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Metaphase and interphase mapping by FISH: improvement of chromosome banding and signal resolution in interphase nuclei by means of iterative deconvolution

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Abstract. FISH images obtained with conventional epifluorescence microscopes are always blurred by glare and out of focus light emissions. In order to restore high contrast images, a procedure based on a modelling of the optical system in the microscope was developed and used for the processing of images acquired with a cooled CCD camera mounted on a fluorescence microscope. This procedure was tested on images of both mouse and human chromosomes stained with DAPI and on images of interphase nuclei hybridized with pairs of cosmid probes. This method improves the definition and the sharpness of the DAPI G-banding and thus facilitates and speeds up the identification of chromosomes. When performed on images of interphase cell nuclei, this procedure allows the discrimination of fluorescent signals which appear partially overlapping on raw images. This significant improvement of spatial resolution is of particular interest for ordering sets of probes on DNA fibers.

Fluorescent In Situ Hybridization (FISH) is commonly used as a rapid procedure for metaphase and interphase gene mapping. In order to assign DNA clones to specific chromosome bands, different protocols have been designed for fluorescence detection and image acquisition, allowing simultaneous chromosome identification and probe detection. Chromosome identification and mapping resolution are strongly dependent upon the quality of the chromosome banding. For precise localization of probes, banding should have good definition, high contrast, and be highly reproducible from one preparation to another for all chromosomal regions.

The possibility of using DAPI for chromosome identification presents several advantages: (i) this fluorochrome is stable under light excitation over a long time; (ii) it does not interfere with hybridization since it is used post hybridization; (iii) its emission spectrum does not overlap with emission spectra of fluorescein, rhodamine and cyanin derivatives; (iv) its affinity for AT-rich DNA sequences confers to this dye the property to produce G bands (Lin et al., 1979). DAPI can thus be used for chromosome mapping providing the DAPI image is properly aligned with collected images of specific probes. However, despite this base pair specific affinity, chromosomes counterstained with DAPI display bands that are difficult to distinguish under an epifluorescence microscope without further image processing. This is in part due to the low axial resolution of conventional microscopes and to out of focus fluorescence emissions. Interphase mapping (Lawrence et al., 1990; Trask et al., 1989) also requires fluorescence images with high resolution and a high signal to noise ratio. Indeed hybridization signals obtained with two probes close to each other on the DNA fiber may often overlap due either to the organization of the chroma-

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tin in the nucleus or to the bias introduced by a 2D analysis of a 3D object. Moreover for 2D analysis the use of conventional microscopes contributes to the observation of overlapped signals and limits the resolution of analysis.

The amount of blur in a fluorescence image is due to imperfections inherent in the optical system of the microscope; thus the latter forms a distorted image of the real object. The relationship between a real object and the image formed in the microscope can be defined by a point spread function (PSF), and degradation of the signal can be represented by a mathematical operation called convolution (Fig. 1). Since the PSF of a given optical system can be determined experimentally, it is possible to design numerical methods to restore a true image of an object. The restoration algorithm consists in inverting the convolution operation and is therefore called a deconvolution (Fig. 1). Thus deconvolution is a mathematical operation which allows the partial restoration of images formed through an optical system. Over the last decades a variety of restoration methods (i.e. iterative deconvolution) have been developed in different domains of applications such as spectral analysis (Jansson et al., 1970), astronomy (Richardson, 1972; Lucy, 1974) and microscopy (Carrington et al., 1990; Shaw et al., 1991; Willis et al., 1993).

For our fluorescence images the PSF of the optical system was determined experimentally in order to apply an optimal correction to the images. The deconvolution procedure can be iterated (iterative deconvolution) until a satisfactory restoration is obtained (balance between resolution enhancement and creation of undesirable artefacts). In addition, a noise reducer was applied on images after each iteration step in order to avoid an amplification of the residual noise during the next iteration step.

In this paper, we show that the use of an image restoration procedure based on a modelling of the optical system in the microscope greatly improves the definition and the sharpness of the DAPI G-banding on human and mouse metaphase chromosomes. We also show that iterative deconvolution, by allowing the discrimination of overlapping fluorescent signals in interphase nuclei, can be used to improve the resolution of interphase mapping. To demonstrate this improvement sets of cosmid probes spanning 250 kb in the human 11q13 region were hybridized to human non-stimulated lymphocyte nuclei and imaged with a cooled CCD camera mounted on an epifluorescence microscope.

Materials and methods

Probes

Plasmids chrom84 and chr85 contain 4.6-kb and 5.3-kb inserts respectively from the long complex repeat unit of the subsatellite region of mouse chromosome 8 (Boyle and Ward, 1992). Cosmids I4, R4B, PHS11, cos9 and cos17 contain a 35-kb insert and are specific for the human 11q13 region. A high resolution physical map is known for the human probes (Brookes et al., 1992; Brookes et al., 1993; Raynaud et al., 1993), thus the physical distances separating the middle of each probe on the DNA fiber are well defined and vary between 50 and 160 kb for the sets of probe pairs used in this study (Table I).

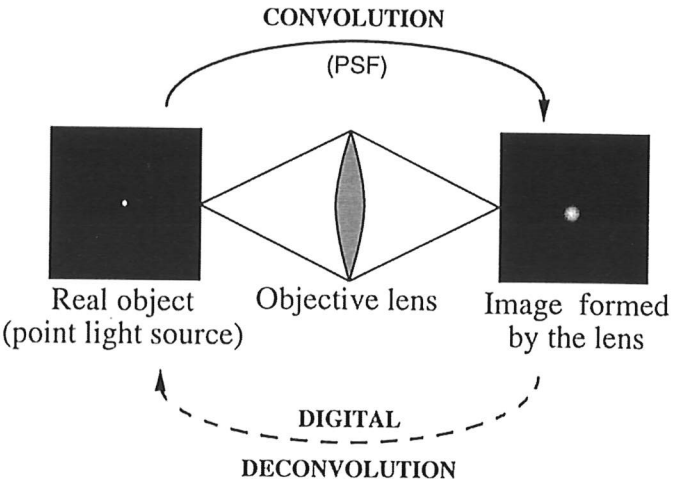

Fig. 1. Image formation by an objective lens. When imaged through a lens an object (here a point light source, left) appears as a smeared spot (right) and is the so called Airy disc. The image formation is described by the point spread function of the lens and can be represented mathematically by a convolution operation. To restore a true image of the object it is necessary to perform the reverse operation, a digital deconvolution.

Metaphase chromosomes preparations

Human chromosomes. Metaphase chromosome spreads from stimulated human female lymphocytes were obtained by standard techniques (i.e.: colcemid treatment, hypotonic shock and methanol-acetic acid fixation).

Mouse chromosomes. Metaphase chromosome spreads from male spleen cells were obtained using a modification of the method described by Sawyer et al. (1987). Spleen cells were cultured in RPMI 1640 medium containing 20% fetal bovine serum, concanavalin A (6 µg/ml) and 2-mercaptoethanol (150 µg/ml) for T-cell stimulation. After 48 h, ethidium bromide (final concentration 25 µg/ml) and colcemid (0.1 µg/ml) were added for 30 min. Standard techniques for hypotonic treatment, methanol-acetic acid fixation, and slide preparation were used (Watt et al., 1986).

Interphase nuclei preparation

Interphase nuclei were prepared from non-stimulated human peripheral blood lymphocytes from one donor. The lymphocytes were isolated via Leukoprep (Beckton-Dickinson) according to the manufacturer's instructions. Hypotonic shock and fixation were performed as for metaphase chromosome preparations.

Hybridization, banding and imaging

Sixty nanograms of mouse repeated sequence or 100 ng of each human cosmid probe were labeled with biotin and hybridized together with 10 µg of sonicated salmon sperm DNA to methanol-acetic acid fixed metaphase spreads or interphase nuclei. Probes were detected using avidin-FITC (Vector-Laboratories) (Lichter et al., 1988) and preparations were counterstained with DAPI (200 ng/ml in a fluorescence-antifading buffer containing 0.23% DABCO). Suppression of hybridization to nonspecific repeated sequences was performed with 3 µg of human Cot-1 DNA (Gibco-BRL).

Preparations of human metaphase chromosomes were stained with DAPI and were subsequently treated and stained for RHG banding. Fixed chromosomes were heated at 87°C for 15 min in physiological saline and then stained with Giemsa (Dutrillaux and Covic, 1974; Dutrillaux, 1977). The G banding obtained with DAPI was then compared to the RHG-banding by imaging the same metaphase spread in the transmission mode at the same magnification.

Preparations were observed under an epifluorescence microscope (Zeiss Axiophot) equipped with a 100 W mercury lamp using the filter set 01 (BP 365, dichroic filter: 395, LP 397) for DAPI fluorescence and the filter set 17 (BP 485, dichroic filter 510, BP 520–560) for FITC fluorescence. Images

Table I. Number of well separated (= resolved) FISH double dots obtained in non-stimulated human lymphocyte nuclei with different pairs of cosmid probes specific for the 11q13 region, before and after deconvolution[a]

Probe pairs	I4 PHS11	PHS11 cos9	R4B cos9	cos9 cos17 A	cos9 cos17 B	I4 cos9	PHS11 cos17
Physical distance (kb)	50	60	85	105	105	110	160
Total number of double dots analyzed	29	40	33	33	33	35	32
Resolved double dots on raw image	1	5	5	13	14	15	22
%	3%	12%	15%	39%	42%	42%	69%
Resolved double dots on deconvolved image	9	15	23	23	22	28	26
%	31%	37%	70%	70%	67%	80%	81%

[a] The total number of double dots analyzed is given for each pair of probes as well as the number of double dots which appear resolved on raw and deconvolved images. These numbers are also expressed as a percentage of the total number of double dots analyzed. Resolved double dots include signals which are completely separated and signals which are juxtaposed. Physical distances are given as distances in kb separating the center of each sequence on the DNA fiber. A control of reproducibility was performed on two independent preparations of lymphocyte nuclei from the same donor with probes cos9 and cos17 (preparation A and B). Hybridization efficiency of each probe when used alone was over 99%. Double signals which appear as singlets on non deconvolved images were discarded from the analysis. Only nuclei with diameter comprised between 8 and 10 µm were considered (>80% of the total nuclei).

were collected with a cooled CCD camera (C4880 Hamamatsu) mounted on the epifluorescence microscope. Metaphase chromosomes were imaged using a 63 ×, 1.25 NA oil immersion objective and an intermediare magnification (optovar) of 1.25 ×. Interphase nuclei were imaged using a 100 ×, 1.3 NA oil immersion objective and an intermediate magnification (optovar) of 1.25 ×. The size of the pixel was 0.095 µm and 0.15 µm, respectively. The exposure time was between 2 and 8 s.

Interphase mapping

Double hybridizations were performed on two different preparations of interphase nuclei with pairs of cosmid probes specific to the human 11q13 region as described above. Hybridization efficiency of each probe when used alone was over 99%. Only nuclei whose diameter was between 8 and 10 µm were considered (> 80% of the total nuclei). Each hybridization was performed with two different probes, each being specific to a unique genomic sequence. In theory, four hybridization signals per interphase nucleus were thus expected. In the case where target sequences are close to each other on the DNA fiber, fluorescent signals corresponding to probes hybridized on the same chromosome will appear as a double dot. However depending on the degree of proximity of the target sequences on the DNA fiber, signals were sometimes observed as partially or fully overlapping. Since only one color was used to detect probes, ambiguous nuclei have not been considered. They include nuclei containing the four signals in the same nuclear territory and nuclei in which only one of the two chromosomes seems to be labeled. The efficiency of the deconvolution procedure was assessed by estimating the percentage of signals that are well resolved before and after the deconvolution procedure (Table I).

Image analysis

In order to improve the quality of images blurred by out of focus haze and glare, deconvolution was performed on gray level 16 bit images with a software developed in our laboratory for the processing of fluorescence images, using an algorithm introduced by Meinel (Eq. 28 in Meinel, 1986). In this program, parameters such as PSF and number of iterations can be chosen; a Gaussian low pass filter (noise reducer) was also introduced in order to obtain the best image quality, time ratio (Usson, 1994). Fluorescein labeled microspheres with a diameter of 0.21 µm were used to determine the PSF of the optical systems. The deconvolution algorithm combined with the noise reducer was iterated until a satisfactory restoration was obtained (balance between resolution enhancement and creation of undesirable artefacts). Images of interphase nuclei were always deconvolved using the same parameters in order to obtain comparable distance measurements for interphase mapping. Image analysis was performed on a Silicon Graphics workstation (Indy R4600). The total time required to acquire and process one image was between 1 and 2 minutes. The source code was compiled using a C compiler for the Silicon Graphics workstation.

Results and discussion

In this paper we show that the quality of the banding pattern obtained with DAPI can be considerably improved and that the resolution of interphase mapping can be increased by suppressing the blur of fluorescence in images obtained with a cooled CCD camera. This was performed by using an iterative deconvolution process coupled with a noise reducer. This type of image restoration offers major advantages over other image processing and filtering procedures: (i) experimental determination of the PSF with fluorescent beads allows one to keep only the relevant information, (ii) noise filtering which can be applied between each iteration prevents noise amplification.

Iterative deconvolution is used on a routine basis in our laboratory for mouse and human cytogenetics. As shown in Fig. 2, this process can be applied with a rather similar efficiency to images of metaphase spreads obtained from these species (Fig. 2a, c). In humans, a comparison between DAPI and RHG bandings has been made in order to assess the quality of the DAPI G-banding (Fig. 2b). The quality of the banding obtained with either method is quite similar. The improvement of the banding quality using the deconvolution procedure is reproducible, however, it greatly depends on the fixation procedure and on the quality and dynamic range of the acquired images.

As already mentioned by Boyle et al. (1992) the quality of the DAPI banding is mainly affected by the denaturation step necessary for the in situ hybridization procedure. It should be noted that the chromosome set shown in Fig. 2d was submitted to denaturation and hybridization steps and that the quality of the banding obtained with our procedure remains very satisfactory even after this denaturation step. In our hands, the identification of chromosomes by DAPI remains possible with even more condensed chromosomes than those shown in Fig. 2. This observation is particularly important since chromosomes of small size are often used for the comparative genomic hybridization (CGH) technique.

In contrast to the vast majority of tools available for image processing, the improvement of images based on the constrained deconvolution procedure relies on a modelling of the

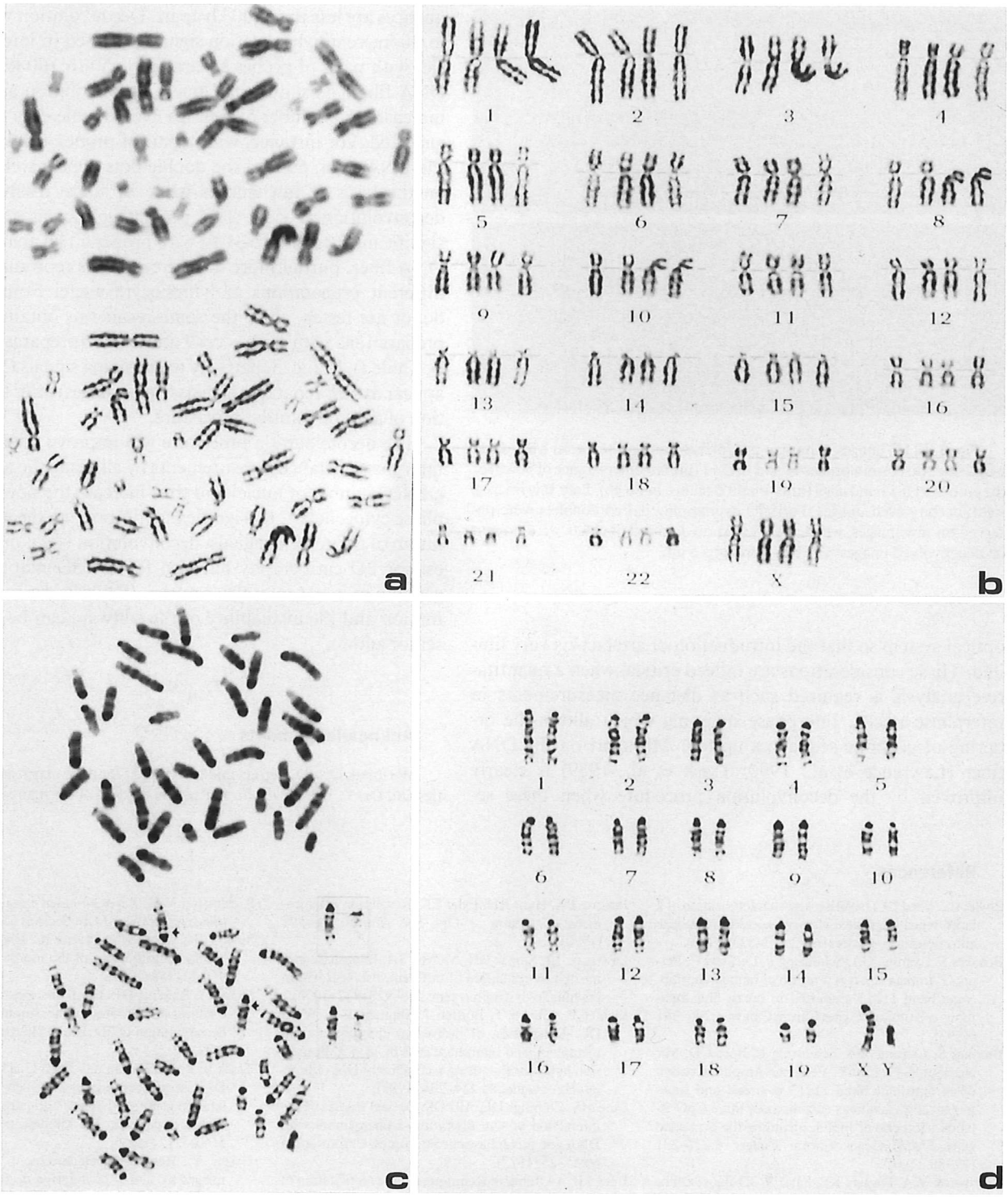

Fig. 2. DAPI banding of human (**a**) and mouse (**c**) chromosomes before and after iterative deconvolution of digital images. Raw (upper halves of (a) and (c)) and deconvolved (lower halves) images are represented. The mouse chromosomes shown in (c) were hybridized with a probe specific for the region A4 of chromosome 8. The inserts on the right-hand side of (c) display the two chromosomes 8 (also indicated with arrows on the full image of the metaphase) with their corresponding FITC images revealing the hybridization signals (far right). The human karyotype shown in (**b**) was established on the basis of human GTG (ISCN 1985), and RHG (Prieur et al., 1971) bandings and the mouse karyotype shown in (**d**) was established on the basis of a mouse G-banding standard ideogram (Evans, 1989). The deconvolved images of (a) and (c) were used to establish the two karyotypes shown in (b: innermost chromosomes) and (d). The G-banding obtained on the human metaphase chromosomes with DAPI was compared to RHG banding on the same metaphase spread (b: outermost chromosomes). The grey scale of DAPI images was inverted to ease the readability.

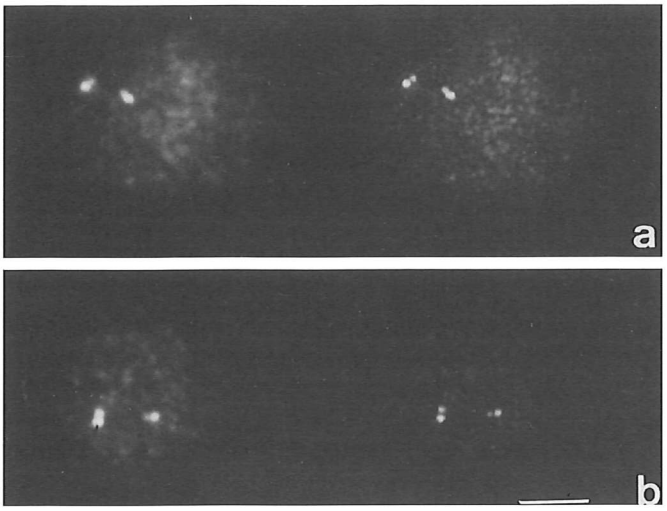

Fig. 3. FITC images of human interphase lymphocyte nuclei hybridized with either (**a**) cosmid probes I4 and PHS11 (mid to mid distance of 50 kb) or (**b**) probes PHS11 and cos9 (mid to mid distance of 60 kb). Left: raw images, right: deconvolved images. Partially overlapping paired doublets were observed on raw images, whereas well separated paired doublets were present on deconvolved images. The bar represents 5 μm.

optical system so that the introduction of artefacts is very limited. These considerations are indeed critical when a quantitative analysis is required such as distance measurements in interphase nuclei. Interphase mapping which allows the ordering of genomic sequences up to 1 Mb apart on the DNA fiber (Lawrence et al., 1990; Trask et al., 1989) is clearly improved by the deconvolution procedure when these sequences are less than 200 kb apart. Deconvolution was applied to fluorescent hybridization signals obtained in interphase nuclei with pairs of probes separated by 50 to 160 kb along the DNA fiber. As shown in Table I, deconvolution significantly increases the number of well resolved double dots that can be analyzed. For instance, with a pair of probes 160 kb apart on the DNA fiber, 69% of the double dots appear well separated on the basis of raw images while 81% are resolved after a deconvolution process. This percentage increases even more significantly (from 3 to 31%) with probes 50 kb apart along the DNA fiber. Furthermore this percentage is reproducible when different preparations of lymphocyte nuclei from the same donor are tested, since the same result was obtained for two preparations with probes cos9 and cos17 (preparations A and B, Table I). In Fig. 3, partially overlapping signals (left images) appear as well separated signals (right images) after the application of a deconvolution procedure.

The deconvolution procedure will improve the accuracy of interphase distance measurements by allowing the analysis of a greater number of nuclei and thus increase the power of interphase cytogenetics. By significantly improving the spatial resolution of fluorescent signals, deconvolution is not only of interest for 2D cartography but also for the 3D mapping of the genome in intact interphase nuclei. Requests for further information and the availability of the software can be sent to the senior author.

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