller or greater than 1.0. When amplifying both test and reference DNA with DOP-PCR, CGH profiles appeared to be more reliable, but not as good as non-amplified normal versus normal DNA (MM Weiss *et al*, 1998, unpublished results). In conclusion, if enough DNA is available for performing CGH without amplifying the DNA it is preferable not to use DOP-PCR. However, if a lack of sufficient DNA requires amplification, both tumour and reference DNA should be amplified in the same way and control experiments need to be performed simultaneously.

DNA LABELLING

A standard method (nick translation) is used to label the DNA and digest the DNA to the optimal fragment length of 500–1500 bp. A small amount (0.5–1.0 µg) of DNA is sufficient.
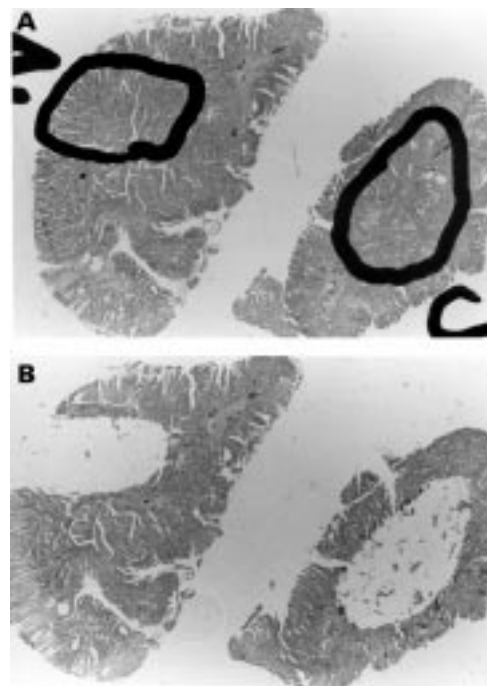
Chromosomes
Cell average
Slide average
0.75 1.25
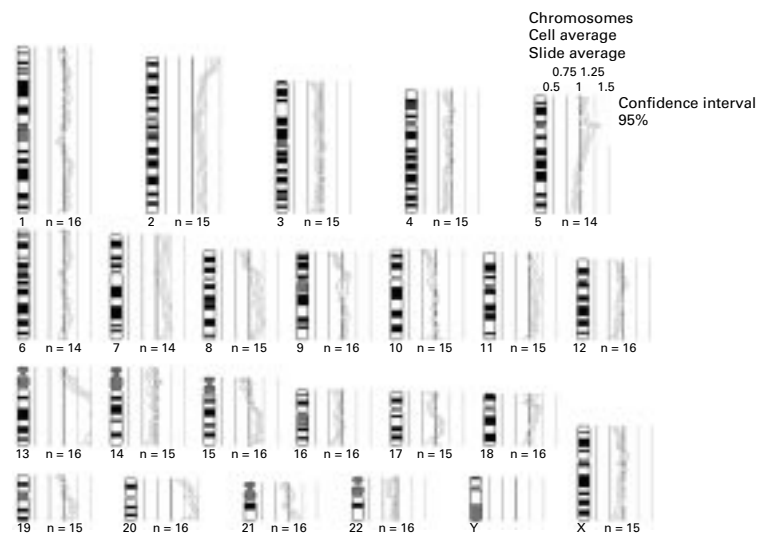0.5 1 1.5
Confidence interval
95%

*Figure 8   Comparative genomic hybridisation (CGH) analysis profile (stomach carcinoma). Relative copy number karyotype showing the quantitative analysis of a CGH experiment. The mean green to red fluorescence ratios of the chromosomes of multiple metaphases are plotted on a graph corresponding to the chromosome ideograms. With CGH analysis, the highly polymorphic centromeric and heterochromatic regions are excluded for technical reasons.[1] The relative copy number karyotype in this figure shows clear gains. The most pronounced aberrations are: gains of whole chromosomes 2 and 7, the long arm of chromosome 8, chromosome 13 (high level gain), chromosome 15, the long arm of 19, and chromosome 20. Losses are seen at the tips of the long arms of chromosome 4, 5, and 12.*

When biotinylated and digoxigenin conjugated deoxynucleotides (dUTPs) are incorporated into the DNA (indirect labelling) after hybridisation, a detection step with fluorochrome conjugated antibodies (avidin–fluorescein isothiocyanate (FITC) and sheep antidigoxigenin –tetramethyl rhodamine isothiocyanate (TRITC), respectively) is required. Deoxynucleotides that have been directly conjugated with fluorochromes render a smoother but weaker hybridisation signal along the chromosomes. It is important that the labelled DNA fragments of both tumour and reference DNA are in the same range of lengths and are within the limits of 500–1500 bp. This has to be checked by electrophoresis on an ethidium bromide stained 1% agarose gel. A protocol for nick translation is given in appendix 2.

BLOCKING

Chromosomal regions with short repetitive DNA sequences occur throughout the whole genome, but in a high number at all centromeres, telomeres, and some specific regions (chromosome arms 1p and 16p, and chromosomes 19 and 22). The lengths of these regions are highly variable between individuals (and thus between tumour and reference DNA), and this can interfere with CGH analysis. Therefore, repetitive DNA regions are blocked with unlabelled Cot-1 DNA (placental DNA from 50 to 100 bp, which is enriched for repetitive DNA sequences). Suboptimal blocking, seen as non-black centromeres, can lead to a reduced amplitude of the green to red ratio (fig 5) and gains and losses may go undetected. As an alternative to blocking, removal of repetitive sequences from the probe may be another solution to this problem.[52]

HYBRIDISATION

Aliquots of 300–350 ng of both tumour and reference labelled DNA (the probes) are mixed with 100 times the same amount of human Cot-1 DNA, precipitated, and resolved in a 6 µl hybridisation mix containing 50% formamide (which decreases the melting temperature of DNA) and 10% dextran sulphate (which increases the effective probe concentration), in 2× saline sodium citrate (SSC), pH 7.0. Sex matching of the normal and the reference labelled DNA is not necessary. In fact, the resulting uneven fluorescence ratio for the X chromosome could function as an extra control of the quality of the experiment (blocking, strength of the signal). However, sex matching should be taken into account when analysing the X chromosome. The probe and the normal metaphase slides are denatured separately: the slides in 70% formamide/2× SSC at 72°C for 5–10 minutes (depending on the metaphase slide batch), and the probes in a water bath at 80°C for 10 minutes. The hybridisation takes place under a coverslip in a humid incubator at 40°C for two to four days. After hybridisation, the slides are washed and counterstained with DAPI (0.35 µg/ml in an antifade solution (Vectashield; Vector Laboratories, Burlingame, California, USA) to produce a banding pattern, which enables chromosome identification and karyotyping. A protocol for hybridisation is given in appendix 3.

**Hardware and software requirements for CGH**
A detailed description of the hardware and software requirements can be found elsewhere.[41 43] In brief, the analysis of CGH preparations comprises the following steps.

VISUALISATION OF FLUORESCENCE

Visualisation of fluorescence of a metaphase for DAPI (blue; for chromosome identification), TRITC (red; normal reference DNA), and FITC (green; tumour DNA) using a fluorescence microscope. The microscope, lamp, objective, and the filters require special attention. Mercury arc lamp light sources are adequate if they are stable and can be aligned to give uniform illumination without chromatic variation. Microscope alignment is crucial, and should be given major attention. The objective should be of the "plan" type, apochromatic, and provide ×63 or ×100 magnification, depending on the resolution of the camera (see below). It should transmit UV light, and there should be no lens autofluorescence. The filters used should minimise the crosstalk between the three fluorochromes, which means that narrow band pass filters are required for excitation and emission. However, as the band width of a filter becomes narrower, the brightness of the remaining signals diminishes, which can result in long exposure times. Therefore, filters should provide the best compromise between crosstalk and brightness. Good filter sets for the often used DAPI/TRITC/FITC fluorochrome combination are commercially available. The lateral shift caused during changing between the filters should be
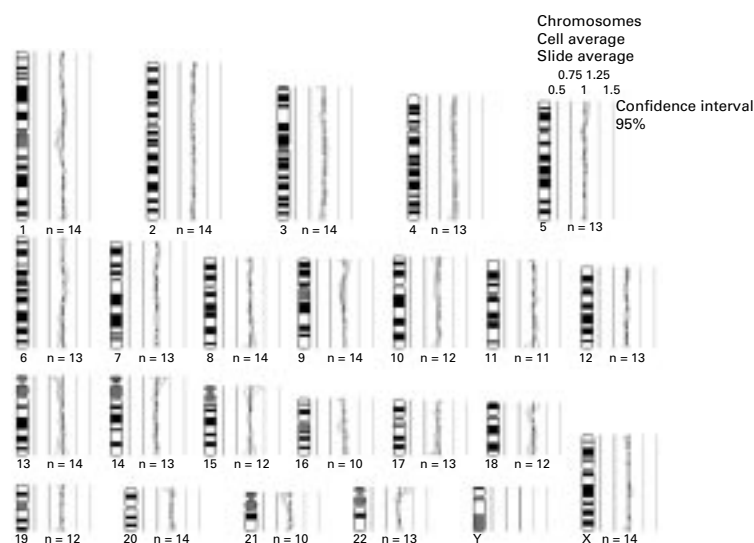
*Figure 9 Comparative genomic hybridisation (CGH) control experiment. Relative copy number karyotype of a CGH control experiment (green labelled normal DNA hybridised versus red labelled normal DNA). All the calculated green to red ratios are located close to the 1.0 line.*

minimal. An automatic filter wheel could prevent this shift, but software correction is also possible.

RECORDING THE IMAGE

The camera should provide a spatial resolution of 0.1 µm at the specimen level, to give an image of ~ $600 \times 600$ pixels for the average metaphase. It should be capable of integrating the signal for the usual time of five to 10 seconds without generating dark current. An 8 bit photometric resolution is sufficient. The camera should have a good linearity. In practice, most commercially available modern PAL-CCD video cameras will meet these criteria. An expensive camera (such as high resolution or cooled CCD) is therefore not necessary for CGH. The computer type is largely determined by the CGH software and can be any UNIX, Macintosh, or Windows 95 based machine with adequate speed. The image capture and storage time for each slide
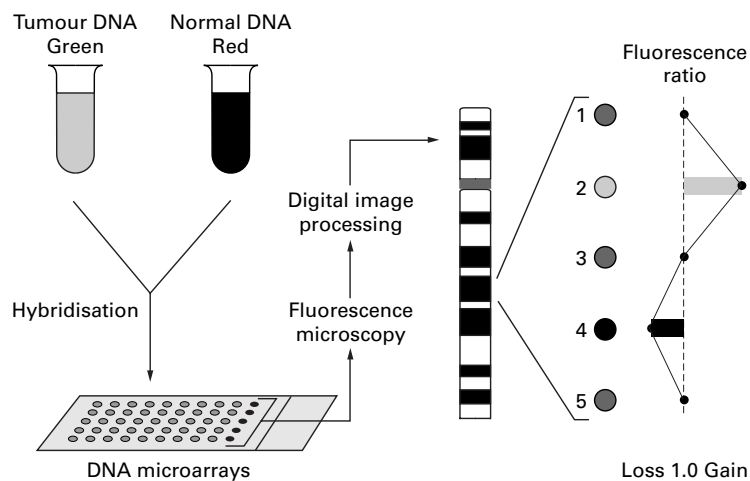


*Figure 10 Principle of microarray comparative genomic hybridisation (CGH). Schematic overview of the CGH microarray technique. Tumour and reference DNA are labelled as for "regular" CGH and hybridised to fragments of DNA (instead of metaphase chromosomes) attached to a glass slide, ordered in arrays. Images of the fluorescent signals are captured and the green to red signal ratios are measured digitally for each target.*

(10–20 metaphases) is approximately one hour. A high quality colour screen of sufficient size (at least 17 inches) is required for adequate display of the colour images and the graphical user interface of the system. When selecting the metaphases to capture, one should keep in mind the following criteria: hybridisation signals should be strong and homogeneous, not granular; background should be minimal (no residual cytoplasm); there should be minimal overlapping of chromosomes; the chromosomes of all the metaphases should be in the same length range (not extraordinarily long or short). Capturing can be done both automatically or manually in most software programs.

IMAGE PROCESSING

Dedicated CGH software (Applied Imaging, Newcastle upon Tyne, UK; MetaSystems ISIS, Belmont, Massachusetts, USA; Vysis Inc, Downers Grove, Illinois, USA; PSI Power-Gene, League City, Texas, USA; Leica, Cambridge, UK; QCGH Leica, Cambridge, UK) should enable the following steps to be carried out: (1) background subtraction; (2) segmentation and removal of non-chromosome objects; (3) normalisation of the FITC:TRITC ratio for the whole metaphase; (4) interactive karyotyping; and (5) scaling of chromosomes to a standard length, either for the whole chromosome or for each arm of the chromosome independently. For the last option it is necessary to position the centromeres correctly, which is also an interactive feature.

The averaged ratios of several well selected metaphases are plotted along ideograms (showing schematically the G banding patterns) of the corresponding chromosomes in a so called relative copy number karyotype, presenting chromosome areas that are lost (deletions) or gained (amplifications). The interpretation of the ratio profiles can be done using either fixed or statistical thresholds. Fixed limits of 0.75 and 1.25 or 0.85 and 1.15 can be used to identify losses and gains, while others use the 95% confidence interval (CI) limits of the ratio profile. In the latter, deviations from normal are interpreted as gains or losses when the 95% CI of the fluorescence ratio does not contain 1.0. Chromosomal regions 1p32–pter, 16p, and 19p should be interpreted with extreme care, and to confirm the CGH findings in these regions, it is recommended that the experiment is repeated with reversed probe labelling. This unreliability is caused by the high amount of repetitive sequences in these regions, which are variable between individuals. The Cot-1 DNA should repress these sequences (see blocking section) but often this approach is only partially successful. Figures 8 and 9 show the result of CGH analysis of tumour (gastric carcinoma) tissue versus normal tissue and of normal tissue versus normal tissue, respectively. In the control experiment, all ratio profiles are around the 1.0 value, whereas in the tumour, deviations can be seen at chromosome arms 2p, 2q, 7p, 7q, 8q, 13q, 19q, 20p, and 20q (gains) and 4q, 5q, and 12q (losses), using a 95% CI.

## New developments

A new technique that is currently being developed in different laboratories is the microarray method. Using metaphase chromosomes for hybridisation limits the detection of events involving small regions (< 10–20 Mb) of the genome. The array technique allows the detection of DNA copy number changes (genomic DNA) in more detail, as well as analysing gene expression using cDNA. Most publications so far concern cDNA arrays.[53–56] Pinkel *et al* recently published an article on genomic DNA microarrays,[57] describing a method for the measurement of copy number fluorescence ratios on a set of clones that are located on chromosome 20, with an average interval of ~ 3 Mb. The technique provides a high resolution (± 40 kb) for measurement of gains and losses of DNA sequences in genomes of mammalian complexity (fig 10). Shalon *et al* hybridised chromosomal probes to arrays of *Saccharomyces cerevisiae* genomic DNA fragments.[58] Microarray CGH will enable the analysis of genetic aberrations in cancer with a high resolution, and will refine the allocation of crucial genes. Moreover, in clinical applications it could enable the identification of the status of certain disease related genes. A detailed discussion can be found elsewhere.[59]

## Conclusions

CGH technology provides genome scale overviews of DNA sequence copy number changes in tumours, using almost any kind of clinical specimen. It maps the origins of amplified and deleted DNA sequences on normal chromosomes, thereby highlighting the locations of important genes. However, to detect translocations, inversions, and small aberrations, additional techniques must be used. Therefore, CGH supplements rather than replaces current methods.

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**Appendix 1**

**DNA isolation protocol for formalin fixed, paraffin wax embedded tissue (10 μm sections, haematoxylin stained) based on affinity columns (QIAamp tissue kit; Qiagen, Valencia, California, USA)**

(1) If the tissue contains water, spin down for five minutes at maximum speed and decant the supernatant.

The next four steps are for unmicrodissected paraffin wax sections only (to remove the wax)

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(2) Add 1 ml xylene, incubate for 10 minutes at 55°C, spin down, and decant supernatant (×3).
(3) Add 1 ml methanol, incubate for five minutes at room temperature, spin down, and decant supernatant (×2).
(4) Spin down for five minutes and decant the supernatant.
(5) Add 1 ml 100% ethanol, vortex, spin down for five minutes, decant the supernatant (×2), and air dry the pellet.

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(6) Add 1 ml 1 M NaSCN and incubate at 37°C overnight.
(7) Spin down for five minutes and decant the supernatant.
(8) Add 1 ml phosphate buffered saline (PBS), vortex, spin down, and decant the supernatant (×2).
(9) Spin down for five minutes, decant the supernatant, and air dry the pellet.
(10) Add 200 μl ATL buffer and 40 μl proteinase K (10 mg/ml), vortex, and incubate overnight at 55°C.
(11) Add 40 μl proteinase K (10 mg/ml) and incubate at 55°C all day, vortex approximately every hour during the day.
(12) Add 40 μl proteinase K (10 mg/ml) at the end of the day, incubate overnight at 55°C.
(13) Add 40 μl RNase A (20 mg/ml), vortex, and incubate for two minutes at room temperature.
(14) Add 400 μl AL buffer, vortex, and incubate for 10 minutes at 70°C.
(15) Add 420 μl 100% ethanol and vortex well.
(16) Place a QIAamp column in a QIAamp tube, apply the total volume of the sample (in steps of maximum 500 μl) on to the column, spin for one minute at 3000 ×*g*, decant the filtrate.
(17) Add 500 μl AW buffer and spin down for one minute at 8000 rpm, decant the filtrate.
(18) Add 500 μl AW buffer, spin down for three minutes at maximum speed and decant the filtrate.
(19) Place column into a new Eppendorf tube (not provided).
(20) Elute DNA with 75 μl AE buffer at 70°C, incubate for one minute at room temperature, and spin down for one minute at 3000 ×*g*.
(21) Measure the DNA concentration the next day.

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**Appendix 2**

**Nick translation for comparative genomic hybridisation (CGH)**

For a 30 μl reaction:

(1) Combine 1 μg of probe DNA, 3 μl of dNTP reaction mixture (0.2 mM dATP, dCTP, dGTP; 500 mM Tris/HCl, pH 7.8; 50 mM MgCl$_2$; 100 μM dithiothreitol; 100 μg/ml bovine serum albumin), 0.5 μl of dTTP (0.2 mM), 1 μl of digoxigenin or biotin conjugated dUTP (1 ng/μl), 3 μl of DNA polymerase I/DNase I (Gibco BRL, Breda, The Netherlands), 0–1 μl diluted DNase I (Gibco BRL). Adjust volume to 30 μl with double distilled water. Note, the amount of DNase I added varies for each DNA sample and is dependent upon the DNase I batch (dilute freshly before use).
(2) Incubate for 1.5–2 hours at 15°C.
(3) Inactivate enzymes at 70°C for 15 minutes.
(4) Check fragment length by gel electrophoresis on an ethidium bromide stained 1% agarose gel (5 μl/sample). Run at 100 V for 30 minutes. Inspect DNA fragment lengths with a UV transilluminator.
(5) For optimum hybridisation conditions, the probe (which is visible as a smear) should be between 500 and 1500 kb in length. If the probe size is larger, add more DNase I and 3 μl DNA polymerase I, incubate at 15°C for 15–30 minutes, and repeat steps 3 and 4.

**Appendix 3**

**Hybridisation**

(1) Mix 8–12 µl of the labelled tumour (biotin) and labelled reference (digoxigenin) DNA.

(2) Add 40 µg Cot-1 DNA to the mixture of labelled DNAs.

(3) Ethanol precipitate sample: add 0.1× volume of 3 M NaAc and 2× volume EtOH, spin at 10 000 ×*g* for 30 minutes.

(4) Decant supernatant and air dry pellet.

(5) Dissolve pellet in 6 µl hybridisation mixture (50% deionised formamide, 10% dextran sulphate in 2× SSC, pH 7.0. Store at −20°C).

(6) Denature metaphase slides in a jar with 70% formamide/2× SSC (20× SSC: 0.3 M sodium citrate, 3 M NaCl, pH 7.0) in a waterbath at 80°C for five minutes (dependent on batch metaphase slides, the temperature in the jar is ~ 72°C).

(7) Dry slides in an ethanol series (70%/96%/100%).

(8) Denature DNA mixture in water bath (80°C) for 10 minutes and transfer to metaphase slide immediately.

(9) Cover with a sealed coverslip (18 × 18 mm).

(10) Hybridise for two to four days in a humid chamber at 40°C.

(11) Remove coverslips carefully.

(12) Wash for 5 minutes in 2× SSC at room temperature.

(13) Wash three times for five minutes each in 0.1× SSC at 45°C.

(14) Wash for five minutes in TNT (TNT: 50 ml 10× TN, 450 ml milli-Q, 1.25 ml 20% Tween-20; 10× TN: 1 M Tris/HCl, 1.5 M NaCl, pH 7.5 at room temperature).

(15) Preincubate for 10 minutes in 100 µl TNB (TNB: 0.5% blocking reagent (Roche Diagnostics, Almere, The Netherlands) dissolved in 1× TN; before use, spin down undissolved particles and take the clear supernatant) under a coverslip (24 × 50 mm).

(16) Incubate for 60 minutes in 100 µl TNB with avidin–fluorescein isothiocyanate (FITC) (1:200) and sheep antidigoxigenin–tetramethyl rhodamine isothiocyanate (TRITC) (1:50) under a coverslip (24 × 50 mm) in a humid chamber at 37°C.

(17) Wash three times for five minutes each in TNT at room temperature.

(18) Wash for five minutes in 2× SSC at room temperature.

(19) Dry slide in an ethanol series (70%/96%/100%).

(20) Cover with 20–25 µl antifade containing DAPI (0.35 µg/µl) and seal with a coverslip (24 × 50 mm).

(21) Analyse slides with a fluorescence microscope and a digital image analysis system.

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