Chapter 2

Comparative Genomic Hybridization on BAC Arrays

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Abstract

Alterations in genomic DNA are a key feature of many constitutional disorders and cancer. The discovery of the underlying regions of gene dosage has thus been essential in dissecting complex disease phenotypes and identifying targets for therapeutic intervention and diagnostic testing. The development of array comparative genomic hybridization (aCGH) using bacterial artificial chromosomes (BACs) as hybridization targets has facilitated the discovery and fine mapping of novel genomic alterations allowing rapid identification of target genes.

In BAC aCGH, DNA samples are first labeled with fluorescent dyes through a random priming reaction with 100–400 ng of genomic DNA. This probe is then co-hybridized to an array consisting of BAC clones, either tiling the genome (~50 kbp resolution) or spaced at intervals (e.g., 1 Mbp resolution). The resulting arrays are then imaged and the signal at each locus is compared between a reference and test sample to determine the copy number status. The DNA samples to be analyzed may be derived from either fresh, frozen, or formalin-fixed paraffin-embedded material, and sample requirements are currently significantly lower than those for oligonucleotide platforms due to the high probe-binding capacity of BAC clone targets (~150 kbp) compared to oligonucleotides (25–80 bp). In this chapter, we describe in detail the technical procedure required to perform copy number analysis of genomes with BAC aCGH.

Key words: CGH, array CGH, bacterial artificial chromosomes, genomics, gene dosage, DNA copy number.

1. Introduction

DNA copy number changes are hallmarks of constitutional diseases and cancer. Somatic alterations in gene dosage lead to the disruption of both oncogene and tumor suppressor gene expression during cancer development whereas variations in DNA copy number have been associated with developmental disorders *(1)*. Therefore, great effort has been employed to define regions of

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copy number change in these diseases in order to uncover pathologically related genes. The advent of conventional comparative genomic hybridization (CGH) allowed researchers to understand the patterns of gene dosage across the entire genome, albeit at a relatively low resolution of ~10 Mbp *(1, 2)*. This technique can identify regions of DNA duplication, amplification, and deletion in diseased cell populations, but will not detect balanced chromosomal alterations such as translocations *(3)*. The capabilities of chromosome-based CGH were improved by the development of array CGH (aCGH), whereby DNA targets are spotted onto a glass surface to serve as hybridization targets as an alternative to using metaphase chromosome spreads *(4, 5)*. Numerous genome-wide CGH arrays have since been produced each differing in the size of the genomic elements spotted and their corresponding coverage of the human genome *(6)*. The first reported genome-wide aCGH approach was using expression cDNA microarrays *(7)*. The utilization of large insert clones (typically BAC clones of ~100–150 kbp size) improved the sensitivity of hybridization targets to their corresponding probes, resulting in high signal-to-noise ratios and accurate assessment of copy number *(8)*. Most recently, oligonucleotide (25–80 bp nucleotide probes) arrays were also developed with the goal of improving the maximal resolution of CGH beyond the size of a BAC clone. Each technology allows the high-resolution profiling of sample genomes with distinct advantages and disadvantages for each. For example, BAC arrays require far less sample input than oligonucleotide arrays allowing the analysis of low-yield microdissected specimens while oligonucleotides have the potential to offer greater resolving power *(1)*. Given the existence of the various types of array technology used in CGH studies, the types of situations that arrays may be utilized are numerous. Although the most frequent use of aCGH is in the detection of somatic changes pertaining to cancer, they are also widely used in delineating alterations in developmental disorders and aiding evolutionary comparisons.

BAC aCGH hybridization is performed by first generating labeled DNA samples by a random priming-based reaction where cyanine dyes are incorporated into copies of the original DNA template. Due to the linear nature of random prime labeling, probe is generated proportionately to the original DNA copy number for each region. By co-hybridizing samples representing a normal (diploid) and sample specimen, mixed with Cot-1 DNA (to block repetitive elements) to the array, we can then infer copy number from the ratios with which each probe binds to a particular segment of the genome (**Fig. 2.1**). The following protocol is adapted from Ishkanian et al. *(8)*.

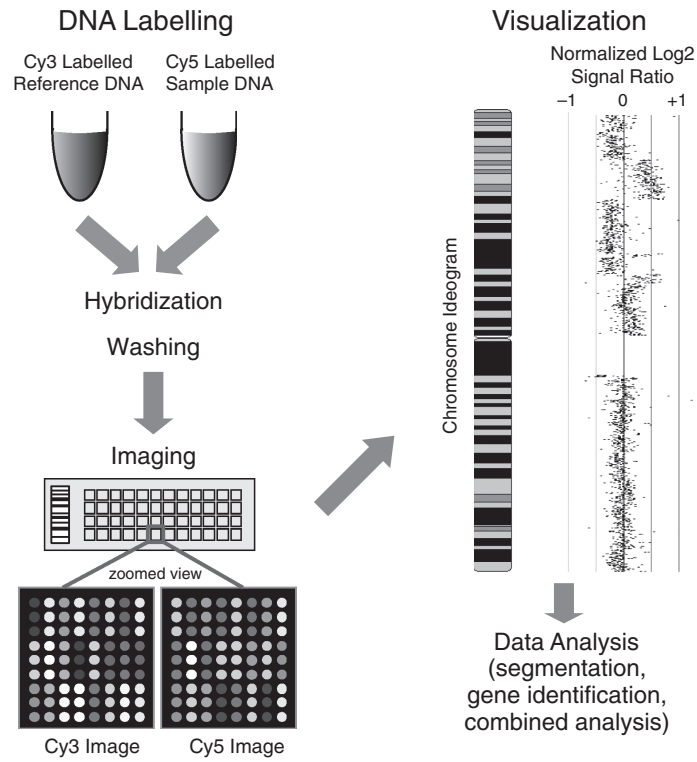

Fig. 2.1. Overview of the array CGH protocol.

2. Materials

2.1. Labeling of Genomic DNA

1. Labeling buffer: Random 8-mer oligonucleotides at 7 μg/μl (Alpha DNA) and Klenow DNA polymerase buffer diluted to 5X concentration (Promega)
2. Klenow DNA polymerase 9 units/μl (Promega), keep on ice at all times
3. Cyanine-3 dCTP 1 nmol/μl (Amersham, GE Healthcare) light-sensitive, pre-aliquot to reduce freeze–thaw cycles
4. Cyanine-5 dCTP 1 nmol/μl (Amersham, GE Healthcare) light-sensitive, pre-aliquot to reduce freeze–thaw cycles
5. RP dNTP mix (2 mM each of dATP, dGTP, dTTP, and 1.2 mM dCTP) (Promega)
6. dH_2O
7. Incubator set at 37°C

2.2. Preparation of Probe

1. Microcon YM-30 filter columns (Millipore)
2. Cot-1 DNA (Invitrogen)

3. DIG Easy hybridization granules, reconstituted at 4.67 g/10 ml (Roche)
4. For option 2: 3 M sodium acetate and 100% ethanol

2.3. Blocking of Repetitive Elements

1. Dry bath incubator set to 85°C
2. Incubator set at 45°C

2.4. Hybridization

1. BAC CGH array (various suppliers)
2. Coverslip 22 × 60 mm (or appropriate size for array used) (Fisher Scientific)
3. Hybridization cassette (Telechem)
4. dH_2O
5. Hybridization incubator set to 45°C
6. Optional pre-hybridization buffer: 4.5 μl 20 mg/ml sheared Herring sperm (SHS) DNA, 4.5 μl 10% BSA, 36 μl DIG Easy
7. For optional pre-hybridization: 100% ethanol, isopropanol, and Coplin jar or slide staining dish

2.5. Washing

1. Wash solution 1: 0.1X SSC 0.1% SDS pH 7.0
2. Wash solution 2: 0.1X SSC pH 7.0
3. Coplin jar or slide staining dish
4. 50-ml conical tubes and Eppendorf 5810R centrifuge (or similar) with swinging bucket rotor, or dedicated microarray centrifuge.
5. Dark slide box (for storing arrays)

2.6. Imaging

1. Microarray scanner. Common systems include the Applied Precision Arrayworx, Axon GenePix 4000B or 4200A, and Perkin Elmer ScanArray.

2.7. Analysis

Most array analysis applications are based on the Microsoft Windows© platform and require a standard modern PC. Due to the significant computational load involved in high-resolution data analysis, at least 2–4 GB of RAM is highly recommended.

3. Methods

3.1. Labeling of Genomic DNA

1. Each CGH array will require 100–400 ng of DNA for both a reference and test sample (*see* **Note 1**). DNA samples should be prepared at a concentration of at least 10 ng/μl or higher.
2. For each array prepare two labeling reactions (one for a reference sample and one for the sample you wish to profile).

Combine 100–400 ng DNA (use an equal amount for both the sample and reference) with 5 µl labeling buffer in a 0.2-ml PCR tube. Bring the total reaction volume up to 16.75 µl and boil for 10 min at 100°C.

3. After boiling place the tubes on ice and add the following: 3.75 µl dNTP mix, 2.5 µl Klenow polymerase, and 2 µl of 1 mM Cy3-dCTP or Cy-5 dCTP (*see* **Note 2**). Mix well by pipetting the solution up and down several times.
4. Incubate the labeling reactions overnight at 37°C.

3.2. Preparation of Probe

3.2.1. Option 1: Rapid Protocol

1. Combine the labeling reaction pairs (one reference and one sample) with 100 µl Cot-1 DNA and add to a Microcon YM-30 column.
2. Spin columns at 13,500*g* for 10 min in the provided tubes.
3. At this point, visual inspection of the DNA pellet in the Microcon can be used to determine if the labeling reaction was successful. A reaction in which both probes have labeled well should appear purple in color, whereas a significant shift to blue or pink represents a failure in one of the labeling reactions and hybridization is likely to be unsuccessful (*see* **Note 3**).
4. Discard the eluate and add 200 µl dH_2O to the column
5. Spin columns at 13,500g for an additional 10 min in the provided tubes.
6. Add 45 µl of DIG Easy hybridization buffer (*see* **Note 4**) to each Microcon and allow the probe to resuspend for 10 min at room temperature (RT). Following incubation at RT flip the Microcon into a new tube (provided with the Microcon) and spin at 3,000*g* for 5 min. (DIG Easy volume is for 22 × 60 mm coverslip, adjust as appropriate for smaller or larger arrays.)

3.2.2. Option 2: Protocol with Labeling Efficiency Analysis

1. Combine the labeling reactions and add to a Microcon YM-30 column.
2. Spin columns at 13,500*g* for 10 min in the provided tubes.
3. Add 50 µl of dH_2O to each column and allow the probe to resuspend for 10 min at RT. Following incubation at RT flip the Microcon into a new tube (provided with the column) and spin at 3,000*g* for 5 min.
4. At this point the probe may be inspected for the efficacy of the labeling reaction. A reaction in which both probes have labeled well should appear purple in color; this is easiest to observe prior to adding water in Step 3. If a NanoDrop

spectrometer is available it can be used to perform a detailed analysis of dye incorporation, with a 1.5-µl aliquot of the labeled material (*see* **Note 5**).

5. Combine the cleaned up probes with 100 µl Cot-1 DNA and precipitate by adding 15 µl of 3 M NaOAc and 375 µl of 100% EtOH.
6. Incubate at –20°C for ~30 min.
7. Centrifuge at maximum speed in a microcentrifuge for 10 min at 4°C.
8. Remove the supernatant and air dry the pellet for a few minutes so that no ethanol remains.
9. Resuspend the precipitated pellet in 45 µl of DIG Easy hybridization buffer (*see* **Note 4**). (Pre-warming the DIG Easy buffer to 45°C speeds up resuspension; DIG Easy volume is for 22 × 60 mm coverslip, adjust as appropriate for smaller or larger arrays.)

3.3. Blocking of Repetitive Elements with CoT-1 DNA

1. Denature the DIG Easy Probe Solution at 85°C for 10 min.
2. Incubate the DIG Easy Probe Solution at 45°C for 1 h to block repetitive elements (Cot-1 DNA was added in **Section 3.2, Step 1**). Avoid incubating for longer than 1 h to reduce probe self-hybridization.

3.4. Hybridization

3.4.1. Optional: Pre-hybridization Only for BAC Arrays Printed on Amine Slides

Depending on the array being used a pre-hybridization step may be required to block the unreacted sites on the slide used to bind the spotted DNA. In the case of aldehyde-coated slides this is not necessary as a chemical inactivation process is performed shortly after array spotting. For amine-coated slides a pre-hybridization step is required (*see* **Note 6**).

1. Place array in boiling water for 15 s to denature spotted DNA. Then dip slide in ice-cold ethanol to prevent renaturing.
2. Allow slide to air dry by placing on an angle leaning against a pipette tip rack sitting on lint-free wipes. Placing the barcode end toward the bottom will prevent the accumulation of any residue on the hybridization area.
3. Pipette 45 µl of pre-hybridization solution onto the surface of a microarray. Gently lower a 22 × 60 mm coverslip over the probe solution avoiding bubbles, some users may find it easier to place the probe solution on the coverslip and lower the slide onto it. (At this point it is important to proceed through Steps 4 and 5 rapidly to avoid evaporation of probe solution.)
4. Place the array in a hybridization cassette (Telechem) and add 15 µl of water to the lower groove (for humidity control).
5. Seal the hybridization cassette and transfer to a 45°C incubator for 1 h.

6. Remove coverslip and dip the slide in isopropanol. Allow the slide to air dry prior to proceeding to hybridization of probe

3.4.2. Hybridization

1. Pre-heat the microarray slide (pre-hybridized according to **Section 3.4.1** if the array is printed on an amine slide) to 45°C on a slide warmer. Although not strictly necessary, this step helps reduce background hybridization which can occur where the probe is deposited if the coverslip is not applied rapidly.
2. Pipette 45 µl of the probe onto the surface of a microarray. Gently lower a 22 × 60 mm coverslip over the probe solution avoiding bubbles; some users may find it easier to place the probe solution on the coverslip and lower the slide onto it. (At this point it is important to proceed through Steps 3 and 4 rapidly to avoid evaporation of probe solution.)
3. Place the array in a hybridization cassette (Telechem) and add 15 µl of water to the lower groove (for humidity control).
4. Seal the hybridization cassette and transfer to a 45°C incubator for 36–40 h.

3.5. Washing

1. Remove slides from cassettes. Place in pre-warmed wash solution for 1 min to loosen coverslip.
2. If coverslip does not fall off in initial wash, remove coverslip by gently sliding partway off of the slide and then gripping the exposed edge to lift off.
3. Wash slides five times for 5 min at 45°C (agitating).
4. Rinse slides three times to remove residual SDS prior to scanning.
5. Dry slides by centrifugation at 800*g* for 5 min in 50 ml conical tubes, or by using an oil-free air stream, or a dedicated microarray centrifuge. It is important that slides are dried immediately after washing due to potential degradation of cyanine dyes by environmental factors (*see* **Note 7**).
6. Store slides and prepare for scanning.

3.6. Imaging

1. After washing, the microarray may be scanned using any commercial microarray scanner. Important factors to consider include scan resolution and scan intensity (*see* **Note 8**).
2. Most scanners offer various output formats; the most universal is to save a single 16-bit tiff image per cyanine dye. Multiple image tiffs are more convenient when using software such as GenePix but may need to be converted for use with third party applications.
3. Softworx and GenePix are the best automated spot-finding applications in our experience. If your software package

allows automated spot-finding after scanning, it is recommended to visually confirm the quality of the spot-finding especially for arrays with printing artifacts as they may often offset grid placement which can result in erroneous data. This is particularly important for spotted arrays which tend to demonstrate more variable spot placement than on slide-synthesized platforms such as most modern oligonucleotide platforms. Following spot-finding, data should be exported. The most common output formats are tab-delimited text files or GPR files (GPR files are GenePix Results format which is a delimited text file with extra information in the header.)

3.7. Analysis

1. With an output file from imaging analysis, the next necessary step prior to delineating gains and losses is normalization. For BAC arrays, any two-channel normalization approach can be applied to remove systematic biases which may be present during the hybridization experiment. Such factors which need to be accounted for include slide gradients and other intensity-related biases. CGH-Norm *(9)* and MANOR *(10)* are two example programs that can be used for normalization.
2. After the data have been normalized, the process of identifying gains and losses, and the genes encompassed, can be performed. There are two necessary components to do this: interactive visualization and statistical analysis. Interactive visualization is important as it provides genomic context to the identified gains and losses. Genomic context includes, but is not limited to, location of transcribed genes and copy number polymorphisms (changes in copy number found in the normal population). Statistical analysis of aCGH data primarily involves segmentation analysis which is a process whereby breakpoints, points at which a change in copy number occurs, are determined for a given sample. There a number of software packages that are freely or commercially available to aid in this process with some software packages providing either interactive visualization or statistical analysis, while others provide both (**Table 2.1**).

4. Notes

1. This protocol has been validated on formalin-fixed, paraffin-embedded samples and high-quality materials. We have observed that results are dependent on DNA quality. There are many factors which affect the usefulness of an archival sample including age of the block and how the tissue was

Table 2.1
Software for the visualization and/or analysis for aCGH

Software	Cost	Interactive visualization	Statistical analysis	Website
aCGH-Smooth	Free		✓	www.few.vu.nl/~vumarray/
BlueFuse for Microarrays	Cost	✓	✓	www.cytochip.com
CGHcall	Free		✓	www.few.vu.nl/~mavdwiel/CGHcall.html
CGHFusion	Cost	✓	✓	www.infoquant.com/index/cghfusion
CGH-Explorer	Free	✓	✓	www.ifi.uio.no/bioinf/Papers/CGH/
CGH Analytics v3.4	Cost	✓	✓	www.chem.agilent.com/Scripts/PDS.asp?lPage=29457
DNACopy	Free		✓	bioconductor.org/packages/2.1/bioc/html/DNAcopy.html
GLAD	Free		✓	bioconductor.org/packages/2.1/bioc/html/GLAD.html
ISACGH	Free	✓	✓	http://isacgh.bioinfo.cipf.es
LSP-HMM	Free		✓	http://www.cs.ubc.ca/~sshah/acgh
M-CGH	Free	✓	✓	folk.uio.no/junbaiw/mcgh/
MD-SeeGH	Free	✓	✓	www.flintbox.com/technology.asp?page=706
Nexus CGH	Cost	✓	✓	http://www.biodiscovery.com/index/nexus
SIGMA^2	Free	✓	✓	sigma.bccrc.ca/sigma2
SpectralWare	Cost	✓	✓	las.perkinelmer.com/Catalog/ProductInfoPage.htm?ProductID=5007-1010
STAC	Free		✓	cbil.upenn.edu/STAC/
VAMP	Free	✓	✓	bioinfo.curie.fr/vamp

stored prior to embedding (i.e., time in formamide) *(11)*. DNA size is a good starting point to determine DNA quality and we recommend DNA with an average size above 300 bp; however, more degraded samples may work and, depending on sample rarity, they may be worth examining. Care must be taken in examining data from archival material as degraded

DNA can generate false copy number alterations *(11)*. The false copy number profile closely follows the GC% of the genome so corrections may also be applied using this information. Additionally, clustering analysis is an excellent way to identify the data demonstrating the false pattern as it is well preserved across different samples.

Reference samples can be a pooled reference from a commercial source (Novagen), a single common reference, or a matched reference sample. We commonly hybridize tumor samples and matched normal samples separately to prevent masking of non-somatic alterations, such as CNVs or cancer-predisposing regions. An example of the value of this can be seen in Coe et al. *(12)* where we identified a copy number alteration in normal cells from a patient with SCLC which contains various apoptosis genes.

2. The choice of which cyanine dye to use for sample and reference material is up to personal choice, so long as a consistent order is used in all experiments to be compared. For cases when the highest quality data is essential, a dye flip reaction can be performed where two aCGH experiments are performed each with the dye used for the sample changed. By combining the two array results, one can generate a result which has reduced noise compared to a single experiment-based result, beneficial in detecting low level or very small alterations with increased confidence *(13, 14)*.
3. In our experience, hybridization quality results can be predicted fairly accurately using simple visual analysis of the probe color. However, we would recommend more stringent quality control provided in probe preparation option 2 (*also see* **Note 5**) in clinical environments or when arrays are more precious than samples.
4. DIG Easy hybridization buffer is a proprietary hybridization solution which demonstrates similar performance to formamide-based solutions. The primary benefit of this solution is the lower toxicity compared to formamide-containing buffers.
5. If dye incorporation is less than 3.0 pmol/μl, poor results are usually observed. Typical random prime labeling yields are approximately 10 μg, which corresponds roughly to 0.5% of bases labeled. In our experience ideal labeling reactions will produce incorporations of 8–25 pmol/μl per dye.
6. The two most common array chemistries are amine and aldehyde. Amine surfaces bind the spotted DNA by charge interactions whereas aldehyde slides bind through a covalent bond formed during slide processing *(15, 16)*. As amine slides retain their reaction chemistry after processing, pre-hybridization is

required to block amine site on the slide from binding the probe resulting in high background signal. The presented pre-hybridization protocol assumes that arrays have been previously processed to crosslink the spotted BACs to the glass slide. If this has not been performed, the DNA should be crosslinked according to the manufacturer's protocol.

7. Cyanine dyes are susceptible to degradation by ozone *(17, 18)*. Cyanine 5 is particularly sensitive and normal atmospheric ozone levels may completely degrade the dye in certain environments (cities, certain times of day, and spring/summer). There are several solutions available to counteract the effects of ozone on cyanine dyes including air filtration systems with carbon filters (various suppliers, www.iqair.us) or ozone catalysts (See The Pat Brown Lab protocols page for details http://cmgm.stanford.edu/pbrown/protocols/index.html) and chemical slide treatments such as Agilent Stabilization and Drying solution, and addition of antioxidants such as cysteamine to wash buffers *(19)*. In addition to environmental controls, we recommend investing in an ozone sensor to monitor atmospheric conditions in your laboratory, and reducing the exposure time of cyanine dyes to air particularly during the slide drying steps where the dye is most susceptible to degradation. This is especially important to consider when using an array scanner with an autoloader which will hold multiple arrays for scanning.
8. There are two main types of microarray scanner: CCD and laser-based systems. Both are appropriate for the analysis of BAC arrays; however, a few differences should be understood. For laser-based scanners such as the Axon GenePix and PerkinElmer ScanArray systems, a scan resolution of 5 μm or 10 μm, whichever is closest to 10 pixel diameters per spot, is optimal (effects both automated spot-finding by improving feature morphology, and data reliability by increasing the number of measurements used to determine a spot's average intensity). Thus, for an array of 120 μm spots, a 10 μm scan resolution is ideal. Scan intensities may be adjusted manually, or often an automatic adjustment option is available. This is a highly recommended setting as the scanner will automatically adjust the images for optimal dynamic range. For particularly dim images, the option of scanning a slide multiple times and averaging the images to remove noise is worth considering.

   For CCD-based systems, resolutions may be offered in increments other than 5 μm, such as the 3.25 μm resolution of the API ArrayWorx. In CCD-based systems, lower resolutions are accomplished by binning pixels together, thus allowing higher intensities to be generated in low-resolution scans (for most purposes a resolution of 9.75 μm is optimal). The

binning effect of low resolutions is important to consider when dealing with low-intensity arrays, where reliable signal may require a lower-resolution scan than with a laser system which runs at lower resolutions by simply skipping alternating points on the slide. Be careful to ensure that the back surface of the slide is clean, as many CCD-based systems scan through the back of the slide and are sensitive to out-of-focus fluorescent artifacts on the back of the slide.

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