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


Laboratoire DyOGen, Unité INSERM U309, Institut Albert Bonniot, La Tronche; Laboratoire de Génétique Moléculaire Murine, Institut Pasteur, Paris, and 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.

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.
Fig. 1. Quantification of the improvement of spatial resolution by iterative constrained deconvolution. Image of a G0/1 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 (Biols). 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 λIVC6 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 λIVC6 insert, and the region separating these probes are known from sequence analysis (Simmler et al., 1996).
**Probe labeling, hybridization, and microcopy**

The HF and Hr inserts were isolated prior to the labeling step. One hundred nanograms of each probe, labeled by nick-transcription with biotin or digoxigenin or with a 1:2 mixture of biotin and digoxigenin, was hybridized on combid 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 Axioshot 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 (dmax) and the distance between the remotest edges of the signals (dmin) were measured. The signal diameter was then calculated as (dmax + dmin)/2. 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/pixel).

**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 – μ = r(ΣSD/n), where r = 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.

**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

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**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)

<table>
<thead>
<tr>
<th>Fragment names</th>
<th>Hr</th>
<th>Hf</th>
<th>IVC6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimates from FISH (kb) in the absence of ICD</td>
<td>1.5 ± 0.3</td>
<td>2.1 ± 0.4</td>
<td>17.0 ± 1.5</td>
</tr>
<tr>
<td>Difference with sequencing</td>
<td>+25%</td>
<td>+11%</td>
<td>-1%</td>
</tr>
<tr>
<td>Estimates from FISH (kb) in the presence of ICD</td>
<td>1.2 ± 0.4</td>
<td>1.9 ± 0.5</td>
<td>17.2 ± 1.2</td>
</tr>
<tr>
<td>Difference with sequencing</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

| Optimal number of measurements | 25–30 | 25–30 | 10–15 |

*Estimated sizes of three fragments (see Fig. 2) are reported with their mean ± standard deviation. Sizes are obtained by measuring the lengths 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).*
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.

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References


