Original Article

Cytogenet Cell Genet 92:59–62 (2001)

Cytogenetics and Cell Genetics

Improvement of FISH mapping resolution on combed DNA molecules by iterative constrained deconvolution: a quantitative study

K. Monier,[a] L. Heliot,[a] C. Rougeulle,[b] E. Heard,[b] M. Robert-Nicoud,[a] C. Vourc'h,[a] A. Bensimon,[c] and Y. Usson[a]

[a] Laboratoire DyOGen, Unité INSERM U309, Institut Albert Bonniot, La Tronche;
[b] Laboratoire de Génétique Moléculaire Murine, Institut Pasteur, Paris, and
[c] Laboratoire de Biophysique de l'ADN, Institut Pasteur, Paris (France)

Abstract. Image restoration approaches, such as digital deconvolution, are becoming widely used for improving the quality of microscopic images. However, no quantification of the gain in resolution of fluorescence images is available. We show that, after iterative constrained deconvolution, fluorescent cosmid signals appear to be 25% smaller, and 1.2-kb fragment signals on combed molecules faithfully display the expected length.

The possibility of identifying two fluorescence in situ hybridization (FISH) signals along a DNA strand is directly proportional to the degree of DNA compaction and the spatial resolution of the optical system. To improve the resolution of fluorescent images, restoration techniques such as iterative constrained deconvolution (ICD), first developed in the field of astronomy, have been applied to images of fluorescent samples. ICD is now widely used in biology to remove the blur of pictures acquired with conventional epifluorescence microscopes. We showed previously that deconvolved images of DAPI-stained chromosomes lead to well-defined, sharp DAPI-G-banding (Monier et al., 1996). By comparing the number of resolved fluorescent signals resulting from hybridization of pairs of cosmid probes to interphase nuclei, we found that ICD allowed 12% to 55% of the signals that appeared to be partially overlapped on raw images to be discriminated, demonstrating that ICD improves the spatial resolution of fluorescent images (Monier et al., 1996). However, the number of resolved double fluorescent signals varied according to the distance separating the probes, making it difficult to estimate an absolute improvement in spatial resolution. We have therefore extended our initial study by comparing the signal diameter obtained from cosmid probes hybridized to interphase nuclei before and after image deconvolution. Moreover, highly and reproducibly decondensed DNA molecules generated by molecular combing were used as a test model to determine the lengths of 1.2- to 17.2-kb DNA fragments before and after submitting pictures to ICD.

Supported by the Ministère de l'Education Nationale, de l'Enseignement Supérieure et de la Recherche, by the Association Française contre les Myopathies (AFM), by the Association de Recherche contre le Cancer (ARC), by the Groupement de Recherche et d'Etudes sur le Génome (GREG), by the University J. Fourier (Grenoble I), the Commission of the European Community (Human Genome Analysis Contract GENO-CT91-0029 and Human Capital and Mobility CHRX-CT93-0177), and by a fellowship to K.M. from AFM. K.M.'s current address is The Scripps Research Institute, La Jolla, CA (USA).

Received 5 September 2000; revision accepted 24 October 2000.

Request reprints from Dr. Karine Monier, The Scripps Research Institute, MB38, 10550, N. Torrey Pines Road, La Jolla, CA 92037 (USA); telephone: 858-784-2354; fax: 858-784-2345; e-mail: kmonier@scripps.edu.

Materials and methods

Interphase nuclei preparation

Human normal fibroblast cells were grown on glass slides in RPMI medium supplemented with 10% fetal calf serum and placed in serum-free medium for 72 h. Isolated nuclei (Protocol 4, Jolly et al., 1997) were rinsed briefly in PBS and fixed with ice-cold methanol/acetic acid.

KARGER

Fax + 41 61 306 12 34
E-mail karger@karger.ch
www.karger.com

0301–0171/01/0922–0059$17.50/0

Accessible online at:
www.karger.com/journals/ccg

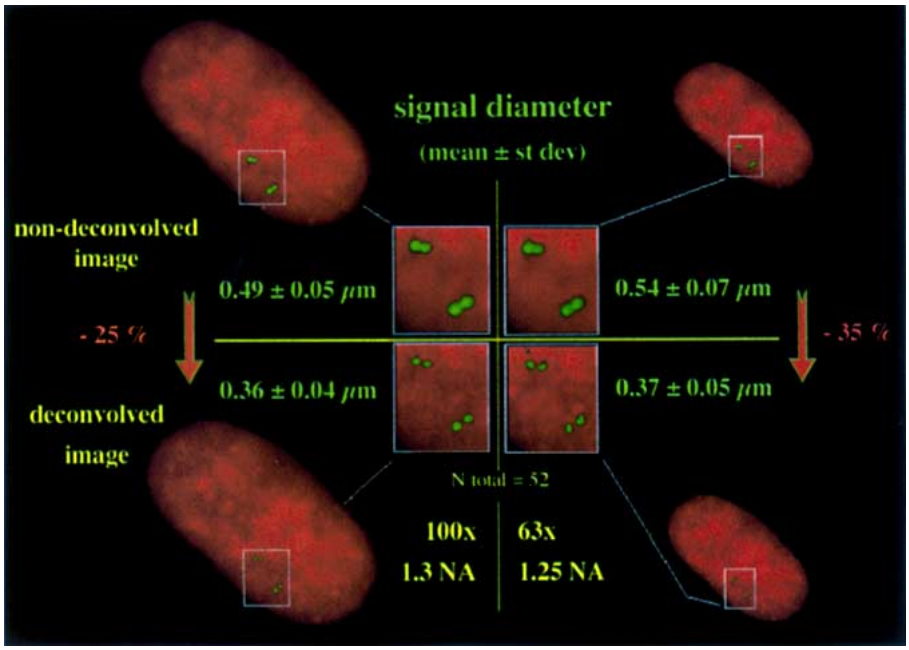

Fig. 1. Quantification of the improvement of spatial resolution by iterative constrained deconvolution. Image of a $G_{0/1}$ fibroblast nucleus (counterstained in red) hybridized with two cosmid probes (green). The physical distance between the center of the two cosmids is 105 kb. In non-deconvolved images (upper part), the signals are barely resolved when imaged with a 100× (left) or a 63× (right) objective lens, whereas after deconvolution the signals appear well resolved (lower part). The mean signal diameters measured on 52 resolved double spots are indicated for both objective lenses before and after deconvolution. A 25% to 35% decrease of the mean signal diameter is observed after deconvolution.

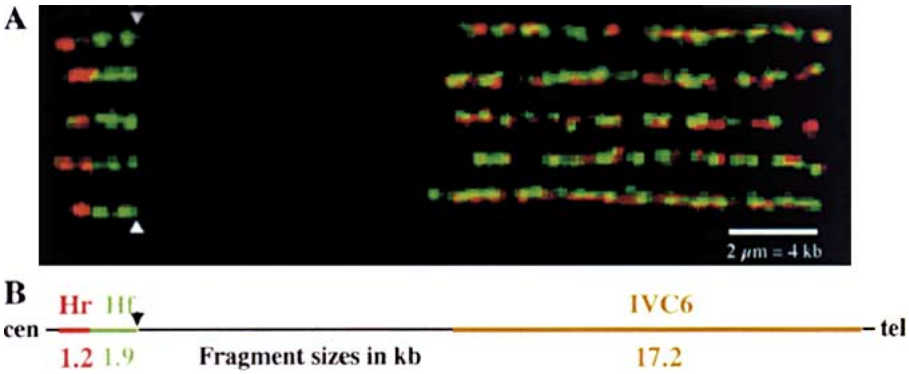

Fig. 2. FISH mapping on combed YAC molecules of two contiguous plasmid probes using a lambda probe. (**A**) CCD images of five representative YAC molecules mapping to the murine X inactivation center (XIC). These YACs are used as a target for the hybridization of probes recognizing genomic targets of, from left to right, 1.2 kb (red signal), 1.9 kb (green signal), and 17.2 kb (doubly labeled signal). The shift between the green and red images was corrected at the pixel size precision (95 nm) using the doubly labeled reference probe. The molecules display reproducible signal positions when aligned according to the right extremity of the small green signals (arrowheads), with no need for a normalization of the length of the signals. (**B**) Oriented physical map of the region determined from sequencing data (drawn to scale).

Preparation of target DNA for molecular combing

Yeast cells were embedded in 1% low-melting agarose blocks (1 μg DNA/100 μl of block) and YAC PA-1 was purified by pulsed-field gel electrophoresis (PFGE) in a 1% low-melting agarose gel, and the gel fragment was digested with β-agarase I according to the manufacturer's instructions (Biolabs). YAC molecules were combed on glass cover slips coated with vinylsilane (Bensimon et al., 1994). Each batch of pretreated cover slips was used to comb control DNA molecules of known size (lambda phage) to ensure that the DNA was stretched to 2 kb/μm prior to FISH.

Probes

Cosmid probes cos9 and cos17 each contain 35 kb of the human chromosome region 11q13 and are separated by 105 kb (the genomic distance between the centers of the probes) (Raynaud et al., 1993). YAC PA-1 and λ IVC6 contain genomic fragments of 500 kb and 17.2 kb, respectively. Hf and Hr are cDNA fragments of 1.9 and 1.2 kb, respectively, both of which are specific to exon 6 of the murine *Xist* gene (Borsani et al., 1991). The Hr cDNA is contiguous to the Hf cDNA and is located 5′ to it. The sizes of Hf, Hr, the λ IVC6 insert, and the region separating these probes are known from sequence analysis (Simmler et al., 1996).

Probe labeling, hybridization, and microscopy

The Hf and Hr inserts were isolated prior to the labeling step. One hundred nanograms of each probe, labeled by nick-translation with biotin or digoxigenin or with a 1:2 mixture of biotin and digoxigenin, was hybridized on combed YAC PA-1, and double-signal color detection was performed using FITC, TRITC, and a three-layer amplification system (Protocol 5a in Monier et al., 1998a). Hybridization on interphase nuclei was performed with biotinylated cos9 and cos17 probes (Monier et al., 1996). Preparations were observed under a Zeiss Axiophot epifluorescence microscope equipped with a 100×, 1.3 NA oil-immersion objective lens (pixel size = 95 nm). Since the size of the pixel is shorter than half the theoretical resolution of the microscope (~ 250 nm), the Nyquist's criterion (sampling step ≤ ½ theoretical resolution) is satisfied. A 12-bit image was collected with a cooled charge-coupled device camera (Hamamatsu C4880) for each fluorochrome by switching between the FITC and TRITC filters.

Deconvolution and measurements

An ICD procedure (Monier et al., 1996) based on Meinel's algorithm was used. The empirical point spread function (PSF) was measured by imaging sub-resolution fluorescent microspheres whose size (0.2 µm) was known. The actual deviation generated by the spreading phenomenon was subsequently used to apply the right correction during the reverse operation. Images were deconvolved using 10 iterations, and a low-pass noise reducer was applied after each iteration. Following deconvolution, the dynamic range of the image was reduced to 8 bits and subsequently stretched linearly to fit the full intensity scale using a noncommercial software program dedicated to FISH image analysis (CytoFISH, Y. Usson). The reduction of the signal diameter generated by deconvolution was determined by comparing the images of 52 double dots measured before and after deconvolution (Fig. 1). To minimize errors, the signal diameter of the dots was not directly measured. Instead, the distances between the closest edges of the two signals (d_{min}) and the distance between the remotest edges of the signals (d_{max}) were measured. The signal diameter was then calculated as $\frac{1}{2}(d_{max} - d_{min})$. For measurements on combed DNA, TRITC and FITC signal images were merged following deconvolution. The lengths of the signals were manually measured on enlarged images. Distances in pixels were converted to micrometers (95 nm/pixel) and eventually expressed in kilobases (2 kb/µm).

Stability of fragment length estimated by combing

For each fragment, the mean (M) and standard deviations (SD) were calculated from 30 measurements. From this original data set, smaller subsets (n = 5, 10, 15, 20, and 25) of data were randomly drawn. The hypothesis of the distribution being normal was assessed using the Kolmogorov-Smirnov test. Each subset presents a Gaussian distribution characterized by a mean and a standard deviation. The probability for the estimated mean (M) to fall within a 5% confidence interval around the real size (µ) was subsequently calculated from the formula $|M - \mu| = \varepsilon(SD/\sqrt{n})$, where ε = normal reduced variable and n = number of measurements. The table of the reduced deviation was used to determine the corresponding probability. The stabilization of the probability was used to define the optimal number of measurements required to get a robust estimate.

Results and discussion

Assessment of resolution improvement by ICD

Two cosmid probes were hybridized to interphase fibroblast nuclei at genomic sites whose centers are separated by 105 kb. Owing to the degree of chromatin compaction in the interphase nucleus, the hybridization signals obtained with these two probes often appear as two partially overlapping dots on raw images, thus preventing accurate measurement of the interphase distance between the two. To assess the improvement ICD made on image spatial resolution, diameters of single dots were measured before and after deconvolution (Fig. 1). As shown in Fig. 1, deconvolution decreases the diameter of the fluorescent signals by 25% when viewed through a 100× objective. This decrease in diameter was confirmed with a 63× objective lens (Fig. 1), as well as by using alternative pairs of cosmid probes. This reduction in image spreading is of the same order of magnitude as that obtained when sub-resolution microspheres are imaged, thus bringing down the theoretical resolution of the microscope from 250 nm to 190 nm when using a 530-nm light source and a numerical aperture of 1.3. The important point of this experiment is that application of controlled ICD to microscopic images does not appear to lead to loss of information nor generate aberrations on the deconvolved images.

Table 1. Estimation of the size of DNA fragments hybridized to combed DNA molecules and measured on images that were submitted or not submitted to iterative constrained deconvolution (ICD)[a]

Fragment names	Hr	Hf	IVC6
Estimates from FISH (kb) in the absence of ICD	1.5 ± 0.3	2.1 ± 0.4	17.0 ± 1.5
Difference with sequencing	+25%	+11%	−1%
Estimates from FISH (kb) in the presence of ICD	1.2 ± 0.4	1.9 ± 0.5	17.2 ± 1.2
Difference with sequencing	0%	0%	0%
Optimal number of measurements	25–30	25–30	10–15

[a] Estimated sizes of three fragments (see Fig. 2) are reported with their mean ± standard deviation. Size estimates are obtained by measuring the length of 30 signals (factor of 2 kb/µm). Accuracy of FISH estimates is given by the difference with real sizes determined by sequence analysis. Estimates are performed on images in the absence or presence of ICD. Note the efficiency of ICD when applied to the size estimation of small fragments (Hr and Hf). The optimal number of measurements to get a stable estimate is given in the last row (see Materials and methods).

Comparison of fragment size estimates with and without ICD

To investigate the performances of ICD, single-copy DNA sequences of known sizes (1.2, 1.9, and 17.2 kb) were hybridized on highly decondensed YAC molecules obtained by molecular combing (Fig. 2) (Bensimon et al., 1994). A lower variability in signal length has been reported, leading to a homogeneous DNA condensation, which is particularly valuable since there is no need for any internal control to normalize the size of hybridization signals (Weier et al., 1995; Monier et al., 1998b). As shown in Table 1, deconvolution significantly improves the estimated sizes of small fragments and reduces the variability of the measurements (compare the standard deviations for the 17.2-kb fragment in Table 1). A total of 25–30 measurements are necessary to get a stable estimated size for small fragments (1.2 and 1.9 kb), whereas 10–15 measurements are sufficient for the 17.2-kb fragment.

The high correlation between DNA fragment sizes estimated by FISH mapping on deconvolved images of combed DNA and their real sizes in the range of 1.2–17 kb and more makes this mapping procedure ideally suited for the study of very small alterations of the genome within tissues or organisms of interest. Analysis of small genetic rearrangements in the

range of 1–2 kb can be achieved with high confidence by combining the use of molecular combing and ICD. Given the extent of DNA decondensation of combed DNA molecules (0.5 bp/nm) and the spatial resolution on images after ICD (190 nm at 530 nm with a 1.3 NA objective lens), the resolution of FISH mapping using combed DNA is expected to be approximately 375 bp.

Acknowledgements

The authors thank Dr. D. Schultz for critically reading the manuscript.

References

Bensimon A, Simon A, Chiffaudel A, Croquette V, Heslot F, Bensimon D: Alignment and sensitive detection of DNA by a moving interface. Science 265:2096–2098 (1994).

Borsani G, Tonlorenzi R, Simmler M, Dandolo L, Arnaud D, Capra V, Grompe M, Pizzuti A, Muzny D, Lawrence C, et al: Characterization of a murine gene expressed from the inactive X chromosome. Nature 351:325–329 (1991).

Jolly C, Mongelard F, Robert-Nicoud M, Vourc'h C: Optimization of nuclear transcripts detection by FISH and combination with fluorescence immunocytochemical detection of transcription factors. Histochem Cytochem 45:1585–1592 (1997).

Monier K, Hazzouri M, Ekong R, Rousseaux S: Optimize FISH on combed DNA molecules. Technical Tips Online (http://www.elsevier.com/locate/tto) T01417 (1998a).

Monier K, Michalet X, Lamartine J, Schurra C, Heitzmann F, Yin L, Cinti R, Sylla BS, Creaven M, Porta G, Vourc'h C, Robert-Nicoud M, Bensimon A, Romeo G: High-resolution mapping of the X-linked lymphoproliferative syndrome region by FISH on combed DNA. Cytogenet Cell Genet 81:259–264 (1998b).

Monier K, Usson Y, Mongelard F, Szepetowsky P, Robert-Nicoud M, Vourc'h C: Metaphase and interphase mapping by FISH: improvement of chromosome banding and signals resolution by means of iterative deconvolution. Cytogenet Cell Genet 72:200–204 (1996).

Raynaud SD, Bekri S, Leroux D, Grosgeorge J, Klein B, Bastard C, Gaudray P, Simon MP: Expanded range of 11q13 breakpoints with differing patterns of cyclin-d1 expression in B-cell malignancies. Genes Chrom Cancer 8:80–87 (1993).

Simmler M, Cunningham D, Clerc P, Vermat T, Caudron B, Cruaud C, Pawlak A, Szpirer C, Weissenbach J, Clavelie J, Avner P: A 94 kb genomic sequence 3′ to the murine *Xist* gene reveals an AT rich region containing a new testis specific gene, *Tsx*. Hum molec Genet 5:1713–1726 (1996).

Weier HUG, Wang M, Mullikin JC, Zhu Y, Cheng JF, Greulich KM, Bensimon A, Gray JW: Quantitative DNA fiber mapping. Hum molec Genet 4:1903–1910 (1995).