Hardware and Software Requirements for Quantitative Analysis of Comparative Genomic Hybridization

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Recommendations are made for hardware and software capabilities that will permit a level of performance of comparative genomic hybridization (CGH) analysis on metaphase chromosomes that is comparable to the best current practice. Guidelines for interpreting the results of CGH analysis in terms of chromosomal gains or losses are also presented.

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Comparative Genomic Hybridization (CGH) analysis (4) has rapidly established itself as a powerful method for analyzing whole genomic DNA (3, 5, 6, 10, 14, 15), and many researchers are interested in acquiring their own CGH analysis capability. Although some chromosomal imbalances, e.g. high copy number amplifications, can be identified by conventional microscopy, comprehensive interpretation of CGH requires digital image analysis. However, at the time of writing there are no commercially available software packages which come close to the capabilities of the in-house systems used by the various research teams which have been at the forefront of CGH developments and application (9, 11, 13).

At a recent workshop on the topic of "CGH Imaging" (organised by the EC Concerted Action on Automation of Molecular Cytogenetic Analyses, and held at the MRC Human Genetics Unit, Edinburgh, 2-3 June 1994), three discussion sessions were devoted to specifying what could be considered to be an "adequate provision" of hardware and analysis software, and what guidelines should be used in the interpretation of CGH data. These are respectively the topics of the three sections of this report, which summarises the consensus that was reached at that workshop.

We hope that these recommendations will prove to be useful for prospective users of CGH. This technical note is not intended to be an introduction to CGH, nor a survey of the literature on the subject, nor is it concerned with preparative techniques (which are discussed in detail in Refs. 7, 8). Readers are referred to original source articles for detailed information on biological preparation protocols, digital microscopy, and analysis software; and also for further discussion of the issues concerning interpretation of the results of CGH analyses, and limitations of the technique.

HARDWARE REQUIREMENTS

Hardware requirements for CGH are generally similar to those for other applications of digital imaging microscopy in the field of molecular cytogenetics. However,

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CGH analysis relies on accurate measurement of intensity ratios between two fluorochromes. Consequently, accurate alignment of the different color images, correction of chromatic aberrations, and specific quantitation of each fluorochrome, are critical features.

Microscope Optics

Recent microscope models from the major manufacturers, equipped for epi-fluorescence, are adequate. The objective should be "plan" and apochromatic with a high numerical aperture. Some commercially available apochromatic objectives are not suitable for CGH analysis since they do not transmit UV excitation. Some recent "fluor" objectives have developed high levels of autofluorescence after short periods of use; users should check for lens autofluorescence regularly and replace the objective when necessary. Mercury arc lamp light sources are adequate if they are stable, and can be aligned to give uniform illumination without chromatic variation. Recent microscope models have improved collector lenses that correct for chromatic aberrations of the illumination, but problems may be experienced with older models.

Filters

Filter sets should be selected to minimize the crosstalk between fluorochromes. Ideally, this would be achieved by using fluorochromes with completely separated excitation and emission spectra, together with narrow excitation and emission filter bandwidths. However, narrow bandwidths result in low signal intensities, and the commonly used FISH labels have overlapping spectra. There will therefore need to be some compromise between crosstalk and brightness. Crosstalk potentially will bias CGH ratio measurements (13); it should therefore be measured for each filter and fluorochrome combination, and if necessary can be compensated by software (1). However, by using appropriate filter sets, separate excitation and imaging of DAPI counterstain, and of FITC and Rhodamine or Texas red genomic hybridization is in practice possible with negligible crosstalk. Texas red is to be preferred to Rhodamine since it has less spectral overlap with FITC. Even smaller spectral overlap of the hybridization labels, as well as better counterstain chromosome banding for chromosome identification, may be obtainable using alternative fluorochromes, and remains a target of current research.

Filters need to be inspected periodically and replaced as necessary, especially UV excitation filters, which in our experience are prone to heat damage after prolonged use.

"Shift" (relative lateral displacement caused by optical or mechanical imperfection of the filter sets) between the different color images should be limited as much as possible. A good solution to this is a fixed triple bandpass beam splitter and emission filter arrangement, with separate excitation filters placed in front of the light source, for example in a manually operated slider or in a computer-controlled filter wheel. Single-bandpass filter sets (each comprising an excitation filter, dichroic mirror and

emission filter) that are moved in their entirety into the optical path to select a particular color often result in large shifts; however, for some modern microscopes well-corrected filter sets are available for which the registration errors are in fact negligible. While the relationship between chromatic shift and measurement error has not yet been determined for CGH, every effort should be made to eliminate shifts altogether, and shifts that are obviously visible (which in practice means shifts relative to the sample of more than about $0.2\mu\text{m}$) are unacceptable. If chromatic shift cannot be avoided by optical means, it should be corrected by software.

CCD Camera

The cameras that have so far been applied for CGH analysis are CCD cameras with digital readout, high spatial resolution, high sensitivity, low noise, high dynamic range—and correspondingly high price. For most CGH purposes, a simpler camera would most probably suffice, although this has yet to be verified. The camera should be capable of integration for 5–10s without generating high levels of dark current. Spatial resolution of $0.1\mu\text{m}/\text{pixel}$ at the sample (resulting in an image size of about 600×600 pixels for an average metaphase spread) is sufficient for CGH analysis; this can typically be achieved with a $\times 63$ or $\times 100$ objective, depending on the camera's pixel size. Photometric resolution of 8 bits is sufficient. The dynamic range requirements are not stringent.

Other Hardware Requirements

We consider that an overall analysis time in the range 10–20 min per metaphase spread is adequate. Given the requirement for significant amounts of user interaction in current systems, achieving such a speed is mostly dependent on the "user's performance", and an exceptionally powerful computer is unnecessary. However, good interaction facilities including a high quality color display screen are essential.

Adequate information storage must be provided for both the raw image data and the analyzed data. CGH analyses may generate image data at the rate of tens or hundreds of Mbyte each day, and so result in substantial demands for on-line and archival storage.

Optional Image Analysis Hardware

The following would prove valuable for CGH analysis, but are not essential and will not be discussed in any detail: 1) Automated metaphase finding (including ranking according to metaphase spread quality and chromosome length); 2) An automated excitation filter wheel (in conjunction with a multi-bandpass beam splitter and emission filter) and autofocussing, both of which would improve the ergonomics of image acquisition considerably; 3) Alternative more stable light source arrangements, such as a mercury/xenon arc lamp, and an optical fiber or other light scrambler, in order to simplify the provision of uniform illumination free of chromatic aberration.

SOFTWARE REQUIREMENTS

Image Acquisition

Image acquisition considerations are similar to those for other quantitative fluorescence imaging applications. Standardization of the image acquisition procedure is essential for CGH analysis, in order to be able to compare images of several metaphases from the same specimen. A method for assessing the "flatness" and any chromatic aberrations of the illumination is essential. Flat field correction is desirable in the case of pronounced chromatic aberrations of the illumination, but is probably unnecessary otherwise (because CGH analysis is concerned only with intensity *ratios*). Pixel shift must be corrected by software if digitized images are not initially correctly registered.

Image Quality Assessment

CGH analysis is critically dependent on the quality of the hybridization and the digital images. Image and hybridization quality can be assessed either visually, or preferably by objective criteria defined by image analysis (e.g., as described in Ref. 11). In the case of visual analysis, this will obviously be done immediately after collecting the images. Some aspects of an automatic assessment depend on features that become available only after part of the CGH analysis has been completed. Features that can be used to assess the quality of the CGH images and build objective exclusion criteria include:

Degree of suppression of repeat sequences in the hybridization. This can be judged by the relative intensity of peri-centromeric repeat sequences.

Intensity of the hybridization signals. With the commonly used high-performance CCD cameras an adequate exposure time (in which some pixels on the chromosomes reach half of the dynamic range of the camera) should be less than 10s for each color.

Signal to background ratio. The ratio of the average signal of the chromosomes to the background intensity outside the chromosomal area should be as high as possible. A quantitative measure of this ratio should be provided to assess the acceptability of the image. A peak signal to mean inter-chromosome background ratio of less than three is clearly indicative of inadequate hybridization.

Homogeneity of the hybridization. This can be assessed by inspection of the reference DNA hybridization intensity, which should be uniform across the metaphase spread.

Homogeneity of the image. This refers to a combination of homogeneity of illumination and absence of hybridization artefacts over the entire metaphase spread, and can be assessed by comparing the centers of mass of the three color versions of the metaphase; these should all lie within a few pixels of each other (11).

Granularity of the hybridization. Good quality hybridizations are uniform rather than granular; this may be assessed visually. Alternatively, the granularity of hybridization images can be quantified by measuring the rela-

tive energy of the higher spatial frequencies, either using a high-pass filter (11) or by analysis of the power spectrum (G. van der Feltz, personal communication).

Other features. Other features that should be assessed include the spreading of chromosomes, proper alignment of the color images, and accurate focussing.

Chromosome Identification

Hybridization results in DAPI banding of very low contrast, and identification of the chromosomes is therefore difficult and time consuming. Semi-automatic karyotyping is highly desirable to allow more rapid CGH analysis (9).

Chromosome Segmentation

Accurate chromosome segmentation is an essential feature of any image analysis of metaphase material. Segmentation using simple gray-level thresholding of the DAPI image will lead to inaccurate definition of chromosome ends. This can be avoided either by pre-filtering the image through a top-hat filter (11), or by basing segmentation on the combined DAPI and reference DNA image (13), or possibly by using a different counterstain with better telomere staining properties.

Quantitation of Profiles of Test and Reference DNA Hybridization Intensities and the Ratio Profile

CGH results are based on the quantitation of reference and test DNA hybridization fluorescence intensities along each chromosome, obtained as integrated intensity profiles similar to those used in automated karyotyping, for example as described by Piper and Granum (12). Profiles measured along a single line through the chromosome will be noisy and are not adequate. The results of CGH analysis are expressed as "ratio profiles", obtained by dividing test DNA profile values by the corresponding reference DNA profile values.

Determination of Chromosome Axis of Symmetry

The basis of profile extraction is the definition of the chromosome axis of symmetry; again this may be obtained exactly as in automated karyotyping (12). Since the accuracy of CGH ratio profiles critically depends on the correctness of the axis, it is essential to provide an interactive facility either to correct the axis or to reject the chromosome if the axis is computed incorrectly.

Background Correction

Accurate CGH analysis requires the removal of background fluorescence before the fluorescence ratios are calculated. Background can be eliminated by subtracting the interchromosomal fluorescence intensity from the chromosomal fluorescence. If background varies across the metaphase, a local estimate is required for each chromosome.

Intensity Normalization

Normalization of fluorescence intensities is necessary for meaningful comparison of data from different metaphase spreads and different preparations. It is also necessary for defining the ratio corresponding to the modal copy number. Possible approaches, each having its own advantages, are normalization so that the total intensities of the metaphase images resulting from the two hybridizations are equal (9, 13), or normalisation of the computed ratio profiles so that the modal ratio profile value for a metaphase is one (11). We emphasize that it is not possible to equate a particular ratio value (e.g., the mode) in a CGH experiment with a particular DNA copy number (e.g., two) without independent evidence, for example from a parallel analysis of the test DNA source material using FISH probes.

Chromosome Length Standardization

This is done routinely in order to average results from homologous chromosomes that inevitably have different physical lengths in different metaphase spreads. Intensity values of the corresponding stretched profiles are interpolated. Since chromosome condensation is not linear, length standardization should ideally be differential, based on matching counterstain banding patterns. An interim solution is to match the centromere positions as well as the telomeres.

Averaging of Ratio Profiles

After normalization, the ratio profiles of homologous chromosomes should be identical. In practice this is not achieved. Averaging reduces random noise and gives better estimation of copy number differences.

Data Presentation

The ratio profiles are plotted as a "relative copy number karyotype" (Fig. 1; see also Fig. 2 of Ref. 9, Figs. 9, 10 of Ref. 11, Figs. 7–9 of Ref. 13). For each chromosome, this shows the computed mean ratio profile, with its variability at each locus, and interpretation thresholds (see "Interpretation" below). Preferably, an overall summary of the variability of the ratio measurements, which relates directly to the quality of the preparation and of the particular chromosomes selected for analysis, should be provided. Chromosome ideograms are helpful additions to the copy number karyotype (see Figs. 9, 10 in Ref. 11), but the current ISCN ideograms (2) are not sufficiently accurate for fine location of ratio change points.

Automation

Many of the methods listed above have been or can be automated, thereby increasing the speed of the analysis. It would also be helpful (and desirable for reason of reproducibility) to have automated data interpretation.

1D vs 2D Analysis

High level amplifications resulting in signals of small extent may be lost in the averaging process inherent in

generating a 1-dimensional ratio profile from the 2-dimensional images. Screening for small high-level amplifications may therefore be improved by additional visual inspection or measurement of the 2-dimensional test hybridization or ratio images.

INTERPRETATION GUIDELINES

The interpretation of CGH results is an area undergoing rapid development. This section provides interim guidelines, which may prove in the future to be conservative, and also indicates the direction of current research.

Data Selection

Hybridizations and digital images which do not fulfill the quality control criteria should not be used for analysis. Only high quality chromosomes should be selected for analysis; chromosomes that are overlapped, or have poor morphology, should not be used.

Relative Copy Number Karyotype

Interpretation should be based on a complete copy number karyotype showing the average ratio profiles derived from several metaphase spreads (Fig. 1, and Fig. 9 of Ref. 11).

It is suggested that a minimum of 5–10 examples of each chromosome (preferably more) should be measured. Clearly, the more variable the data, the more samples are required. It must however be emphasized that analysis of many chromosomes from poor hybridizations is not a substitute for using high-quality hybridizations. Further studies are required to develop procedures for determining if an adequate number of examples of each chromosome has been included in the analysis, based on an objective measure of the data variability.

A measure of the distribution of individual ratio profiles should always be provided on the copy number karyotype (Fig. 1). Since all CGH ratio profiles for a chromosome ought to be identical or nearly so, obvious outliers (which indicate poor material or faulty digital analysis) should be excluded from the computation of the average.

Determination of Non-Modal Regions

Even after appropriate normalization, CGH analysis procedures result in measurements of *relative* copy number only. Our common experience is that in almost all cases there is a clear modal ratio with value close to 1.0, which can be used as the basis for the interpretation of non-modal values. At the present time, most interpretations are based on whether the mean profile at a locus (6, 11), or the mean ratio value for an entire chromosome arm (9), is either higher than an upper threshold, or less than a lower threshold. Upper thresholds of between 1.10 and 1.25, and lower thresholds between 0.75 and 0.90, are currently used (the narrower limits 0.90–1.10 being appropriate for directly conjugated nucleotide labeling of the test and reference DNA). Independent confirmation of chromosomal gains or losses have shown that these

FIG. 1. Relative copy number karyotype from a CGH experiment comparing a lung cancer test DNA with a normal male reference DNA. The average ratio profile and its 95% confidence interval (defined as $\pm$ the standard error of the mean multiplied by 1.97) are shown for each chromosome. The number of samples of each chromosome is shown at the left of each graph. Thin underscoring shows where the entire confidence interval lies below a lower ratio threshold of 0.9, indicating regions with relative losses (proximal 1p, 6q, 9, proximal 14q, distal 19p,

distal 19q, and 22), while bold underscoring indicates regions for which the entire confidence interval lies above an upper threshold of 1.1, indicating relative gains (1q, 6p, 11p, 13q). Interpretation should take account of the fact that CGH analysis is uninformative in regions of the genome with high concentrations of repeat sequences (here, for example, we discount the apparent deletions of 14p, 21p, and Y), and also may be unreliable near the chromosome telomeres (as at 8qtel in this example).

thresholds are robust in the sense that false positives do not arise (11), and normal vs. normal hybridizations show ratios that always lie within the thresholds. Particularly the wider thresholds (0.75–1.25) may however be insufficiently sensitive in cases where a chromosomal imbalance is present in only a proportion of the cells contributing the test DNA (a common occurrence with tumor samples). It is recommended that for a given set of experiments, the chosen thresholds should be validated.

However, this current practice takes no account of the quality of the estimate of the mean ratio, particularly the notion that a larger sample should permit tighter limits to be applied.

Statistically validated interpretation is an active research topic. Possibilities are: 1) Internally controlled interpretation, where only the variability of the current experiment is taken into account. Here there are two possibilities: A) Thresholds are defined by the 95% con-

fidence interval based on that 50% of the genome which has average ratio closest to the modal ratio value (11). In this case, the thresholds vary between experiments according to the distribution of ratio values measured on apparently modal regions of the genome; B) A non-modal or unbalanced region is one where the computed confidence interval surrounding an individual mean ratio point is either entirely above an upper threshold, or entirely below a lower threshold. Thresholds could either be fixed (13), or could be determined for each experiment by a method such as 1A above; the former method is employed in Figure 1; 2) Interpretation by comparison with the variability of a simultaneously hybridized control (normal vs. normal DNA) experiment, in which the variabilities of both test vs. reference and control data are compared.

CONCLUSIONS

These guidelines were discussed and agreed by research groups that have developed and used CGH image analysis during the past 2 years. It is inevitable that rapid development will continue and that additional issues will need to be added to the specification of minimal requirements. However, the underlying concepts of CGH analysis on metaphase targets are unlikely to change radically, and we hope that this note will remain a useful summary of the technical issues for the foreseeable future.

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