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Analysis of chromosomes in molecular tumor and radiation cytogenetics: approaches, applications, perspectives.

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Limitations of conventional cytogenetics

For the study of pathological processes the analysis of chromosomes has become of ever growing importance. Today, entire journals are dedicated to this task; thousands of publications so far have shown a close correlation between chromosome aberrations and malignancy (Dal Cin and Sandberg, 1989). Presently, information is available on more than 14,000 neoplasms with an abnormal karyotype (Mitelman *et al.*, 1990). Accordingly, one should have expected that cytogenetics would play a central role not only in tumor research but also in clinical pathology of tumors, as well as therapy planning and control. This, however, until now has not been the case. A major reason for this may be the practical difficulties of conventional chromosome diagnostics:

1) Using chromosome banding procedures, the human workload is too high for extensive applications in a clinical environment.
2) Due to the limitations of microscopic banding analysis, tumorrelevant changes can be detected only if the alterations exceed approximately 2000 to 4000 kilobasepairs (kb) of DNA. Thus, tumorrelevant small alterations (e.g. small translocations, amplifications below a certain amount; submicroscopic deletions; viral integration sites, Doerfler, 1989) cannot be detected in this way.
3) Tumor cells are often difficult to cultivate; even then, they may contain only a few analysable metaphase spreads; it remains unclear whether these few metaphase spreads are representative for the entire tumor cell population.
4) A detailed diagnosis is possible only with metaphase spreads, NOT with interphase nuclei. In particular, specific chromosome aberrations cannot be simultaneously diagnosed in tissue sections viewed by pathologists.

Similar limitations are encountered in the use of conventional staining procedures for the study of chromosome aberrations induced by ionizing irradiation. Translocations may be particularly useful to monitor the effects of a single exposure even many years after such an event has taken place, as well as cumulative effects of multiple or chronic exposures. Since in a variety of cases, malignant tumors are correlated with specific translocations, the correlation with dose of the amount of such translocations in irradiated cells might contribute to better risk estimates for the induction of malignant tumors by ionizing radiation. However, due to the statistical requirements (numbers of cells to be evaluated), the human workload is too high to routinely allow biological dosimetry, particularly in the most important low dose range. Again, a diagnosis of specific aberrations is possibile only with metaphase spreads (e.g. from cultured peripheral lymphocytes), NOT with interphase nuclei. This makes it difficult to analyse the clastogenic effects of *a local* irrradiation exposure of tissues which cannot easily be cultivated *in vitro*.

Attempts to apply automatic image analysis of banded chromosomes (Piper and Lundsteen, 1987; Piper and Granum, 1989; Lundsteen and Piper, 1989) to translocation monitoring in tumor and radiation cytogenetics, so far have met with considerable problems.

New Approaches: General Strategy

To overcome the problems outlined above, a new strategy has been proposed for a rapid and efficient chromosome analysis: only the chromosomes or chromosome subregions relevant for the solution of a given specific problem are highlighted. This approach has been applied not only to metaphase spreads but also to interphase nuclei. Its realization depended on two important technical developments of the last decade, namely the invention of highly sensitive non-isotopic *in situ* hybridization protocols and the generation of numerous human chromosome specific DNA clones (for review see Lichter *et al.*, 1991). The latter development was largely facilitated by the generation of DNA libraries for individual human chromosome types using fluorescence activated chromosome sorting. Quantitative sorting and cloning was first performed with the human sex chromosomes (Davies *et al.*, 1981; Cremer C., *et al.*, 1982, 1984) and later applied to all human chromosome types (Van Dilla *et al.*, 1990). The concept of interphase cytogenetics (Cremer T. *et al.*, 1986) was based on evidence that chromosomes occupy distinct territories (chromosome domains) in the cell nucleus (Cremer C., *et al.*, 1980; Cremer T., *et al.*, 1982; Schardin *et al.*, 1985; Manuelidis, 1985). If only a few chromosome regions of interest are decorated, automated image analysis may be greatly facilitated. With few exceptions, such a strategy has not been feasible using conventional dyes; recently, however, selective "painting" of whole human chromosomes or of chromosomal subregions has become possible. In this new approach DNA probes with a suitable sequence are chemically labelled, e.g. with biotin, aminoacetylfluorene, mercury or digoxigenin; then they are hybridized *in situ* to the complementary sequences of the chromosomal DNA (*in situ* hybridization, ISH). To visualize the binding sites, absorbing dyes, or fluorescence techniques (fluorescence-*in situ*-hybridization, FISH) may be used.

Using conventional fluorescence microscopy, unique DNA-targets as small as 1kb can be detected (for review see Lichter *et al.*, 1991).

In situ suppression (CISS-) hybridization protocols ("chromosome painting") have been designed to suppress the signals of interspersed repetitive sequences which in addition to site specific sequences are present in many cloned DNA probes (Landegent *et al.*, 1987; Cremer T. *et al.*, 1988a; Lichter *et al.*, 1988a; Lengauer *et al.*, 1991; Pinkel *et al.*, 1988; Ried *et al.*, 1990). This achievement makes it possible to use cloned DNA sequences from the human genome, e.g. plasmid, bacteriophage-, cosmid- or yeast artificial chromosome (YAC-) clones either individually or in appropriate combinations for site specific *in situ* hybridization without the need of elaborate subcloning schemes in order to remove unwanted interspersed repetitive elements from these probes. In this way it has become possible to specifically "paint" entire individual chromosomes or chromosome subsets, individual chromosome arms or chromosome band, down to the level of individual genes and intragenic regions (for review see Lichter *et al.*, 1991).

Applications

In the following, a few examples shall be discussed for the application of this new concept in molecular tumor cytogenetics and radiation cytogenetics.

I. Molecular Tumor Cytogenetics

In recent years chromosome specific tandem repetitive sequences located in the constitutive heterochromatin have been cloned for most human chromosomes (Willard and Waye, 1987). Such clones have provided useful tools in FISH-experiments to define specific numerical chromosome aberrations. For example, specific repetitive DNA sequences for human chromosomes 1, 7 and 18 were used to study numerical aberrations of these chromosomes in human glioma cell lines both in metaphase cells and in interphase nuclei (Cremer T. *et al.*, 1988a, b). The majority of these glioma cell nuclei was observed to have three or more hybridization "spots" for each chromosome type, whereas in normal diploid cells, only two hybridization spots were detected in the majority of cases. Such a rapid assessment of numerical aberrations in interphase nuclei has already been applied to a variety of other malignant tumors (Devilee *et al.*, 1988; Hopman *et al.*, 1988, 1991; Anastasi *et al.*, 1990; Van Dekken *et al.*, 1990; Kolluri *et al.*, 1990; Poddighe *et al.*, 1991) including paraffin embedded tissue sections (Emmerich *et al.*, 1989a). An automated image analysis of numerical aberrations should be straightforward if an appropriate contrast can be achieved between the gray level value of the hybridized spot-regions and the gray level values of the surrounding nuclear areas.

To test the feasibility of a threshold oriented automated classification, images of female human lymphocyte nuclei following FISH with an alphoid DNA probe specific for the pericentromeric region on the X chromosomes were analysed. Our preliminary results (Cremer C. *et al.*, 1992) suggest that a rapid and reliable automated classification may indeed be feasible (one to a few seconds per nucleus using a 80386 microprocessor).

For a more general application of ISH methods in the detection of malignancy, it is important to identify also tumorrelevant translocations at all stages of the cell cycle. For this purpose entire human chromosomes have been decorated by CISS-hybridization and applied for the detection of chromosome aberrations in metaphase

and interphase tumor cells (Cremer T. *et al.,* 1988a, 1990). Interphase nuclei with a reciprocal translocation including a segment of the painted chromosome show three domains, i.e. the domain of the unaffected chromosome and two domains reflecting the painted chromosome material in the resulting two translocation chromosomes. The relative sizes of these domains reflect the relative size of the painted chromosomes and chromosome segments in metaphase spreads. Image analysis provides a means to correctly segment the painted chromosome domains by gray-level thresholding (Cremer T. *et al.,* 1988a and our unpublished data). In cases where the painted interphase chromosome domains appear inhomogeneously stained or less compact or where the domains overlap each other a correct determination of the number and size of the painted domains may not be possible. This problem can be greatly reduced by painting exclusively the specific breakpoint regions of interest. For this purpose probes are useful which either span the breakpoint or flank it at both sites. The specificity of this approach was further enhanced by painting the two sites in different colors (Arnoldus *et al.,* 1990; Trask *et al.,* 1991; Lichter *et al.,* 1991). Recently, Ried *et al.,* (1991) have developed a triple color *in situ* hybridization protocol for the specific metaphase and interphase detection of translocations in Burkitt lymphoma cells with breakpoints in 8q24. In addition to the painting of the entire chromosome 8 in blue color using a chromosome 8 specific library, individual clones flanking the breakpoint were used to paint both sites in green and red, respectively. In normal nuclei the green and red signals are colocalized in the normal two blue chromosome 8 domains. In Burkitt tumor cell nuclei, however, this colocalization is only observed in the remaining normal chromosome 8 domain, while the other green and red signal becomes clearly separated by the translocation event. This example may suffice to illuminate the possibilities of a highly specific breakpoint analysis in interphase nuclei. Criteria such as the number, color, size and relative positioning of painted chromosome regions can be used to distinguish tumor nuclei unequivocally from normal nuclei. In principle, an automated, rapid classification should become feasible also in these cases by digitizing the different color images separately; from this, the number, size and relative location of the differently colored spots may be obtained. It will be an important task of automated molecular tumor cytogenetics to set up reliable and rapid automated classification algorithms of multicolored interphase nuclei. The possibility to combine chromosome painting with immunophenotyping of tumor cells (Tiainen *et al.,* 1991) adds another important aspect which may help to improve classification schemes for various tumors in the future.

II. Molecular Radiation Cytogenetics

The recent developments of fluorescence *in situ* hybridization procedures have also opened new perspectives for a rapid classification of irradiation-induced translocations in human cells (Pinkel *et al.,* 1986; Lucas *et al.,* 1989; Cremer T. *et al.,* 1990; Popp *et al.,* 1990a, 1992). Using probes from chromosome specific DNA libraries established by flow sorting of human chromosomes, selective staining of whole human metaphase chromosomes was performed following ^{60}Co-γ-irradiation of stimulated human lymphocytes (Cremer T. *et al.,* 1990). In this way, quadratic dose-effect curves for translocations were obtained by microscopic observation. By counting the number of hybridized interphase chromosome domains, nuclear dose

response curves were obtained which again suggested a quadratic dependence on dose of aberrations of the specific chromosomes painted. As a first clinically relevant application, chromosome aberrations were detected by "painting" procedures in lymphocyte metaphase spreads of patients which had incorporated the radioactive X-ray contrast medium "Thorotrast" about 40 years ago (Popp *et al.,* 1990a). Thorotrast contains the radioisotope ^{232}Th and is permanently stored within the reticulo endothelial system. It decays mostly with α-emission. In two patients studied so far, about 2% of the painted lymphocyte chromosomes were aberrant (translocations; deletions; insertions). In two age matched controls treated at the same time in the same hospital without receiving a Thorotrast injection, however, only about 0.3% of the painted chromosomes showed alterations. A rapid automated detection of radiation-induced chromosome translocations is feasible in appropriately painted metaphase spreads, using simple thresholding procedures. The evaluation is based on the assumption that (in the case of one chromosome type painted) a normal metaphase spread exhibits two painted areas whereas an aberrant metaphase spread containing translocation(s) involving painted chromosome material shows more than two. From the number of recognized signals and their size, the metaphase spreads are classified as "normal" or "aberrant". This approach has the particular advantage that instead of 46 (or more) objects in a metaphase spread, only a few have to be segmented. The results so far indicate a false classification rate of a few percent obtained in this way (Cremer C. *et al.,* 1992). In the future a system can be envisaged which combines automated digital fluorescence microscopy with an automated metaphase finder to relocate the metaphase classified as "abnormal" for the further visual inspection by an experienced cytogeneticist. The workload of the cytogneticist could thus be greatly reduced in particular in the low dose range, where the frequency of aberrant metaphase spreads is low. The accuracy of such a system will then largely depend on the rate of false negatives, i.e. metaphase spreads which are classified as "normal" by the system and are therefore not reviewed although they contain a translocation detectable by the cytogeneticist. A low rate of false negative could probably be achieved by defining strict criteria for the automated classification of "normal" cells, thus accepting a higher rate of false positives, i.e. cells classified "abnormal" by the system but normal by the reviewing cytogeneticist.

A preliminary analysis of lymphocyte nuclei following irradiation and chromosome painting, suggests that threshold segmentation procedures may be used for a classification of chromosome aberrations also in interphase nuclei, if a sufficient contrast between hybridized and non-hybridized areas can be realized (Cremer C. *et al.,* 1992).

In cases where chromosomes can be isolated in suspension, e.g. from human peripheral lymphocytes, a considerable further speed up of automated chromosome analysis may become possible by a laser-technological approach, called Slit-Scan Flow Cytometry (Lucas *et al.,* 1991). In this technique, oriented chromosomes very rapidly pass one after another a focused laser beam. As a result, one obtains time resolved fluorescence profiles (presently up to about 1,000 per second). These profiles may be different for normal and aberrant chromosomes. In case of homogeneous staining for example, a normal chromosome is represented by a bimodal profile with one significant dip (1 centromere), while a dicentric chromosome is represented by a

profile with two significant dips (Cremer C. *et al.*, 1989). To improve the reliability of dicentric detection, the centromeric regions may be highlighted by FISH in suspension (Celeda *et al.*, manuscript in preparation). Fluorescence hybridization of entire chromosomes in suspension (Dudin *et al.*, 1987) may eventually allow the rapid slit scan monitoring of chromosome translocations (Hausmann *et al.*, 1991).

Perspectives

1. The recent development of molecular cytogenetics has already largely closed the gap which so far existed between the analysis of chromosome changes at the light microscopic level and at the DNA level (Ferguson-Smith, 1991). The cytogenetic "resolution" (in terms of the minimum amount of a specific piece of chromosomal DNA identifiable in an individual cell) has been increased a thousand times as compared with conventional cytogenetics (a few kb as compared to a few Mb of DNA contained within a small chromosome band). With image intensifier systems, it might become possible to detect even unique DNA segments smaller than 1 kb routinely (H. Tanke and D.C. Ward, personal communications). In metaphase spreads, these approaches will facilitate the accurate identification of small translocations and deletions. In interphase nuclei as well as in nuclei of terminally differentiated cells they make possible for the first time a cytogenetic analysis of specific numerical and structural aberrations of any chromosome. Multicolor *in situ* hybridization procedures (Nederlof *et al.*, 1990; Trask, 1991) will help to test individual cells simultaneously for a variety of numerical and structural aberrations. In this way tests can be developed which are optimized for the particular needs of a cytogenetic analysis, for example in prenatal diagnosis or in certain types of tumors. More simple and rapid protocols for multicolor *in situ* hybridization are urgently required to speed up the implementation of the new possibilities in routine clinical and tumor cytogenetic laboratories. Individual bacteriophage and cosmid DNA probes directly labelled with fluoresceine have already successfully been used in FISH-experiments (Wiegant *et al.*, 1991; Ried *et al.*, 1991). The commercial availability of fluorochrome labelled probes at moderate costs for specific diagnostic purposes will be of great importance for cytogenetic laboratories who do not have the equipment and expertise presently required for the successful performance of molecular cytogenetics experiments. Various groups are concerned wiht the use of polymerase chain reaction (PCR) to amplify probes (e.g. Lengauer *et al.*, 1990; Lichter *et al.*, 1990; Weier *et al.*, 1990; Celeda *et al.*, 1992; Dùnham *et al.*, 1992). In this way more elaborate cloning procedures for probe generation can be avoided.

2. The preliminary results obtained so far suggest that rapid, automated image analysis procedures for painted chromosomes may be feasible and may contribute to a considerable reduction of the human workload. Highly sensitive, fast registration devices suitable for multi-color-fluorescence imaging have to be developed together with appropriate software. For some applications digital transmittance light microscopy of permanently colored *in situ* hybridization specimens will be advantageous. It should, however, be taken into consideration that multicolor analyses will only become feasible in the fluorescent mode. For

example, a chromosome painted in one color, may be banded simultaneously in another color, the colocalization of two colors at a given ratio may distinguish a chromosome target from other targets which are only painted in a single color, as well as from another target painted with these two colors at a distinctly different ratio etc. We expect that in the not too distant future, the possibilities to detect specific genes, mRNAs and proteins simultaneously in the same cell using multicolor fluorescence approaches will not only be of great importance in research laboratories but also become more and more important in many diagnostic settings. In the light of these considerations the development of appropriate systems for digital fluorescence microscopy at a reasonable price appears to be a challenge for the optical and computer industry.

3. So far chromosomal *in situ* suppression hybridization and image analysis has been largely applied to human cells. However, this approach opens new avenues also in animal tumor and radiation research as soon as appropriate DNA probes become available. In this respect the establishment of chromosome specific libraries using fluorescence activated flow sorting from mouse and rat appears as an important goal. In addition to the applications discussed in this review, a wide spectrum of other applications has already been started or can be foreseen in human, animal and plant cytogenetics. Some of these applications, such as the physical mapping of genes both on mitotic chromosomes and in interphase nuclei (Landegent *et al.*, 1987; Lawrence *et al.*, 1988; Trask *et al.*, 1989; Lichter *et al.*, 1990; for review see Lichter *et al.*, 1991), studies of chromosome evolution (Wienberg *et al.*, 1990), as well as studies of the threedimensional structure and arrangements of chromosomes in nuclei of various cell types (Borden and Manuelidis, 1988; Manuelidis and Borden, 1988; Van Dekken *et al.*, 1989; Emmerich *et al.*, 1989b; Popp *et al.*, 1990b; Geiger *et al.*, 1991; Cremer T. *et al.*, 1991; Bradl *et al.*, 1992) will likely improve our understanding of fundamental biological processes. Questions to which extent cell type specific threedimensional arrangements of chromatin may exist in the cell nucleus and whether such arrangments may change during malignant transformation provide research topics of particular interest for which appropriate tools have now been developed in our and other laboratories. Other applications concerning improved cytogenetic analyses of domestic animals and economic plants will likely provide great practical benefits.

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