Non-encymatic, low temperature in situ hybridization of metaphase chromosomes for magnetic labelling and sorting

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Abstract. Sorting of metaphase chromosomes has become an important tool in molecular cytogenetic analyses. To establish DNA libraries, nowadays only a few sorted chromosomes are required due to new PCR techniques. However, to investigate the functional significance of chromosomal non-histone proteins, bulk sorting is still necessary. Here, we describe basic experiments for magnetic sorting of chromosomes by means of CIMS (Continuous Immuno Magnetic Sorting). This device is based on magnetic deviation in a variable inhomogeneous magnetic field that allows well-defined separation forces so that the morphology of the chromosomes is well-preserved as visualised by light microscopy. For magnetic labelling of the chromosomes, antibody activated paramagnetic beads were used and a new non-enzymatic, low temperature in situ hybridization technique ("cold ISH") was developed which allowed labelling and sorting under "physiological" conditions. "Cold ISH" means that only the probe DNA was thermally denatured whereas the chromosomes were subjected to physiological temperature and hybridization buffer conditions. As a model system the Chinese hamster cell line DON was used and hybridized with genomic hamster DNA. The labelling efficiency, i.e. the percentage of chromosomes carrying a magnetic bead was determined microscopically. Further experiments were performed with magnetic beads bound by a secondary antibody directed against DNA to detect the influence of the CIMS itself. Experiments with high temperature in situ hybridization (= thermal denaturation of probe and target sequences) revealed results comparable to that of "cold ISH". In both cases, on average about 20 % of the chromosomes were magnetically labelled using different buffer conditions and concentrations. Control experiments were performed without probe DNA and with "unspecific" DNA probes to verify the influence of occasional attachments of beads and DNA entanglement effects. The results indicate that "cold ISH" is a feasible method to label chromosomes. This was confirmed by experiments with the specific DNA probe pUC l.77 which was used to label the region 1q12 on human lymphocyte metaphase chromosomes specifically. Effects of triple strand binding and the application of "cold ISH" for highly repetitive DNA probes are discussed.

Keywords: Metaphase chromosomes, in situ hybridization, low temperature, physiological conditions, magnetic labelling, continuous immuno magnetic sorting.

1. Introduction

For sorting of metaphase chromosomes, sorting techniques as for instance 1g-sedimentation [1-3], velocity sedimentation [4,5], and flow cytometry [6-9] have found widespread applications. Since Hoe sorting allowed high purity sorting (up to 98% [10, 11]), it became the standard technique to establish chromosome specific DNA libraries [12-14]. In a flow sorter each particle of a given chromosome suspension is analysed individually. Although this can be done in less than 1 msec, it takes hours and days to sort a considerable amount of one chromosome type with acceptable purity [14]. Therefore, much effort was spent to increase the sorting velocity of a flow cytometer [15] or to develop protocols for alternative techniques, e.g. free-flow-electrophoresis [16].
近日，要求按照DNA库的标准进行高纯度染色体排序已通过PCR技术克服[17]。此新称为DOP-PCR[18]允许在使用少量（甚至更少）染色体的基础上放大特定染色体DNA序列。这些染色体可以使用流式细胞仪在几分钟内以高纯度进行排序。

然而，对于需要进行更大染色体群的高纯度排序的其他生物调查，例如非组蛋白与染色体的关联[19,20]。足够的材料和已隔离染色体的特定组成与染色体的非组蛋白和它们的蛋白序列有关。这将允许“大规模”的生产，以期了解其功能。

这可能意味着需要开发“特定染色体蛋白库”。

A preliminary estimate of the amount of chromosomes that may be necessary to be sorted out shows that new methods of bulk sorting may be helpful, with a higher throughput rate than flow cytometers have (optimally in the order of some 103 particles per second). Novel protein sequence analysis techniques need 5-10 pmol of a protein under optimal running conditions and 50-100 pmol typically in routine[21]. This means, that about $10^{12} - 10^{14}$ chromosomes have to be sorted out.

Recently the CIMS technique (free-flow-magnetophoresis)[22] has been introduced for cell sorting with high purity and high throughput rate[23, 24]. It is based on magnetic deviation of particles specifically labelled by magnetic beads via antibody-antigen binding[25]. With a "one lane" CIMS device a throughput rate of some 109 particles per hour can presently be achieved[26]. Using a multiple lane CIMS device, in principle, the sorting of the required number of particles would technically become feasible. For the application to chromosome sorting, however, the magnetic beads used in CIMS have to be specifically bound to the chromosomes, for instance by means of in situ hybridization[27, 28].

The feasibility of in situ hybridization for fluorescence and magnetic labelling of individual chromosomes isolated in suspension has been shown earlier[29, 30]. The protocols were based on established fluorescence in situ hybridization techniques for chromosomes fixed on slides. In these protocols the application of a high percentage of formamide (50% - 70%) or equivalent denaturing chemical agents in the hybridization buffer are used as an essential means to reduce the denaturation temperature and to increase the stringency. The subsequent washing steps (up to 13) are associated with a considerable loss of suspended chromosomes which appears to be unacceptable for bulk sorting. Therefore, new in situ hybridization techniques (Fast-FISH) have been developed that omit formamide or other equivalent denaturing chemical agents in the hybridization buffer. As a consequence, the number of washing steps was reduced to 1-2[31-33]. So far, preliminary results of hybridization of isolated chromosomes in suspension using Fast-FISH were observed[34]. The Fast-FISH technique has the advantage that the hybridization time can be reduced to about 15 - 30 min. The temperatures for denaturation and specific renaturation are considerably different to formamide protocols. Systematic studies of hyperchromicity effects (J. Rauch, D. Wolf, M. Durm, M. Hausmann, C. Cremer, manuscript in preparation) of DNA and chromosomes showed that strand separation effects are detectable in temperature ranges around 80°C. In order to preserve the native structure of the chromosomal proteins for functional tests, the target chromosomes should not be exposed to considerably higher temperature conditions than physiological ones.

The statistical evaluation of several series of hyperchromicity measurements (J. Rauch, D. Wolf, M. Durm, M. Hausmann, C. Cremer, manuscript in preparation) revealed significant changes at temperature ranges around 40°C which can be interpreted to represent conformation changes of the chromosomes. These results suggested new possibilities for in situ hybridization which may be explained e.g. on the basis of DNA triple stranded structures[35 - 37].

In this article we show that a low temperature, non-enzymatic in situ hybridization protocol ("cold ISH") can be used for magnetic labelling of isolated metaphase chromosomes for sorting by CIMS. Being aware that the base sequence of the DNA probe might have a significant influence on the efficiency of "cold ISH", e.g. on the formation of triple structures[38 - 40], a model system was used in which genomic DNA was hybridized to the entire chromosome complement so that appropriate DNA probe sequences - if they
exist - were available.

To test the possibility of specific labelling by means of a „cold ISH” protocol, human metaphase spreads were used since so far DNA probes specific for Chinese hamster chromosomes have not been available. The preliminary results using a highly repetitive DNA probe (pUC 1.77 [41]) for fluorescence labelling of the 1q12 region on human lymphocyte metaphase spreads suggested the feasibility of „cold fluorescence in situ hybridization”.

2. Material and Methods

Preparation of hamster chromosome suspensions

Cells of the Chinese hamster cell line DON [42] were grown in 750 ml Falcon flasks in sterile minimum essential medium (MEM, Flow Laboratories, Scotland), after adding 10% fetal calf serum, 1% non-essential amino acids (Whittaker Bioproducts, USA) and 1% penicillin/streptomycin solution (Whittaker Bioproducts, USA). 24 h after the last medium change, mitotic cells were synchronised by a Colcemid block of 4 h (0.045 µg Colcemid/ml medium) and then harvested by a shake off. The mitotic cells were pelleted by centrifugation (350 g, 20 mm), frozen (-20°C, 5 min) and 2 ml of a hypotonic buffer (10 mM Tris/HCl pH 7.5, 5 mM MgCl₂, 10 mM NaCl) was added to the pellet. The incubation period of the resuspended cells was 17 min. After centrifugation (350 g, 20 mm), to remove the hypotonic solution, 400 µl hexylene buffer (25 mM Tris/HCl pH 7.5, 750 mM 1.6 hexylene, 5 mM MgCl₂, 5 mM CaCl₂, prior to adding to the chromosomes, the buffer was adjusted to pH 3.2) was added [43]. To release the chromosomes, remaining mitotic cells were mechanically disrupted using a 22 G needle on a syringe. The suspension was stored at 4°C. This allowed long term storage of chromosomes [7].

Preparation of human metaphase spreads on slides

Metaphase chromosomes were obtained from human lymphocytes isolated from peripheral blood by standard techniques [44]. The lymphocytes were stimulated by Phytohemagglutinin M (2.5 µg/ml lymphocyte medium) and cultivated for 72 hours followed by a Colcemid block (27 µM) (Boehringer Mannheim) for the last two hours. The cell were treated according to a modified hexandiol method [29] and the metaphase spreads were fixed on slides by means of methanol/acetic acid (3:1, v:v).

Preparation of genomic hamster DNA

Genomic DNA was used to hybridize the chromosomes. The DNA was extracted from cultured cells of the DON cell line by the Quiagen Kit (Diagen), according to product information. Using the cells of one 750 ml Falcon flask (10⁶ - 10⁷ cells) this preparation resulted in 100 µl solution of genomic DNA. The genomic DNA concentration of this solution varied between 20 and 60 µg/ml, as measured by hyperchromicity registration. The genomic DNA was labelled with digoxigenin by nick translation (Nick Translation Kit, Boehringer Mannheim) according to product information. Remaining nucleotides were removed in a sephadex G-50 column (Pharmacia Fine Chemicals) according to product information.

Preparation of the human DNA probe pUC 1.77

The chromosome 1 subcentromeric highly repetitive probe DNA probe pUC1.77 (commercially available from Boehringer Mannheim) was labelled in vitro in the presence of digoxigenin 11-dUTP by nick translation of the entire plasmid pUC 1.77 [41]. The DNA lengths typically varied around 200 - 500 bp.
Labelling techniques

a) Labelling of DON chromosomes by secondary antibody-bridge

Before labelling, the chromosome isolation buffer was carefully replaced by a PBS buffer (0.15 M NaCl in 0.01 M Na-phosphate, pH 7.4): 1 ml PBS buffer was carefully added by dropping and mixing it to the isolation buffer. After centrifugation (350 g, 40 mm) 75% of the supernatant fluid was removed and 1 ml of the PBS buffer was added dropwise to the pelleted chromosomes. The chromosomes were resuspended by pipetting. After centrifugation (350 g, 40 mm) the chromosomes were suspended in 200 µl of the PBS buffer. 20 µl of the mouse antibody AC 30-10 directed against the sugar-phosphate backbone of single and double stranded DNA (concentration 0.1 µg/ml, Pro Gen Heidelberg, Germany) was added. After the incubation period (1 h, 20°C) the chromosomes were washed in a PBS solution to remove remaining antibodies. The pellet was suspended in 300 µl PBS.

Magnetic beads, 4.5 µm in diameter, precoated with goat anti-mouse IgG antibodies (Dynabeads M-450, Dynal, Norway), were washed three times in a PBS/BSA solution [44] and added to the suspended chromosomes. The ratio of magnetic beads to chromosomes was 10:1. In control experiments only the precoated magnetic beads were mixed to chromosomes, not exposed to the antibody AC 30 - 10. During the incubation period (more than 35 min, 4°C) the suspensions were periodically resuspended. The mixtures were exposed to CIMS. Aliquots of each sample before and after separation were analysed by light microscopy after fluorescence staining of DNA with DAPI.

b) Labelling of DON chromosomes by high temperature in situ hybridization

Before hybridization, the chromosome isolation buffer was carefully replaced (see a) by the hybridization buffer. 1*SSC (1* standard saline citrate: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.5) or 0.15 M NaCl (pH 7.5) were used. In two cases 2 µl 1 M MgCl₂ were added for chromosome stabilization. The concentrations of the chromosomes were varied between 3*10⁶ and 2* 10⁷ chromosomes/ml. 200 µl of a chromosome suspension and 100 µl of the genomic DNA solution were mixed. Then simultaneous denaturation of both genomic probe and chromosomal DNA was performed at 84°C for 10 min in the absence of denaturing chemical agents such as formamide [32]. For renaturation, the mixture was incubated at 37°C for 3 - 4 h and incubated at 4°C overnight. Control experiments were performed analogously omitting the DNA-Probe or substituting the DNA-Probe by nucleotides.

c) Labelling of DON chromosomes by "cold in situ hybridization"

Before hybridization, the chromosome isolation buffer was carefully replaced (see a) by the hybridization buffer. 1*SCC or 0.15 M NaCl at pH 7.5 were used. 3*10⁶ up to 1*10⁷ chromosomes were suspended in 1 ml of these buffers. The chromosome suspension was kept at room temperature for 10 min and in some cases at 37°C for the same time. Only the genomic probe DNA was denatured at 84°C for 10 min. After denaturation 200 µl chromosome suspension (room temperature) and 100 µl solution of genomic probe DNA (84°C) were mixed. The temperature of the mixture was estimated to be about 40°C as calculated from heat capacities. For renaturation, the mixture was stored at 37°C for 3 - 4 h. The entire labelling procedure was performed in the absence of denaturing chemical agents such as formamide.

In another experiment the genomic DNA was frozen at -20°C after denaturation. After thawing the genomic DNA, the temperature of the solution was less than 10°C. 100 µl of this genomic DNA solution were added to 200 µl of the chromosome suspension kept at room temperature. For renaturation the mixture was stored at 37°C for 3—4 h.

Control experiments were performed analogously omitting the DNA or substituting the DNA by nucleotides. In addition the same protocol was applied in experiments with commercially available cot1-DNA, CT-DNA (calf thymus), and MB-DNA (hering sperm) instead of genomic Chinese hamster DNA.
d) Labelling of human metaphase spreads by "cold in situ hybridization"

20 ng of the labelled DNA probe were diluted in 10 µl hybridization buffer (1×SSC) in a 0.5 ml Eppendorf tube. Thermal denaturation was performed at 84°C for 10 min in a waterbath. In a second experiment the buffer of the Fast-FISH protocol was used: 20 ng of the labelled DNA probe, 1 µl hybridization buffer (10×: Tris-HCl 100 mmol/l; MgCl₂ 30 mmol/l; KCl 500 mmol/l; gelatine 10 mg/l; pH 8.3 (20°C)), and 1 µl 20× SSC were diluted in deionized H₂O to make up a final volume of 10 µl in a 0.5 ml Eppendorf tube. Thermal denaturation was performed in a waterbath at 95°C for 5 min.

For hybridization, the slides were placed onto a plate of a thermocycler specially designed for in situ hybridization experiments (Cyclogene HL-1, thermo-DUX GmbH). The slides were preheated at 37°C for 5 min. The hybridization mixture was pipetted on the slides which were covered by a cover glass and sealed with rubber cement (Fixogum, Marabu, Tamm). Hybridization took place at 37°C for 3 hours.

Visualization

a) Visualization of the hybridization sites on isolated DON chromosomes

2 µl of mouse anti-digoxigenin antibodies (concentration 0.1 mg/ml, Boehringer Mannheim), 198 µl 1×SSC, and 2 µl 1 M MgCl₂ were mixed. After centrifugation of the chromosome suspension (350 g, 40 min) the pellet was resuspended in the solution of the antibodies. Following an incubation period of 1 h at room temperature, the remaining antibodies were removed by centrifugation (350 g, 40 min). Magnetic beads (1×10⁷) that were precoated with goat-anti mouse IgG antibodies (Dynabeads M-450, Dynal, Norway) were washed according to product information and suspended in 300 µl PBS. The suspension of the beads was added to the pellet of the chromosomes and the mixture was well resuspended. During the incubation period (more than 35 min, 4°C) the suspension was periodically resuspended. This mixture was exposed to CIMS. Before and after magnetic separation aliquots of the samples were analysed by light microscopy after fluorescence staining (DAPI).

b) Visualization of the hybridization sites on human metaphase spreads

The coverslip was carefully removed and the slides were incubated in the washing buffer (1×PBS (pH 7.2); 0.2% Tween 20) for 5 min at room temperature. For fluorescence labelling with antidigoxigenin-fluorescein Fab fragments (Boehringer Mannheim) the stock solution (200 µg/ml) was diluted in 1×PBS (pH 8.4) to a final concentration of 10 µg/ml. Approximately 70 µl of this solution were pipetted on each slide, which was covered with a plastic coverslip and placed into a huminified steel chamber. The closed chamber was incubated in a waterbath at 37°C for 1 hour. Then the slides were washed again in 1×PBS (pH 7.2) for 2 min in the dark. The chromosomes were counterstained with propidium iodide (0.2 µg/ml). After air drying in a prewarmed chamber at 370°C the slides were mounted with Vectashield mounting medium [45].

CIMS instrumentation and separation process

After magnetic labelling, the suspension was injected by a peristaltic pump into the separation chamber of the CIMS apparatus [46] with a separation medium inside. In all experiments, the buffer of the sample mixture (PBS/BSA) was also used in the separation chamber. The medium flowed in a vertical direction upwards as a thin film under the conditions of a strictly laminar flow, which was controlled by a second peristaltic pump. The sample, being transported in a fine stream by the separation medium, passed an inhomogeneous magnetic field, perpendicularly oriented to the direction of the flow. The sample was injected at a rate of 4.5 ml/h and passed the magnetic field with a velocity of a 2.1 mm/sec. For sorting, the current of the electromagnet was adjusted to 5.0 A, inducing a maximum magnetic field of 75 mT. This field strength resulted in deviation forces of about 10⁻¹²N, using Dynabeads M-450 (Dynal, Norway). According to the magnetic moments, induced by the magnetic field, magnetic beads and magnetically labelled chromosomes were deviated into the direction of the magnetic forces while the non-labelled, weakly magnetizable particles, i.e. chromosomes without magnetic beads and cell debris passed the
magnetic field with negligible interaction.

Varying the geometry of the wedge type magnetic poles and the current of the electromagnet, different deviation forces were adjusted to obtain several sorting conditions so that the stabilities of the binding-bridges were studied. Using Dynabeads M-450 (Dynal, Oslo Norway), the deviation forces could be varied from $10^{-17}$ N up to $10^{-11}$ N [26]. After the passage through the area of the magnetic field, the thin film of the separation medium was split into fractions which were collected into separate vials.

**Microscopy and evaluation**

For visualization, a fluorescence light microscope (Leitz Orthoplan) was used equipped with a 50 W mercury arc lamp. The DAPI stained suspended chromosomes were excited via a band pass filter (270 - 380 nm) and detected via a 430 nm long pass filter. The fluorescein labelled and propidium iodide counter-stained metaphase spreads were excited via a band pass filter (450 - 490 nm) and detected via a 515 nm long pass filter. Colour images were recorded by a cooled colour CCD camera (CF 15 MC, Kappa Meßtechnik, Gleichen). For registration and interactive image analysis, the commercially available software package Optimas was used running on a PC 80486 under Windows with the operating system MS-DOS.

To determine the labelling rate for the different experimental conditions, typically one hundred to a few hundred chromosomes (depending on the specimen concentration) were inspected and the number of chromosomes attached to magnetic bead was counted. Chromosome aggregates (Figure 1d) were not taken into consideration. To give an error estimate, the following assumptions were made: If $N$ is the counted number of chromosomes, the statistical error can be estimated by $N^{1/2}$. Thus the error $\Delta$ of a ratio $N_1/N_2$ can be estimated by

$$\Delta = N_1/N_2(1/N_1 + 1/N_2)^{1/2} \cdot 100\%$$

**Results**

**Labelling by secondary antibody-bridge**

To show the principal applicability of CIMS to magnetic separation of metaphase chromosomes, they were labelled with Dynabeads of 4.5 µm diameter by antibody AC 30 - 10 forming a binding-bridge between the chromosomes and the magnetic beads. The control experiments were performed analogously. In Table 1 the experimental results are summarised.

After the incubation period, the suspension of each experiment was analysed by light microscopy. Before CIMS up to 37.2% of the chromosomes were identified to carry a magnetic bead. In case of the controls only to 4.6% of the chromosomes were attached to magnetic beads. After separation, the fractions were also analysed by light microscopy. All chromosomes of the control experiments, unspecifically attached to magnetic beads, were detached during the separation process. Only magnetic beads without any chromosomes were found in the deviated fractions. With the antibody AC 30 - 10, 69% magnetically labelled chromosomes were on average found in the deviated fraction. In the non deviated fraction 1% and less was attached to a magnetic bead. After CIMS and fluorescence staining with DAPI, the chromosomes still showed a well preserved morphology as judged from the light microscopic appearance (Figure 1a).

These results indicated that using CIMS, it was possible to distinguish between specific labelling and unspecific attachment. The separation forces appeared to be strong enough to break single O - H bridges or hydrophobic and electrostatic interactions between the precoated magnetic beads and the unlabelled chromosomes.
Non-encymatic, bw temperature in situ hybridization of metaphase chromosomes...

M. Kraus, M. Hausmann, R. Hartig, M. Durm, F.-M. Haar, C. Cremer

<table>
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Mean (No 1-5) 20.1±1.3
Mean (No 6-8) 2.9±0.5

Tab.1. Magnetic labelling of chromosomes by antibody AC 30-10.

No = experiment number (* = control experiments without antibody AC 30-10); c = chromosome concentration; n = absolute number of counted chromosomes; r = ratio of magnetically labelled to total number of chromosomes in percent ( - = not detected)

Labelling by high temperature in situ hybridization

Isolated chromosomes were hybridized with digoxigenin labelled genomic DNA. In these experiments both, the DNA probe and the chromosomal target, were thermally denatured. The control experiments were performed according to the same protocol but without probe DNA or with nucleotides, respectively. Table 2 summarizes the results.

Between 16% and 26% of the chromosomes were magnetically labelled by in situ hybridization. The labelling rate was decreasing for an increasing number of chromosomes estimated for a constant DNA probe amount. The results were considerably higher than in the control experiments, where 3%—6% of the chromosomes were attached to magnetic beads. After in situ hybridization and CIMS 67%—80% of the chromosomes in the deviated fractions carried a magnetic bead. In the non-deviated fractions only 0.1% - 3.4% appeared to be attached to a bead. In all cases the chromosomes showed a well-preserved morphology as visualised by light microscopy after DAPI staining (Figure 1b).

Labelling by “cold in situ hybridization”

Isolated chromosomes were hybridized at Low temperature with digoxigenin labelled genomic DNA. In these experiments, only the DNA probe was thermally denatured. Again control experiments without probe DNA or only with nucleotides were performed. Furthermore three "control" experiments with different "unspecific" DNA probes were evaluated. In these cases two aliquots of each experiment were counted on two different days. Table 3 summarizes the results.

After "cold ISH" the morphology of the suspended Chinese hamster chromosomes was well preserved as visualized by light microscopy after DAPI staining (Figure 1c). The labelling rate varied between 7%—16% (experiments type A) and 28%—75% (experiments type B). Type A experiments had a low concentration of probe DNA resulting in a lower binding ratio at all. Experiments of type B, again suggested the correlation of increasing DNA probe concentration or decreasing chromosome amount with an increasing labelling ratio. In all cases the binding ratios were higher than in the control experiments without probe DNA where 3%—5% of the chromosomes were attached to magnetic beads. After CIMS the chromosomes of a control experiment were completely detached from the magnetic beads.

The control experiments with "unspecific" DNA probes revealed compatible results with the type A experiments for CT- and MB-DNA. The results obtained by the experiments with cot 1-DNA were compatible to "background" experiments without DNA probe or with nucleotides only. On average, the binding rate
Fig. 1. Analysis of the magnetically labelled and sorted chromosomes by light microscopy after DAPI staining. a) shows the chromosomes labelled by secondary antibody bridges. In b) the chromosomes were labelled by high temperature in situ hybridization. c) shows the magnetically labelled chromosomes after "cold ISH". In d) aggregates of chromosomes are shown. These aggregates limited the separation purity in the separated fractions and were excluded from evaluation. (Arrows indicate magnetic beads associated with chromosomes).

Tab. 2. Magnetic labelling by high temperature in situ hybridization after CIMS.

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Mean (No 1-3) 18.5±1.7

The "cold ISH" protocol with 1*SSC was applied to fluorescence in situ hybridization of human lymphocyte metaphase spreads. Figure 2a showed an example for labelling with the subcentromeric, highly repetitive DNA probe pUC 1.77 coupling to the chromosome region 1q12. Although the method resulted in a visible background on all chromosomes by fluorescein, two intensive fluorescent "spots" were visible on the two largest chromosomes. These "spots" were compatible to the major binding sites of pUC 1.77 [41]. In Figure 2b preliminary results are shown for the same probe but for "cold ISH" with a hybridization buffer used in fast-FISH experiments [32, 33]. Compared to the 1*SSC conditions the background was considerably lower and the binding sites appeared to be brighter. Minor binding sites often labelled in Fast-FISH as a consequence of low stringency conditions were not visible under "cold ISH" conditions. Further results will be published elsewhere (Durm et al., manuscript in preparation).
Tab.3. Magnetic labelling by "cold in situ hybridization".
No = experiment number (\* = control experiments); $c_1$ = concentration of probe DNA (DON = genomic DNA of Chinese hamster cell line DON); $c_2$ = chromosome concentration; n = absolute number of counted chromosomes; r = ratio of magnetically labelled to total number of chromosomes in percent (- = not detected)

Fig.2a. a) Digitized print of a human metaphase spread after "cold ISH" with pUC 1.77 in 1\*SSC and fluorescein staining of the binding sites indicated by circles. The largest chromosomes are carrying clearly visible "spots" in the subcentromeric region. These positions are compatible with the major binding site of pUC1.77 on the region 1q12
Fig. 2b. b) Digitized print of a human metaphase spread after "cold ISH" with pUC 1.77 using the Fast-FISH hybridization buffer [32] and fluorescein staining of the binding sites indicated by circles. The chromosomes were counterstained with propidium iodide. Compared to a) FITC-background on the chromosomes was reduced. The typical subcentromeric binding sites for pUC 1.77 on chromosomes are highlighted.

Discussion

Basic experiments for in situ hybridization of isolated metaphase chromosomes with respect to magnetic separation were described. A new, low temperature, non-enzymatic in situ hybridization technique ("cold ISH") was introduced. Such techniques might have considerable advantages in cases where the native protein structure should be maintained. The binding efficiency of "cold ISH" was compared to in situ hybridization with high temperature denaturation of both target and probe DNA. Magnetic labelling by microbeads was used as the parameter for positive in situ hybridization signals. The application of CIMS offered the possibility of signal (i.e. beads bound by in situ hybridization) to noise (i.e. beads occasionally attached) discrimination by magnetic deviation of the beads. The experiments with the antibody AC 30-10 showed that the deviation forces were not strong enough to destroy the antibody-antigen labelling bridge but they were strong enough to detach chromosomes "unspecifically" attached to magnetic beads.

Compared to fluorescence labelling, the advantage of the relative large magnetic beads was that magnetic labelling is not quantitatively correlated to the amount of probe DNA bound. This means, that if a certain relatively small amount of DNA is hybridized to the target, a magnetic bead can be fixed and thus the labelling site can be visualised. In quantitative fluorescence labelling, however, due to signal noise the effect might not be so significant. According to our experience with fluorescein labelling (B. Hagmann, M. Durm, M. Hausmann, C. Cremer; unpublished results), the chromosomes showed an increased fluorescence after "cold ISH" as compared to control experiments but the signal to noise discrimination was not significant in these experiments. In this case further developments appear to be necessary in combination with improved image analysis techniques such as time gated fluorescence measurements [47]. This concept was supported by the experiments with the pUC 1.77 DNA probe. However, the application of the Fast-FISH buffer [32,33] increased the signal to noise ratio considerably, although the optimal conditions
concerning renaturation time, pH etc. have still to be found. Systematic experiments and quantitative image analysis offer possibilities to further optimize "cold ISH" conditions for specific DNA probes. This will be the aim of future investigations.

Different mechanisms are conceivable which would allow a specific binding of single-stranded probe DNA to chromosomal target DNA under the conditions of "cold ISH" used. The design of the experiments reported here was based on the assumption that the formation of triple stranded DNA (single stranded probe DNA and double stranded native DNA) might occur under "cold ISH" conditions. If so, its mechanism would differ from the mechanism for high temperature ISH (single stranded probe DNA plus single stranded target DNA hybridize to result in double stranded probe-target DNA). Since appropriate sequences for triple strand formation might be relatively rare, genomic Chinese hamster (DON) DNA was used as a probe in the test experiments in order to obtain the appropriate DNA labelling sequences.

Treatment under "cold ISH" conditions of isolated DON-chromosomes with cot1 DNA, with CT-DNA (calf thymus), or with MB-DNA (hering sperm) resulted in percentages $r$ of magnetically labelled chromosomes between about 5% and 11%. The mean $r$ value ($< r >$) was about 8%. This is about four times lower than $< r >$ following "cold ISH" using genomic DON-DNA which was about 35%. The error ranges estimated suggested that this difference was significant. In individual experiments, however, the variability was relatively high. An $r$-percentage up to 11% in the "cold ISH" experiments with CT- and MB-DNA might be explained in different ways: For example, it would mean the consequence of a high degree of "unspecific entanglement" under conditions of ISH in suspension. Alternatively, it may be speculated that the Chinese hamster target DNA and the CT and MB probe DNA share certain sequences prone to form interspecies triple DNA strands.

Experiments of "cold ISH" offer new perspectives in molecular cytogenetics. The approach reported here might open a way towards in situ hybridization under near-physiological conditions. An application to interphase cytogenetics might contribute to the development of techniques to study the in vivo compartmentalization of the cell nucleus [48, 49]. However, the protocol should be optimized to increase the binding efficiency. More experiments have to be evaluated to obtain a better statistical significance. For specific sorting by CIMS, specific DNA probes should be used.

The experiments of "cold ISH" and CIMS can be seen as a basis for future developments in specific bulk sorting of chromosomes to establish chromosome specific protein libraries. It is anticipated that the low pH used in the chromosome preparation buffer might denature DNA or remove some protein structures. Hyperchromicity measurements of pure DNA solutions support this assumption. In contrast to DNA, however, chromosomes (= DNA + proteins) behave completely different. Significant conformation changes are always found for temperatures above 75°C independently from the pH of the buffer (J. Rauch, D. Wolf, M. Hausmann, M. Durm, C. Cremer, manuscript in preparation).

In the experiment described here, the low pH was only used to store the chromosome suspension. In principle chromosome preparation can be performed around neutral pH [50], if the hybridization procedure, magnetic labelling and sorting are performed immediately afterwards.

Because "cold ISH" requires neutral pH conditions, preparation at neutral pH is not an obstacle against this technique. Additionally, the use of pH below 6.0 causes the formation of aggregates of material of high RNA content that are difficult to remove from final preparation [51]. Such aggregates (Figure 1d) limited the purity of the deviated fractions to about 87% in our experiments on the magnetically labelled chromosomes but they may be minimised by improved preparation techniques.

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