Chapter XX

## Comparative Genomic Hybridization (CGH) in Genotoxicology

**Baumgartner A1,2.**

1Department of Paediatric Cardiology, Cytometry Group, University of Leipzig, Heart Centre, Strümpellstr. 39, 04289 Leipzig, Germany

2University of Bradford, Biomedical Sciences, Richmond Road, Bradford, BD7 1DP, United Kingdom

Email: [a.baumgartner@bradford.ac.uk](mailto:a.baumgartner@bradford.ac.uk)

Phone: +44--1274--23--5510

Fax: +44--1274--30--9742

Summary

In the past two decades comparative genomic hybridization (CGH) and array CGH have become crucial and indispensable tools in clinical diagnostics. Initially developed for the genome--wide screening of chromosomal imbalances in tumour cells, CGH as well as array CGH have also been employed in genotoxicology and most recently in toxicogenomics. The latter methodology allows a multi--endpoint analysis of how genes and proteins react to toxic agents revealing molecular mechanisms of toxicology. This book chapter will provide background on the use of CGH and array CGH in the context of genotoxicology as well as a protocol for conventional CGH to understand the basic principles of CGH. Array CGH is still cost intensive and requires suitable analytical algorithms but might become the dominating assay in the future when more companies provide a large variety of different commercial DNA arrays / chips leading to lower costs for array CGH equipment as well as consumables such as DNA chips. As the amount of data generated with microarrays exponentially grows, the demand for powerful adaptive algorithms for analysis, competent databases as well as a sound regulatory framework will also increase. Nevertheless, chromosomal and array CGH are being demonstrated to be effective tools for investigating copy number changes / variations in the whole genome, DNA expression patterns, as well as loss of heterozygosity after a genotoxic impact. This will lead to new insights into affected genes and in the underlying structures of regulatory and signalling pathways in genotoxicology and could conclusively identify yet unknown harmful toxicants.

## Key Words:

Comparative genomic hybridization, CGH, array CGH, microarray, genomic imbalances, genotoxicology

## Running head

## CGH in Genotoxicology 1. Introduction

For cytogenetics and clinical diagnostics, the introduction of a highly versatile molecular cytogenetic technique named fluorescence *in situ* hybridization (FISH) in the mid--1980s was revolutionary in order to evaluate cytogenetic aberrations and abnormalities on a molecular basis ***(1)*.** Since then, this technique has been further improved and carried out countless times. The plethora of applications being used range from fibre FISH ***(2)***, multiplex FISH ***(3)*** and spectral karyotyping ***(4)*** to combined binary ratio labelling (COBRA) FISH ***(5)***. Also, 3D FISH applications have been more recently developed to study, for example, nuclear chromosome compartments in interphases ***(6, 7)***. Depending on the assay in use, the resolution varies from 5kb to 5Mb when examining fluorescent signals at the level of chromatin strands, interphases or metaphases ***(8)***. All the above assays have in common that complementary probes or probe sets differentially labelled with one or more fluorescent dyes are hybridized onto target DNA that needs to be investigated, such as metaphase chromosomes or interphase nuclei. Changes within the target DNA can then be visualised and evaluated. However, it has proved extremely difficult and challenging to prepare metaphase spreads from certain cells or tissues like solid tumours. Thus, it was just a matter of time until the very extensively used multicolour FISH technique was modified to serve as the basis for comparative genomic hybridization (CGH). Virtually at the same time, two groups, one in the USA at the University of California in San Francisco ***(9)*** and the other group in Heidelberg, Germany ***(10)***, recognized the principles of CGH.

## 1.1. Chromosomal comparative genomic hybridization (CGH)

The conventional chromosomal CGH method exclusively compares whole genomes for copy number changes, e.g. gain and loss of chromosomal DNA sequences. The major difference between CGH and the commonly employed FISH technique is the use of metaphase chromosomes serving only as a hybridization matrix for comparative genomic hybridization and are not representing target structures to be analysed. Metaphase chromosomes spread on glass slides therefore always originate from cultures of stimulated lymphocytes from healthy individuals, either self--prepared or commercially bought. The quality of the chromosome spreads, the condensation degree of the chromosomes and the density of the spreads on the slide are important criteria for the selection of these slides for CGH. Two sets of DNA--probes are co--hybridized onto the chromosomal matrix (metaphase chromosomes). These DNA--probes are generated from isolated genomic DNA from cells of a healthy individual or individuals (control DNA) on one hand and from specific target cells / tissue, e.g. a solid tumour, on the other hand ***(11)***. As a prerequisite, the DNA--probes have to be differently labelled with two kinds of fluorochromes, for instance the control DNA in green [e.g. fluorescein isothiocyanate (FITC)] and the test DNA in red (e.g. Cy3 or rhodamine). After hybridization, the ratio of fluorescence intensities along the metaphase chromosomes now exclusively displays the cytogenetic information regarding genomic changes in the test genome in relation to the control DNA (**Figure 1**).

< Insert **Figure 1** here >

Thus, there is no need for culturing and preparing metaphase spreads from target cells such as tumour cells in order to analyse copy number changes or numerical abnormalities in the genome of the studied cells. As the two sets of DNA--probes are applied evenly on the metaphase chromosomes, which serve as a hybridization matrix, the result is a balanced mix of the fluorescent dyes, e.g. appearing yellowish due to an even blending of green and red fluorescence intensities. Areas within the studied cells’ chromosomal setup, which are either lost or gained / amplified, lead to an imbalanced mixture of the two probe sets at corresponding complementary sequences along the metaphase chromosomes. Subsequently, image analysis is employed to qualitatively and quantitatively evaluate information about copy number variations such as deletions, duplications and amplifications, within the studied cells’ genome. The lowest detection limit of over--expressed DNA in conventional CGH was found to be 0.25 Mb. In addition, for assessing minor amplifications the rule of thumb states: the smaller the duplication the higher the copy number (≥ 20 copies). The maximum resolution after losing one homologue is approximately 2 Mb, for the loss of both homologues the resolution decreases to 1 Mb ***(12)***. Section 3 of this chapter shows a standard protocol for chromosomal CGH. DNA probes employed in this protocol are directly labelled with fluorochromes by using Nick Translation.

Initially developed to investigate chromosomal changes in the genome within solid tumours, the chromosomal CGH method has also been employed to study the impact of genotoxins to the genome. Corso and Parry developed the cell line MCL--5 for the use with the CGH assay by transfecting the human B cell--derived lymphoblastoid cell line AHH--1 TK+/-- with cDNAs from CYP1A2, CYP2A6, CYP2E1, CYP3A4 and EPHX1 in plasmids, thus, expressing human cytochrome P450 enzymes as well as microsomal epoxide hydrolase. Being metabolically active makes these cells very useful as a screening tool for mutagenicity testing of chemicals ***(13)***. In the following years toxicological studies have been undertaken determining copy number variations within the whole genome using various types of cell lines such as K562, MCF--7 and MCF--10A or by using animal tumour cells (e.g. rat gastric tumour cells). In particular, the resistance to various cytostatic drugs ***(14)***, the gastric tumour inducer and alkylating agent N—methyl--N’--nitrosoguanidine (MNNG) ***(15),*** xenoestrogens ***(16)*** and the soy isoflavone genistein ***(17)*** were evaluated. An interesting approach to evaluate environmental toxins such as mycotoxins and viruses in relation to carcinogenicity was carried out by Wong and colleagues. Using cells from human hepatocarcinomas from different geographic locations around the globe, they associated with different risk factors such as aflatoxin intoxication or hepatitis B (HBV) / C (HCV) virus infection ***(18)***. Subsequently, patterns of chromosomal gains and losses were successfully determined by chromosomal CGH. One finding indicated that HCV--related samples from Japan had a characteristically high incidence of a 11q13 gain in the tumour’s genome (***18***). With the completion of the Human Genome Project sequence information became publicly available, revolutionizing biochemical research to carry out investigations on a genome wide scale by using microarray technology ***(19)***.

## 1.2. Array CGH

In the late 1990s, the chromosomal CGH method was consequently further developed ***(20)*** using microarrays of genomic DNA fragments instead of whole metaphase chromosomes as hybridization targets which significantly increased the resolution of the methodology. Early assays employed clones with large human DNA inserts in plasmids like bacterial artificial chromosomes (BAC), P1--derived artificial chromosomes (PAC) or yeast artificial chromosomes (YAC) but also other sources of well characterized DNA sequences like cosmids or sub--cloned cDNA. The physical length of a single BAC being spotted as an element on such an array can be up to 200 kb long. These so--called BAC--arrays reached from 3,000 up to 30,000 spots and the resolution was found to be rather limited at around 3 Mb ***(21)***. Such arrays are mostly being produced in--house using self--made or commercially bought arrayers.

Soon after or even in parallel to the development of BAC--arrays, oligonucleotide--based microarrays (**Figure 2**) were introduced with elements of 25 to 85mer high--density synthetic oligonucleotides or expressed sequence tags (EST) on up to 250,000 printed spots per slide / chip providing a higher resolution of 50--100 kb ***(22, 23)***. On an array, the oligonucleotides are covalently bound to the surface of a glass slide or any other solid substrate and are conjugated with fluorochromes. After hybridization of the sample -- fragmented genomic DNA also being labelled with fluorescent molecules -- onto the oligonucleotides, the ratio of intensities of both fluorescent dyes can be visualised and subsequently evaluated. The amount of fluorescence conjugate bound to each microarray spot corresponds to the level of genes expressed in the examined cell ***(23, 24)***. In the dawn of oligonucleotide arrays, a feasibility study showed that this technology can only provide a facile overview of gene expression responses relevant to drug metabolism and toxicology ***(25).*** But with growing numbers of mapped oligonucleotides resolution and efficiency increased leading even to variants of this array--based method. The exon array CGH assay for instance evaluates exogenic copy number variations, hence, only targeting exons within genes ***(26, 27).*** Although developed for pre--implantation genetic diagnosis to assess for numerical abnormalities on single blastomeres array, CGH can also be employed to focus on the genotoxic impact at the single cell level ***(28)*** using the single--cell array CGH assay where only one specific cell such as a blastomere is analysed for its genomic copy number variations ***(29, 30)***.

A disadvantage of the above described microarray CGH methods is unapparent at first as it is intrinsic to its set--up. Due to the particular use of chromosomal DNA fragments as target structures no information on zygosity can be obtained -- this also applies to conventional chromosomal CGH. Thus, manufacturers of array--based chips are adding single nucleotide polymorphism (SNP) nucleic acids sequences (25 bp long with a centred SNP) together with oligonucleotides onto microarrays. Moreover, straight SNP arrays are able to assess DNA sequence variations within chromosomes, individuals or even species ***(31)***. Besides distinguishing loss of heterozygosity events, these high density SNP arrays also proved to be highly efficient for evaluating genome--wide copy number changes such as genomic amplifications or homozygous deletions ***(32, 33)***.Modern state—of—the—art high resolution CGH arrays are nowadays capable of detecting and assessing segmental DNA copy--number variations at a kilo base pair resolution (spatial resolution at around 35 kb) with more than 900,000 SNPs and close to one million probes for the detection of copy number variation, e.g. the Genome--Wide Human SNP Array 6.0 from Affimetrix ***(34, 35)***. Nevertheless, despite the growing number of very sophisticated microarray chips, common BAC arrays with a spatial resolution in the 150 kb range, exhibit the highest signal—to--noise ratio when compared to oligonucleotide array platforms and might be better suited to detect single-copy aberrations ***(36)***.

For genotoxicity evaluations and toxicogenomics, both types of microarrays – BAC and oligonucleotide arrays, have been employed using mainly but not exclusively cell lines as target cells for toxicants. By using BAC array CGH containing approx. 6,500 BAC clones representing 0.5 Mb genomic resolution, Herzog *et al*. ***(37)*** evaluated copy number changes and chromosomal instability in mouse lung adenocarcinoma cells induced by the potent human lung carcinogen nicotine--derived nitrosamine ketone NNK (4--(methylnitrosamino)—1--(3--pyridyl)—1--butanone) present in tobacco smoke ***(37)***. Signiﬁcantly, more gross chromosomal changes were found in NNK--induced tumours compared with spontaneous tumours ***(37).*** A 32 K BAC re--array collection (CHORI) tiling path array CGH platform was used to characterize various cell lines with and without amplifications of the EMSY gene. This gene plays a role as a potential oncogenic driver in the development of breast cancer. The ten cancer cell lines were treated with cis--platinum or olapirib to analyse for increased sensitivity to genotoxic therapies, i.e. for platinum salts or poly(ADP--ribose) polymerase (PARP) inhibitors, in the presence of an amplified EMSY gene ***(38)***. A cell line from normal human foetal colonic mucosa has been established by Soucek and colleagues and has been characterized for their mechanism of spontaneously acquired immortality ***(39)***.

Oligonucleotide arrays have been employed to evaluate genotoxins as well. Usually, cell lines but also human and animal tumour cells deriving from primary cells are the cells--of--choice to study the impact of genotoxins to the DNA, genes and underlying regulatory and signalling pathways. At around the turn of this century, the NIEHS Microarray Centre developed a so--called ToxChip with spotted human cDNAs containing clusters of up to 12,000 different cloned genes ***(40)***. This array CGH chip technology allowed the screening and classification of toxicants due to their gene expression pattern, i.e. mechanism of action. Accrued toxicant signatures then permitted the evaluation of unknown compounds by comparison with well--known toxicants. Heinloth *et al*. ***(41, 42)*** used these chips to assess the effect of 5 Gyγ--radiation, 7.5 J/m2 UV--radiation and oxidative stress (75 µM *tert*--butyl hydroperoxide) on dermal fibroblasts. The three treatments resulted in distinct patterns, indicating an involvement of ATM in regulation of transcription factors such as SP1, AP1 and MTF1 ***(41)*** as well as cyclin E--associated kinase activity reduction ***(42)***. For a detailed study on multidrug resistance a custom--designed ABC--Tox microarray was also developed to focus exclusively on ABC--transporter ***(43)***.

Genistein a major soy isoﬂavone has multiple properties and its impact on breast cancer is still controversially discussed. Therefore, MCF--10A cells were treated with genistein for three months and then evaluated using conventional chromosomal CGH and also high--density oligonucleotide microarray CGH in order to detect small copy number changes. A characteristic deletion on 9p21 was found. In general, long--term exposure might increase chromosomal imbalance ***(17)***. Castagnola *et al*. ***(44)*** focussed on oral potentially malignant lesions of the mucosal epithelium with regard to cigarette smoke and alcohol consumption. A human oligonucleotide array showed significant copy number aberrations in the genome of these cells highlighting the potential transformation risk towards carcinoma ***(44)***. Young Gprc5a--KO mice were treated with the tobacco carcinogen NNK and it was found that lung tumourigenesis was augmented by NNK in this mouse model. Microarray analysis revealed that gene expression changes induced by tobacco carcinogen(s) may be conserved between mouse and human lung epithelial cells ***(45)***. In an extensive study, another group looked at the impact hepato­carcinogens like the mycotoxin aflatoxin as well as non--hepato­carcinogens on F344/N rat liver cells over a 2 year period. Gene expression patterns revealed that the formerly untested compounds myristicin and isosafrolemay act in an hepatocarcinogenic manner ***(46)***.Gene expression profiles can also be monitored in the yeast *Saccharomyces cerevisiae* strain S288C to assess the genotoxic impact of the mycotoxin citrinin suggesting that this compound found in food such as cereals and bread significantly induces oxidative stress response genes but also other genes associated with metabolism, cell response, defence, virulence and energy ***(47)***. Huang *et al*. ***(48)*** used a microarray expression and genotyping assay to assess gene expression and genotypes (heterozygous or homozygous) in the MCF--10F cell--line after treating with the steroid hormone 17β--oestradiol (E2), which is capable of inducing complete neoplastic transformation of the human breast MCF--10F epithelial cells. Functional profiling revealed progressive alterations in the integrin signalling pathway, apoptosis inhibition and gain of tumourigenic cell surface markers. Oestrogen exposure thus triggered phenotypic and genomic changes causing tumourigenesis, which confirms the role of E2 in cancer initiation ***(48)***.

The development of microarray platforms for other species such as the very recent transcriptomic microarray platform for the Manila clam ***(49)*** will additionally allow the rapid evaluation of toxicants in our environment, more efficiently protecting wild life.

# 1.2.1. Array CGH evaluation

A high--throughput method like array CGH evaluates in great detail copy number changes within the DNA across the whole genome, thus, the evaluation of a huge amount of data becomes increasingly challenging. Fluorescently--labelled DNA--probes consisting of equal rations of test and reference DNA, co--hybridize to the mapped array DNA fragments on a slide or a chip. Array CGH intensity ratios, i.e. their transformation on the binary logarithmic (log2) scale, provide the most suitable information about genome--wide changes in copy number. In a perfect but theoretical situation without normalization or measurement errors, where for example, all tumour cells have identical genomic alterations and are uncontaminated by cells from surrounding normal tissue, the normal copy--neutral ratio would correspond to = 0 because reference and test DNA fragments both have two copies. It would indicate equal proportions of both DNAs and, thus, no copy number changes would be seen in the resulting profiles. The log2--transformed mean intensity ratios of single--copy losses and gains would exactly be = -1 and = 0.6, respectively. Multiple--copy gains or amplifications frequently found for oncogenes in tumours would relate to = 1, = 1.3 and so on. Loss of both copies on the other hand or deletions, which are often associated with tumour--suppressor mutations, would correspond to a negative infinite value (--∞). In this hypothetical situation, the genomic alterations can be easily deduced from the data without statistical techniques. However, under real world conditions, the log2 values may vary significantly from the calculated theoretical ratios. The main cause for this discrepancy can be found in the contamination of tumour samples with normal cells from the surrounding tissue as well as in the dependence between the fluorescence intensities of neighbouring DNA fragments on the array ***(50)***.

Hence, efficient and robust statistical algorithms need to be developed in order to reliably characterize the CGH profiles. A recent popular approach to analyse and characterize array CGH data is the hidden Markov modelling of these data. This model favours a division of the signal into segments of constant copy number and a subsequent classification, which describes each segment as neutral, a loss or a gain. A disadvantage of this technique is the sensitivity towards outliers triggering over--segmentation with the consequence that segments then incorrectly stretch across very short regions ***(51)***. A modified hidden Markov model combines the necessity to account for the dependence between neighbouring DNA fragments with an adopted Bayesian approach, which assumes informative priors for the model parameters. The strong point of this Bayesian hidden Markov model is its reliability on essentially no tuning parameters; only the input of normalized log2 ratios is required, which is very convenient for the end user with little or no statistical training ***(50)***.

## 1.3. Toxicogenomics

In the wake of microarray technologies new scientific fields have emerged, opening new ways for researchers. Toxicogenomics was one of them as it investigates how the genome reacts to hazardous substances and identifies genes that respond to groups or categories of chemicals by using high--throughput “omics” technologies such as genomic--scale mRNA expression (transcriptomics), cell-- and tissue--wide protein expression (proteomics) as well as metabolite profiling (metabolomics) in combination with bioinformatics and conventional toxicology ***(52, 53)***. In essence, toxicogenomics studies the relationship between the structure / activity of the genome and the detrimental biological effects of exogenous agents, enabling a multi--endpoint analysis of previously inaccessible information about the functional activity of biochemical pathways and differences among individuals and species ***(54)***.

Microarray technology in toxicological research allows the evaluation of toxin--modulated gene expression at mRNA level and, thus, reveals molecular mechanisms of toxicology. With this technology the identification of genes and their products being involved in resistance or sensibility to toxic compounds became possible ***(55)***. A comparison of CGH technologies across microarray platforms such as BAC array, genotyping oligonucleotide array and RNA expression microarray showed similar variations per probe. They all performed at an optimal level to detect known copy number variations when using for instance HL--60 cells. The evaluation of the performance of these platforms becomes more important in the future as the array CGH technology continues to evolve ***(36)***. Aardema and McGregor anticipated in 2002 that this new technology will spawn new families of biomarkers permitting the characterization and efficient monitoring of cellular perturbation. This will then lead to a better knowledge of the influence of genetic variation on toxicological outcomes and will allow the definition of environmental causes of genetic alterations and their relationship to human disease. Such an integrated approach will most likely amalgamate the fields of cell pathology, toxicology, molecular genetics and genetic toxicology generating a comprehensive understanding of genetic control of cellular functions and of cellular responses to alterations in normal molecular structure and function ***(54)***.This integrated approach for example has much to contribute to the early prediction of drug toxicity and adverse drug reactions ***(56)***.

Toxicogenomics bridges genotoxicity and carcinogenicity with methods being commercially available for high throughput analyses. In the 21st century, computational toxicology for safety testing within a regulatory setting will significantly contribute to a substantial reduction of animal testing and human clinical trials (***53***). Based on the analysis of gene expression using array--based toxicogenomics, it will become possible to screen for carcinogenicity and at the same time differentiate the possible mode of action of a detrimental compound ***(53)***.

# 1.3.1. Public databases

The public Comparative Toxicogenomics Database (http://ctdbase.org/) provides a centralized resource based on literature linking the chemical to the gene and the disease, as well as the gene to the disease to better comprehend the interaction of genes and gene products with environmental chemicals, and thus their effects on human health. This database integrates information about sequence, reference, species, microarray and general toxicology and is also capable of visualizing cross--species comparisons of gene and protein sequences providing information for building complex interaction networks ***(57, 58)***. Up to date the database holds close to 16 million toxicogenomics relationships. Other databases include web resources such as the toxicological data network TOXNET (http://toxnet.nlm.nih.gov/), the European chemical substances information system ESIS (http://esis.jrc.ec.europa.eu/) or the carcinogenic potency database CPDB (http://potency.berkeley.edu). An overview of these and other databases can be found in a reviewed and published list of toxicology databases ***(59)*** or at http://alttox.org/ttrc/resources/databases.htmL.

## 1.4. Regulatory Guidelines

In toxicology, it is crucial that the screening of toxicants as well as understanding of toxicity pathways is based on methodologies, which are sound, proven and embedded in regulatory guidelines. The field of “omics” has become an important tool for the evaluation of general and reproductive toxicology, the carcinogenicity potential of pharmaceuticals and several other types of toxicity, eventually replacing the use of animals. Much progress has been made in the last decade to standardize procedures; however, challenges remain for the assessment of pharmaceuticals for regulatory purposes in terms of off--target toxicological effects or issues of interpretation ***(60)***. The use of microarray technology in toxicology marks the advent of toxicogenomics allowing monitoring the expression level of thousands of genes on a genome wide scale ***(61)***.

In 2007, the U.S. National Research Council (NRC) released a report, "Toxicity Testing in the 21st Century: A Vision and a Strategy" that proposes a paradigm shift for toxicity testing involving a fundamental change in toxicity assessment towards the identification of serious perturbations of toxicity pathways ***(62, 63)***. Perhaps with this proposed vision, toxicity testing will be significantly reduced or maybe the use of animals even completely eradicated in toxicity testing. It will focus much closer on human significance and higher throughput enabling a wider coverage of toxicants, life stages and sensitive subpopulations ***(64)***. Methodologies such as microarrays will help to transform current toxicology tests towards an *in vitro* toxicity pathway test approach as proposed by the NRC ***(64, 65)***. Functional genomics as envisioned by the NRC then involves the assessment of gene alterations as well as protein and metabolite profiling upon chemical exposure. This will allow the identification of target pathways, establish dose--relationships and map underlying structures for pathway activations in a toxicological context ***(64, 66–68)***.

The microarray technology is a particularly powerful tool to identify new genotoxic substances and their mechanisms of action. It also allows determining no--effect levels and different susceptibility levels for tissues and cell types. However, besides the consideration of cost and effort, this microarray technology also requires careful planning and choice of analysis methodology to get optimal results ***(69)***. Certain parameters such as suitable signal—to--noise ratios, low standard deviations (SD) of intensity ratios as well as an optimized cot--1 DNA to probe DNA ratio are essential for a successful employment of this technology. It has to be taken into account that genomic array CGH uses a more complex probe mixture and shows lower copy number variations compared to pure expression arrays. Thus, it is crucial to indicate the percentage of spots that provided acceptable values of intensity ratios (typically >97%, signal--to--noise ratio >2 and SD <10% for the duplicates) ***(70)***. It is further important to define threshold values to avoid false negative results, i.e. to eliminate false positives without removing true positives. Such a threshold for deletions and duplications can be described as the mean ± 4SD ***(71)***. This cut--off level results in one false positive for every four analyses (for an array with 3,500 loci) as 99.9936% of the fragments fall within the normal range. For a valid diagnosis, this value of four times SD has to be below the detection limit of an autosomal duplication since the log2 of the ratio of duplication ( = 0.6) is closer to the normal ratio ( = 0) than to the ratio of a chromosomal loss ( = -1). Thus, this threshold can be defined as the difference between the log2--transformed mean intensity ratio of duplicated loci and twice the SD, i.e. 4SD ≤ (() -- 2SD) or SD ≤0.096. It is therefore imperative for quality control that data are obtained with criteria that address the quality and threshold values. In particular, it is crucial to report the number of clones on an array with successful hybridization, a minimum threshold for normal clones and that the cut--off threshold for the SD of the log2--transformed intensity ratios did not exceed 0.096 ***(70)***.

Exponentially increasing amounts of data from highly adaptive and high--throughput microarray assays will entail a crucial role for bioinformatics with a demanding need for standardization. In 2001, the “Minimum Information About a Microarray Experiment” (MIAME) database ***(72)*** was set up to help structuring and channelling the enormous amounts of generated data for biostatistical analyses or even meta--analyses. Hence, bioinformatics is becoming more and more essential when using array chip technology to understand the mode of action and the regulatory cellular networks being involved in the toxicity pathway function ***(64)***. Targeting gene expression patterns of murine lung cells from more than 250 microarrays, Taylor *et al*. ***(73)*** were able to show by using a set of network algorithms that nuclear factor erythroid 2--related factor (Nrf2) is a direct regulator of proteins involved in oxidative stress response after exposure to reactive oxygen species. Bioinformatics has also been utilized to predict novel transcriptional targets of Nrf2. Thus, network inference algorithms operating on high--throughput gene expression data have the potential to identify regulatory and signalling pathway relationships implicated in disease ***(73)***.

Standardization, quality control and sound biostatistical algorithms are key to publish valid and comparable results, especially with regard to toxicological and toxicogenomic databases, which may hold results of a vast quantity of experiments from different laboratories around the world.

**2. Materials**

***2.1. DNA labelling***

1. Nick translation buffer (10X): 500 mM Tris--HCl, pH 7.5, 100 mM MgSO4, 1 mM Dithiothreitol, 500 µg/mL Bovine Serum Albumin. For 20 mL, combine 10 mL 1 M Tris--HCl (pH 7.5), 2 mL 1 M MgSO4, 0.2 mL 0.1 M Dithiothreitol and 10 mg Bovine Serum Albumin. Mix and adjust to final volume with pure water and freeze aliquots at --20 °C.
2. DNase I solution: 0.4 mU/µL DNase I, 40 mM Tris--HCl, pH 7.5, 6 mM MgCl2, 2 mM CaCl2. Prepare always fresh.  
   For 15 mL of DNAse buffer (10X), combine 6 mL 1 M Tris--HCl (pH 7.5), 0.9 mL 1 M MgCl2 and 0.3 mL 1 M CaCl2. Mix and adjust to final volume with pure water and freeze 1 mL aliquots at --20 °C. For use, dilute 10X DNAse buffer after thawing with pure water to a 1X concentration. Then mix the DNAse I enzyme (Invitrogen) with 1X DNAse buffer to yield a final enzyme concentration of 0.4 mU/µL.
3. TAE buffer (50X): 2 M Tris--base, 1 M Acetic acid, 50 mM EDTA, pH 8.0.
4. 1.5% Agarose gel
5. 0.5 µg/µL Genomic DNA
6. 40 mM dNTPs (dATP, dCTP, dGTP, dTTP)
7. 1mM dNTPs (dATP, dCTP, dTTP)
8. 1 mM Cy3--dCTP
9. 1 mM Cy3--dUTP
10. 1 mM Fluorescein--12--dATP
11. 1 mM Fluorescein--12--dCTP
12. 0.5 U/µL DNA Polymerase I
13. Absolute ethanol
14. 1 kb DNA ladder
15. Loading dye
16. 0.5 µL/mL Ethidium bromide
17. 20 mg/mL Glycogen

***2.2. Conventional CGH***

All aqueous solutions have to be prepared with pure water, e.g. milliQ water.

1. 70% Formamide solution, pH 7.0: 70% (v/v) deionized Formamide in 2X SSC. For 100 mL, mix 70 mL deionized Formamide, 10 mL 20X SSC and 10 mL pure water; adjust to final pH with 1 M HCl and then to final volume with pure water.
2. 50% Formamide solution, pH 7.0: 50% (v/v) deionized Formamide in 2X SSC. For 100 mL, mix 50 mL deionized Formamide, 10 mL 20X SSC and 30 mL pure water; adjust to final pH with 1 M HCl and then to final volume with pure water.
3. SCC buffer (20X), pH 7.0: 3 M NaCl, 0.3 M Sodium citrate, adjust to pH 7.0 with 1 M NaOH. For 500 mL, dissolve 87.7 g NaCl and 44.1 g Sodium citrate dihydrate in 400 mL of pure water; adjust to the final pH and then to final volume.
4. Master Mix: 71.4% (v/v) deionized Formamide, 14.3% (w/v) Dextran sulphate, 2.86X SSC. Mix 5.5 mL deionized Formamide, 1 mL 20X SSC and 1 g Dextran sulphate and heat for 2–3 hours at 70 °C to completely dissolve the dextran sulphate; adjust to pH 7.0 with 1 M HCl and fill up to the final volume of 7 mL with pure water. Store 1-mL aliquots at --20 °C.
5. PN buffer, pH 8.0: 0.1 M NaH2PO4 (monobasic), 0.1 M Na2HPO4 (dibasic), Adjust to pH 8.0 with titration by adding monobasic solution (pH ~4.5) to the dibasic solution until a pH of 8.0 is reached, then, after measuring the reached volume, add 0.1% (v/v) IGEPAL® CA--630 (0.1 mL per 100 mL phosphate buffer).
6. PNM buffer, pH 8.0: 5% Non--fat dry milk powder, 0.02% Sodium azide, make a suspension with PN buffer, adjust pH with 1 M NaOH while stirring, stir overnight at 37 °C, centrifuge for 10 min at 3000 x g, sterile--filter the supernatant using 0.45 µm filter to avoid bacterial growth, aliquot and store working solution at 4 °C and rest of aliquots at --20 °C.
7. DNA counterstaining solution (antifade): 0.05 µg/mL 4′,6—Diamidino—2--phenylindole dihydrochloride (DAPI) , make a solution with 2X SCC buffer and store aliquots at --20 °C.
8. Antifade solution: 0.2 M Tris--base solution, pH 8.0, 90% (w/v) Glycerol, 2.33 g of 1,4--Diazabicyclo[2.2.2]octane (DABCO), store aliquots at --20 °C.
9. RNase A solution: 10 mM Tris--HCl, pH 7.5, 15 mM NaCl, 10 mg/mL RNase A, boil the stock solution for 15 min and let it slowly cool down to room temperature, store aliquots at --20 °C. Use a 1:100 dilution in 2X SSC, pH 7.0, as a freshly prepared working solution.
10. Pepsin stock solution: 10% Pepsin (200 mg pepsin in 2 mL pre--warmed water (37 °C); freeze 50--µL aliquots at --20 °C).

As a freshly prepared working solution, use 10–50 µL of pepsin stock solution per 100 mL of 0.01 M HCl, pH 2.3 (pepsin end concentration of 10--50 µg/mL).

1. 1.5 % Agarose gel
2. Loading dye
3. Cot--1 DNA
4. Salmon sperm DNA
5. Sodium acetate
6. Magnesium chloride
7. Formaldehyde

**3. Methods**

## 3.1. DNA labelling

Occasionally, the amount of isolated DNA from test cells or single cells is not enough. Then, it is recommended to amplify the DNA before DNA labelling by using degenerate oligonucleotide primed PCR [DOP—PCR; ***(74)***] or primer extension pre--amplification [PEP; ***(75)***].

# 3.1.1. Nick translation

Nick translation is used to incorporate fluorescently labelled deoxynucleotides into the DNA. Two enzymes, DNase I randomLy nicking the DNA and bacterial DNA polymerase I, are employed in this assay. The latter enzyme possesses a 5’🡪3’ exonuclease activity to remove 1–10 nucleotides starting from a nick in the DNA as well as a 5’🡪3’ polymerizing gap--filling activity, elongating the DNA from the 3’ hydroxyl terminus mediating nick translation along the strand. By encountering a nick on the opposite strand, a double--strand break will be generated. For DNA polymerization, this template--dependent enzyme requires deoxynucleotides (dNTP) and bivalent Mg2+ ions. To enhance the efficiency of DNA labelling the employed DNA polymerase I lacks the 3’🡪5’ proofreading activity, which is found in the native enzyme. This assay produces a range of differently sized probes resulting in smaller fragments over time.

1. Prerequisites: Set the water bath to 16 °C, set the heating blocks to 37 °C and 65 °C, cool down absolute ethanol at --20 °C, prepare agarose gel and buffers during the procedure
2. Mix reactions from **Table 1** in 500µL vials, e.g. Eppendorf vials, always pipette enzymes on ice (*see* **Note 1**)
3. Incubate reactions at 16 °C for 90 min and then put them on ice
4. Run 10 µL volume of each reaction on a 1.5% agarose gel – the range of the length of the labelled DNA fragments on the gel should run as a smear from 0.3–2.3 kb (*see* **Section 3.1.2**)
5. Continue incubation at 16 °C for another 15--30 min if the fragments are still too large or stop reaction at 65 °C for 15 min accordingly
6. Remove excess nucleotides, by using Nucleotide Removal Kit commercially available (e.g. Bio Spin 30 columns [Bio--Rad] or the QIAquick from QIAGEN)
7. Ethanol--precipitate the labelled DNA (*see* **Section 3.1.3**)
8. Keep the resuspended DNA in the fridge before use. For longer storage, store at -20 °C

< Insert **Table 1** here >

# 3.1.2. Agarose gel electrophoresis

1. Prepare a 1.5% normal melting point agarose gel in 1X TAE buffer
2. Mix 10 µL of reaction volume with 1 µL of loading dye – as a marker use a 1 kb standard [e.g. GeneRuler, Fermentas]
3. Mix 4 µL of water with 1 µL of 1 kb ladder and 1 µL of loading buffer [e.g. Blue/Orange Loading Dye (6X) from Promega)
4. Fill the wells of the gel with the standard and the reactions
5. Use 1X TAE buffer as the electrophoresis running buffer
6. Run the electrophoresis at 100 V, 300 mA for approximately 20 min
7. Stain the DNA, by using 0.5 µL/mL of ethidium bromide or SybrGreen I, either during preparation of the gel or by submersing the gel in an ethidium bromide solution
8. Document the agarose gel – the length of the labelled DNA fragment should be in the range of 0.3--2.3kb

# 3.1.3. Ethanol precipitation

1. Add to each reaction 1 µL of 20 mg/mL glycogen (Invitrogen) per 50 µL of volume and mix thoroughly. Glycogen will help to precipitate the DNA in an ethanol solution.
2. Add -20 °C cold ethanol (2.5 times the reaction volume)
3. Mix by inverting the vial three times.
4. Put vials for a minimum of 30 min in the freezer (better for 1 h or overnight)
5. Centrifuge at highest speed at 4 °C for 30 min using a table--top centrifuge.
6. Discard the supernatant, place the vials upside down on paper tissue
7. Dry the pellet for 5 min using a Speed--Vac centrifuge (no heating) or at 37 °C for 20–30 min in a drying cabinet
8. Resuspend each DNA pellet in pure water (20--80 µL) and shake at 37 °C for 30 min

## 3.2. Chromosomal CGH

Prerequisites: Set the water bath to 72 °C and warm up both water and Coplin jar with 70% formamide at the same time, set heating blocks to 37 °C and 76 °C, cool down 70% ethanol at --20 °C, warm up washing solutions a) 2X SSC, b) 50% formamide and c) 0.2X SSC to 42 °C. Avoid exposure to light when working with fluorochromes.

# 3.2.1. DNA precipitation

1. Mix reaction (as given in **Table 2**) in a 500µL vial, e.g. Eppendorf vial (*see* **Note 2)**

2. Precipitate at -20 °C overnight (alternatively at --80 °C for 30 min)

3. Centrifuge with highest speed at 4 °C for 30 min using a table--top centrifuge

4. Remove the supernatant

5. Wash the pellet with 250 µL of 70% ethanol (--20 °C cold)

6. Centrifuge with highest speed at 4 °C for 10 min using a table--top centrifuge

7. Remove the supernatant and place the vials upside down on tissue paper

8. Dry the pellet for 5 min using a Speed--Vac centrifuge (no heating) or at 37 °C for 20--30 min in a drying cabinet

9. Resuspend the pellet in 3 µL of pure water. This is the ‘probe DNA’.

< Insert **Table 2** here >

# 3.2.2. Preparation of the hybridization mix

1. Add 7 µL of Master Mix (*see* **Note 3**) to the resuspended probe DNA (from step 9 of section 3.2.1)

2. Mix thoroughly

3. Incubate at 37 °C for 30 min

# 3.2.3. Denaturation and pre--annealing of the probe DNA

1. Denature the probe DNA at 76 °C for 7 min

2. Incubate at 37 °C for 45 min to allow for pre--annealing of cot--1 DNA to repetitive DNA sequences, e.g. *alu* sequences, in the reference and test DNA (*see* **Note 4**)

3. In the meantime warm up formamide solution and start denaturing metaphase slides (*see* **Section 3.2.5**) 15 min before the end of the pre--annealing step

# 3.2.4. Pre--treatment of metaphase chromosomes with RNase A and Pepsin

*The following pre--treatment steps are optional; however, they might be crucial for optimal hybridization of the DNA probes, especially when self--made slides are used.*

1. Apply 150 µL of 100 µg/mL RNase A solution, on to the slides

2. Cover the slides with a plastic cover slip, e.g. a piece of Parafilm

3. Incubate at 37 °C for 60 min in a wet box

4. Shortly dip slides in 2X SSC

5. Wash three times for 5 min in 2X SSC at room temperature

6. Place slides in an empty Coplin jar and fill it up with pepsin working solution

7. Incubate at 37 °C for 3–10 min (see **Note 5**)

8. Rinse shortly in PBS. Wash twice for 5 min each in PBS at room temperature

10. Incubate for 5 min in PBS substituted with 50 mM MgCl2

11. Incubate for 4 min in 1% formaldehyde to bind the chromosomes to the glass surface and to cross--link residual proteins on the slide, this also includes the deactivation of remaining pepsin (*The incubation time might vary from 1–15 min depending on the metaphase quality*)

12. Rinse shortly in PBS, then wash for 5 min in PBS at room temperature

14. Run slides through 70%, 90% and 100% ethanol series for 2 min each

15. Air--dry slides

# 3.2.5. Denaturation of the target DNA

1. In a Coplin jar with 70% formamide, denature metaphase slides (e.g. CGH Metaphase Target Slides from Abbott Molecular) at 72 °C for 5 min. (*Caution: Temperature decreases by 1 °C per slide)*

2. Transfer the slides into a Coplin jar with 70% ethanol (-20 °C cold) and incubate for 2 min (cold 70% ethanol is favoured in this step; dehydration and the drastic temperature change helps to keep the strands of the denatured target DNA separated).

3. Incubate the slides for 2 min each in 90% and absolute ethanol (at room temperature)

4. Allow the slides to air--dry

# 3.2.6. In situ hybridization

1. Shortly pre--warm metaphase slides on a warming block at 37 °C

2. Apply the hybridization mix on to the metaphase slides at an area with a high amount of metaphases

3. Cover with a 22x22 mm2 glass cover slip and seal it with rubber cement

4. Incubate at 37 °C for 48–72 hours in an incubator

# 3.2.7. Washing steps

*Caution: Protect the slides from light exposure in all the subsequent steps*

*Do not ever let the targeted area(s) on the slides dry out*

1. Carefully remove the rubber cement

2. Let the cover slip slide off in 2X SSC (this takes approx. 5 min)

3. Wash for 30 min in 50% formamide solution at 42 °C

4. Rinse shortly in 2X SSC

5. Wash for 5 min in 2X SSC at 42 °C

6. Wash twice for 5 min each in 0.2X SSC at 42 °C

7. Place slides for 5 min in PN buffer at room temperature (*See* **Note 6**)

# 3.2.8. DNA counterstaining

1. Incubate slides in DNA counterstaining solution for 30–60 s at room temperature

2. Wash shortly in pure water

3. Place slides for 5 min each in PN buffer at room temperature

4. Drain the buffer, apply 40 µL antifade solution and cover--slip the slides

5. Store slides in the dark at 4 °C until evaluation

# 3.2.9. Evaluation

For the evaluation of the CGH slides a fluorescent microscope equipped with a set of suitable excitation and emission filters, CCD camera and a computer is considered a prerequisite. In principles, it is possible to roughly analyse CGH slides manually; however, this is rather inaccurate. Analysis software is highly recommended to get an accurate quantitative and qualitative evaluation of the CGH. Companies like MetaSystems, Altlussheim, Germany offer evaluation software (http://www.metasystems--international.com/isis/cgh) to analyse a set of metaphase for numerical abnormalities as well as copy number variations as seen in loss and gains of chromosomal material. Data interpretation then becomes more precise as the results from many metaphases are integrated for each chromosome (*see* **Note 7**).

A reliable evaluation requires the assessment of a number of metaphases (usually 5 to 15) with certain characteristics. The spreading has to be optimal with an even staining and only a few or none overlapping chromosomes. Besides a low unspecific background, the hybridization pattern along the chromosomes has to be even with consistent intensities. The correct settings of upper and lower thresholds are therefore very important. For karyotyping, the inverted black--and--white image of the counterstained metaphase is used. The chromosomes are subsequently sorted for identification according to their number, size and banding patterns by an experienced investigator. Some programmes allow automatic sorting of the chromosomes but the resulting karyotype should always be additionally checked. The fluorescent intensities along the axis of the chromosomes finally result in an intensity profile. Average profiles for all 24 different human chromosomes are then calculated by using the profiles from all analysed metaphases -- at least four copies of each chromosome should be analysed. Centromeres, the p--arms of acrocentric chromosomes (13, 14, 15, 21 and 22) and heterochromatin regions, i.e. 1q12, 9q12, 16q11.2 and Yq12, are usually excluded from interpretation (***76***–***79***).

## 4. Notes

1. Avoid exposure to light when working with fluorochrome labelled nucleotides. Use drawers, covers or boxes to protect the slides from direct sun light. Use yellow fluorescent lights in the lab if possible.
2. This DNA precipitation mix already contains all the DNAs needed for the hybridisation process: both the labelled reference as well as the test DNA, human cot--1 DNA in excess (to block non--specific hybridisation of the probe to non--target DNA, hence the pre--annealing step later on in the protocol) and salmon sperm DNA (which acts as a carrier DNA reducing the background by associating to non--biological sites during hybridisation). Sodium acetate and ethanol are used to precipitate the DNAs.
3. The Master Mix contains 3 crucial ingredients necessary for successful hybridisation of DNA probes to target DNA. Formamide destabilises hydrogen bonds within the DNA’s double helix; as a consequence reducing the melting point of the DNA. Dextran sulphate is a polyanionic derivative of the glucan dextran acting as a crowder substance in the Master Mix. It helps to accelerate the hybridisation process by reducing the access to water to nucleic acids. Monovalent cations like Na+from the SSC buffer interact with the negatively charged phosphate backbone of the DNA, thus, increasing the affinity between the DNA probe and the target DNA.
4. This incubation is crucial as cot--1 DNA pre--anneals in this step with moderately repetitive sequences in the probe--DNA reducing later on unspecific background signals on the target DNA throughout the genome. Cot--1 DNA consist of a fraction of DNA with a re--association coefficient of co \* t = 1 mol s l--1, combining time of incubation and DNA concentration in this term.
5. The incubation time depends in the quality and the age of the metaphase slides. It is recommended to use different time intervals to determine the optimal incubation time as well as different concentrations of pepsin as indicated in section 2,2. Once established for a batch of slides, use the optimal concentration and time of exposure to digest superficial proteins for the rest of the slides. Avoid over--exposure to pepsin as the chromosomes can detach from the glass surface of the slide.
6. If DNA probes have been indirectly labelled with reporter molecules like biotin or digoxigenin by using correspondingly conjugated nucleotides for the mixture in Table 1 then the detection procedure must be carried out after the above washing steps. For this example, no detection procedure is necessary as the DNA probes are directly labelled with fluorochromes.
7. The resolution of an experiment is in general defined by the number of bands that can be distinguished (high resolution ranges from 550 – 850 visible bands). Hence, it is directly proportional to the length of the metaphase chromosomes. The condensation degree which defines the length of the metaphase chromosomes can be influenced by the concentration and the duration of exposure of colcemid to the cells during the culture of PHA--stimulated peripheral blood lymphocytes. Colcemid arrests the cells in metaphase by depolymerising the microtubules of the spindle fibre. Commercially available metaphase spreads are usual showing long prometaphase chromosomes suitable for high resolution CGH.

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**Figure Captions**

**Figure 1**:

Chromosomal CGH – Representation of gain (on p--arm) and loss (terminal on q--arm) of chromosomal material in the test genome in relation to the reference / control genome. After hybridization DNA probes made from genomic DNA of the test and the control cells, the fluorescence ratio of 1.0 = indicates two copies of homologue sequence throughout the chromosome / genome -- for both the control and test genomic DNA. The loss of one or even two homologues leads to ratios of 0.5 = or 0 = , respectively. The latter then only shows the fluorescence of the fluorescently labelled control DNA.

**Figure 2**:

Array CGH. Fluorescently labelled cDNAs generated from RNA of both control and test cells are hybridized onto a DNA chip (e.g. oligonucleotide microarray). Subsequent evaluation of the resulting dot matrix on the chip produces the gene expression profile of the studied cells.

**Table Captions**

**Table 1:**

Exemplary mixtures for labelling normal and test genomic DNA by using Nick translation. The test DNA can be for instance from a tumour.

**Table 2:**

Exemplary mixture of various probe DNA for CGH

**Table 1:**

|  |  |  |
| --- | --- | --- |
| Reagents | Reference DNA [µL] | Test  DNA [µL] |
| Water (pure, autoclaved) | 53.4 | 53.4 |
| Genomic DNA (0.5 µg/µL) | 12 | 12 |
| Nick--Translation Buffer (10x) | 10 | 10 |
| dATP (40 mM) | -- | 2 |
| dCTP (40 mM) | -- | -- |
| dGTP (40 mM) | 2 | 2 |
| dTTP (40 mM) | 2 | -- |
| dATP (1 mM) | 0.8 | -- |
| dCTP (1 mM) | 0.8 | 0.8 |
| dTTP (1 mM) | -- | 0.8 |
| Cy3--dCTP (1 mM) [PerkinElmer] | -- | 4 |
| Cy3--dUTP (1 mM) [PerkinElmer] | -- | 4 |
| Fluorescein--12--dATP (1 mM) [PerkinElmer] | 4 | -- |
| Fluorescein--12--dCTP (1 mM) [PerkinElmer] | 4 | -- |
| DNase I Solution (5--10 µL depending on DNA) | 5 | 5 |
| DNA Polymerase I (0.5 U/µL) [LifeTechnologies] | 6 | 6 |
| Total Volume | 100 | 100 |

**Table 2:**

|  |  |
| --- | --- |
| Reagents | [µL] |
| Reference DNA, fluorescein—labelled | 20 |
| Test DNA, rhodamine—labelled | 20 |
| Human cot--1 DNA (1 mg/mL) | 40 |
| Salmon sperm DNA (10 mg/mL) [Sigma] | 1 |
| Sodium acetate (3 M, pH 5.0); 1/10 volume | 8.1 |
| Ethanol, absolute, --20 °C; 2.5x volume | 202.5 |
| Total Volume | 291.6 |