Chromosome Analysis by Image Processing in a Computerized Environment. Clinical Applications

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Dealing with a routine regional cytogenetic activity, we have developed and adapted to clinical work a semi automatic karyotyping machine. Attempts for an accurate automated chromosome classification using a neural network have led to partial results. A specific adaptation to cancer cytogenetics is under development (determination of the modal number, translocations analysis with densitometric curves, automatic identification of markers). A specific program allows quantification of chromosome labelling with radioactive probes. Exchanges of digitized karyotypes are feasible with labs using automated karyotyping machines. A local network connects several karyotyping and metaphase finding stations. Guidelines for an international data bank concerning abnormal chromosome images have been elaborated.

On the other hand the ISH techniques have been applied to the following topics: identification of human chromosome aberrations in amniotic and chorionic cells, chromosome studies of human gametes and embryos (including sex determination), identification of markers in cancer cells.

INTRODUCTION

Chromosome analysis by image processing

The aims of the European concerted action on automated cytogenetics were described in August 1985: collaborate on various issues of automated cytogenetics; optimize the use of national resources; contribute to the standardization of cytogenetic analytical and preparative techniques; promote common European scientific and economic interests in the international competition in the field; initiate further European research activities (e.g. in image processors and image analysis); recommend systems and methods to the European industry as well as for potential users.

Several European groups have been involved in the development of systems for automated cytogenetic analysis. A first collaborative study has been published in September 1989⁽¹⁾. An evaluation comparing some machines is available. One of the main purposes was to develop automated karyotyping machines. Prenatal cytogenetic diagnosis became during the recent years the main routine application of chromosome analysis by image processing. 4500 prenatal tests have been carried out for 5 years with a system which has been elaborated in our lab⁽²⁻⁶⁾.

Molecular cytogenetics

Fluorescent in situ hybridization (F.I.S.H.) after labelling by non radioactive probes has

recently been introduced in clinical cytogenetic⁽⁷⁻²²⁾. Advantages of F.I.S.H. versus radioactive in situ hybridization are short delays and accuracy for chromosomes or part of chromosomes identification. Chromosome studies of human gametes and embryos are important to elucidate the mechanisms of chromosome aberrations and for genetic counseling. Sex determination by gonosome identification is of definite interest in the field of recessive linked diseases. On the other hand molecular cytogenetic prenatal diagnosis is under evaluation. Study of chromosomes abnormalities and markers in cancer using I.S.H. is also available. Automated and molecular cytogenetics are currently applied to a part of our patients (table 1).

Additional techniques are performed on spermatozoa, oocytes and early embryos from I.V.F. protocols.

Table 1. CLERMONT FERRAND CYTOGENETIC CENTER
1990 STATISTICS
(number of cases)

Patients (total)	1869
Cytogenetic prenatal diagnosis	918
Amniotic fluid	798
Chorionic villi	24
Foetal blood	96
Postnatal constitutional diseases	799
Hematological diseases	188
Solid tumors	39

MATERIALS AND METHODS

1/AUTOMATED CLASSICAL CYTOGENETICS

A karyotyping station is made up of the following components (Figs. 1, 2): IBM/PC computer equipped with a 1024×1024 digitizer (256 levels of grey intensity); video camera; high resolution vide screen; high resolution videoprinter; optical disk as a storage device. The karyotyping steps are summarized in Fig. 3. Several karyotyping workstations are connected within a local network (Figs. 4-5).

Automated chromosome classification

Several methods have partially failed in front of the complex structure of chromosomes. Today neuronal networks technology has turned the tide and can solve problems in complex pattern recognition.

Classical methods

The most common are probabilist. They need statistical studies to evaluate chromosomal patterns. The basic criteria usually used to classify chromosomes are the following: length, surface area and position of the centromere. The study of the banding pattern is an essential element. The complexity and the variability of banding make it difficult to develop a fully-

Fig. 1. Basic diagram of a karyotyping system

Fig. 2. Karyotyping stations

KARYOTYPING MENU

Fig. 3. Karyotyping menu

Fig. 4. Cytogenetic network

automated system of identification. We choose first to carry out a statistical study of the R-Banding pattern commonly used in the majority of French laboratories. The statistical study involved two phases: collecting data and processing them. The only initially available elements were the digitized images of classified and non classified mitoses recorded on the storage device. One of the most stable and most characteristic parameters is the position of the extremes of grey

Fig. 5. Chromosome analysis in a computerized environment (present status)

levels. We have therefore limited ourselves to the study of the position of extremes, keeping in mind the possibility of taking other characteristics into account if this initial study does not provide a sufficiently clear criterion of classification. Indeed, a single parameter of identification is not necessarily valid for the totality of chromosome pairs. The numerical data were obtained from densitometric curves of chromosomes. Before the statistical study, the curves were "smoothed" and standardized. Average curves were then calculated. The comparison of a sample of standardized curves with an average curve shows that the extremes allow us to summarize the banding pattern, which is what characterizes a given chromosome. Each curve schematized as a "key" can therefore be tried out in the 24 sets of confidence intervals schematized as 24 "locks" (Fig. 6).

Neuronal networks in chromosome classification

Neuronal networks presently housed in software simulations that are run on ordinary computers can make common sense choices and they can learn from experience⁽²³⁾. A neuronal network is an information processing structure modeled after the function and physiology of cells

Fig. 6. Keylock system for chromosome identification

Fig. 7. Neuronal network

(neurons) in the human brain (Fig. 7).

It emulates the biological processes of the brain to solve pattern recognition problems because biological neural systems have demonstrated the capability to organize pattern classification solutions solely from experience, i.e., internally learned from knowledge from prior data to extend its learned capabilities to deal with noisy pattern inputs and to be incrementally trained. With a neuronal network architecture, the main problem to be solved is to select a set of features which are discriminating. The neuronal network has proved its efficiency for chromosome classification using densitometric curves. The learning accuracy rate reaches 100%. The system can train quickly to learn pattern categories of chromosomes whose complexity makes them impractical to define formal approaches and the applications are also very flexible. The data base was collected using chromosomes from 3 different laboratories. It contains metaphases from blood and amniotic fluid samples. The images are digitized and analyzed. In order to avoid memory overflow, they are compressed using the Huffman statistical method of encoding. The metaphase plates are automatically thresholded. The overlapping or touching chromosomes are interactively separated. The features measured are area, perimeter,

isoperimetric deficit, total and relative density, histogram of grey level distribution, window's size, centrometric index, chromosome sectioning and densitometric profile. For each feature, the patterns are classified into one of the four groups: correctly identified chromosomes, incorrectly identified chromosomes, dubiously identified chromosomes, unidentified chromosomes. The system presents its results by response type, by class or by learning data file. One aim is to form 2 groups of classes for each type of feature: one of correctly identified classes, one of dubiously identified classes. A complete karyotype is finally set up on the video screen.

II/FLUORESCENT IN SITU HYBRIDIZATION

We use DNA libraries from flow sorted human chromosomes. Some of the probes are commercially available. The slides are treated with RNase at 37°C for 45 min, rinsed in 2 SSc, dehydrated in ethanol and air dried. They are denatured in 70% formamide/2SSc at 72°C for 3 min; the probe is denatured for 6 min at 75°C in the hybridization mixture. After hybridization, washing and blocking the detection of the biotinylated probe, linked fluorescence is amplified by successive treatments with biotine labelled goat antiavidin. The cell nuclei and metaphases are counterstained with propidium iodide/DAPI and examined using a Nikon fluorescence microscope. Finally a classical chromosome staining is performed. We use alternatively biotin labelled probes and CISS-hybridization procedures.

III/CHROMOSOME ANALYSIS OF HUMAN GAMETES AND EMBRYOS

1/Ovocytes (24-25)

The samples are obtained from 122 patients (aged from 21 to 42) where IVF has been carried out for sterility of different etiologies having lasted for varying periods. Two stimulation protocols were applied:

a/Human menopausal gonadotropin (HMG) after desensibilisation by a synthetic Gonadotropin Releasing Hormone agonist (busereline): busereline is started on day 2 of the cycle and 0.3 mg s/c are administrated twice a day until human chorionic gonadotropin HCG) injection. HMG administration starts after 16 to 22 days of desensibilisation, 300 IU IM daily during two days, 150 IU IM daily during five days.

b/Clomiphene - citrate and HMG after programming by norethisterone: a 10 mg per day administration of norethisterone is started on days 20 of the menstrual cycle before the scheduled IVF cycle for 10 to 20 days. The day following the last day of norethisterone administration was day 0 of IVF stimulation cycle. Follicular recruitment is achieved by 100 mg per day of clomiphene citrate from day 2 to day 6 and 225 IU of HMG on day 4 and 6. From day 8 of the cycle, the dose of HMG is adapted following monitoring of follicular growth. The response to ovarian stimulation is monitored from day 8 by daily ultrasound and 17 beta Estradiol serum assay. Ovulation is triggered by administration of 5000 IU of HGC. The oocytes are retrieved 35-36 h after HCG injection by coelioscopy or vaginally using ultrasound guidance. The cumulo-oocyte complexes are inseminated with 40-80000 spermatozoa. 465 uncleaved oocytes 48 h after insemination were submitted to a cytogenetic analysis. Chromosome preparation is performed according to a modification of the Tarkowski's air-drying technique⁽²⁶⁾.

2/Spermatozoa (27-28)

Sperm capacitation

The semen sample collected is filtered to remove the gel particles. It is then kept at 16°C in a dark chamber. Spermatozoa count, motility and forward progression are determined. We use 2 protocols for spermatozoa capacitation:

The standard sperm penetration assay (SPA) is performed as follows: 0.1 ml of the semen is washed 3 times with 5 ml of supplemented BWW medium and centrifuged at 600 g for 5 min. The pellet containing the spermatozoa is resuspended in capacitation medium at 3.3% of BSA to a final concentration of 10^7 spermatozoa/ml. The sperm suspension is preincubated at 37°C, 5% CO₂ and 90% humidity, during 3-4 hours for capacitation. The BWW medium is supplemented by human serum albumin (HSA), NaHCO₃ and sodium lactate.

The second protocol is performed as follows: 0.1 ml of sperm suspension is added to 1 ml of TEST-yolk buffer. The semen buffer mixture is preincubated for 24 to 72 hours at 4°C, and then placed at 37°C for 1 hour. The samples are washed 3 times with 4 ml of supplemented BWW and centrifuged at 600 g for 5 minutes. The final pellet is resuspended and adjusted to a concentration of 2×10^5 sperm/ml.

Hamster superovulation and egg preparation

Mature female golden syrian hamsters (*Mesocricetus auratus*) (2n=44 chromosomes) are superovulated by one intraperitoneal injection of Pregnant Mare's Serum Gonadotropin (PMSG, 0.2 UI/g body weight). Fifty seven hours later, an intraperitoneal injection of human chorionic gonadotropin (HCG, 0.2 UI/g body weight) is performed. Seventeen hours later, oocytes are collected and separated from cumulus cells with supplemented BWW medium containing 0.1% hyaluronidase (Boehringer). The free oocytes are washed in BWW solution. The zona-pellucida is then removed using a similar solution containing 0.1% trypsin (Sigma chemical). The oocytes are washed in BWW medium. The eggs are judged mature and suitable for fertilisation when the first polar body is observed.

Insemination and egg culture

Each hamster gives 30 to 50 oocytes, 20 to 25 zona-free eggs are placed in 200 microliters of a suspension containing motile capacitated spermatozoa. Three hours later the eggs are observed. We tested 2 different media: the first one is Ham's F10 medium (Gibco) supplemented with 15% fetal calf serum and the second is Chang medium (Irvine scientific). Both types of cultures are incubated for 13-15 hours under 5% CO₂ in air at 37°C. The next morning, eggs are transferred into culture medium containing 0.4 mg/ml colcemid (Gibco) and incubated for 6 to 8 hours to arrest cells at mitotic stage.

Cytogenetic technique

The eggs are placed in a hypotonic solution (sodium citrate at 0.1%) for 6 to 14 min. It is important to supervise the evolution of the hypotonic shock, since the oocytes are fragilized by the enzymatic treatment. Ten to fifteen eggs are fixed with ethanol and acetic acid (3:1). The chromosome preparations are stained with Giemsa 4% in 96% phosphate buffer pH 6.8 for 10 min. They are examined with a Zeiss microscope.

3/Early human embryos

In our study we undertake to establish the karyotypes of non transferable, non freezable embryos. 47 embryos from 34 patients are analysed. 48 hours after insemination normal embryos contain 4 to 8 similar blastomeres without cytoplasmic inclusions. In our sample, embryos with less than four blastomeres or presenting morphological or cytological abnormalities are selected.

A culture with colcemid is performed during 7 hours; chromosome preparations are obtained using a modification of Tarkowski's technique. The bad spreadings and the overlapping of two blastomeres metaphases are not taken into account. For ISH repetitive satellite DNA chromosome probes commercially available are used (chromosomes X, Y, 13-21, 18).

For *routine cytogenetic prenatal diagnosis*, cell culture and chromosome preparation are performed according standard methods. A R banding technique is finally performed in every case.

IV/STUDY OF BLADDER CANCER CELLS (29-31)

A human malignant urologic cell line has been established in vitro from a moderately differentiated transitional cell carcinoma; the line has been cytogenetically characterized.

Cell culture

The patient was untreated with radiation or intravesical chemotherapy when the cytogenetic study was performed on a first bladder resection. Tumor sample was first disaggregated mechanically and then digested in a 0.8% type II collagenase solution, diluted in Ham F10 medium, for 4 hours at 37°C. After washings in the culture medium, all the material collected by centrifugation was distributed to culture flasks. No attempt is made to separate pieces of tissue from single-cell suspensions, since we observed that most outgrowths started from cell clusters and small pieces of tissue.

After 24 hours of incubation, the non-attached material is gently removed and fresh medium is added. Until growth was established and passaging was started, medium is changed once a week. The culture medium used is Ham F10 medium supplemented with 5% fetal calf serum and serum substitute (IBF), L. glutamine and antibiotics (penicillin 100 IV/ml and streptomycin 100 µl/ml. At each passage, confluent flasks are treated with 0.25% trypsin and detached cells are used simultaneously for chromosome analysis and passaging. The cells propagated in culture show a mixed epithelial-fibroblastic morphology.

Cytogenetic analysis

Cytogenetic examination is carried out on primary cultures, after 2 days of culture and sequentially on subcultures during seventy passages. Mitoses are obtained by 2 hours exposure to colcemid added to a final concentration of 0.1 µg/ml. The cells are harvested by trypsin treatment and resuspended in a hypotonic solution of sterile water, fetal calf serum (5:1). After incubation at 37°C for 30 minutes, metaphase cells are fixed in 3:1 methanol; glacial acetic acid at 4°C.

RESULTS AND DISCUSSION

I/CLASSICAL AUTOMATED CYTOGENETICS

We have used routinely a karyotyping system for 5 years in an hospital laboratory. 4500 prenatal tests were carried out during this period (amniotic fluid, chorionic villi, foetal blood). The chromosomes of 16 mitoses are compared for each patient. In each case, 4 mitoses are visualized in the form of hardcopies. The average time for a prenatal diagnosis is 45 minutes. For all the cases examined taken together, the cost is reduced by a factor of five. The precision is comparable to that of conventional methods. Semi automated karyotyping is also feasible in hematological disorders and solid tumors.

The choice a karyotyping system depend upon several factors, scientific and non-scientific (funding), and is complicated by the many systems now available. The possible user of a karyotyping system should keep in mind the following questions: are performances data based on routine clinical use? does the system match the requirement and needs of the individual laboratory? will the system purchased today soon become out dated? how much space is needed? is a special environment necessary? finally is the system "user friendly"?

The acceptance by the daily users is a problem, not entirely solved; some of the non specialized technicians are reluctant to the new approach. The most frequent complaint is that slides allowing a correct interpretation with the optical microscope are not always suitable for the automated system. In contrast, the manipulation of the system itself raise no problems other than technical incidents. Difficulties are associated with the final quality of the prints: more research on this point has to be performed. Finally completely automated classification has not given until now perfect results 70 to 80 per cent of the chromosomes are correctly classified in constitutional diseases and the karyotype has always to be achieved interactively.

Perspectives of automated cytogenetics in a workstation environment (Fig. 13)

The future evolution of our system includes the following aspects:

General specificities

—Exploitation for the daily use of the system by cytogeneticists, biologists, technicians and other scientists in a medical context

—Development and research approach. Elaboration of a platform concept to allow new fonctions to be added

—Opening to the "external world": exchange of images, functions and messages

—Integration of other programs (metaphase finding, ISH quantification, etc.)

—Portability and durability

Functional specificities

—Hardware and software adaptation at the level of processing, acquisition, storage, etc.

—Processing automation with an interpretable micro-language

—Vectorial approach in processing

—External functions assimilation

—Interface with the administrative side

Fig. 13. Chromosome analysis in a workstation environment

- Management of image databases
- Data import/export according to different existing systems
- Elaboration and updating of the usable documents
- Complete independance towards hardware
- Management of images one by one or by set
- Ergonomic interface for access to different functions

Technical specificities

- Use of a standard software (UNIX, C, X)
- Exhaustive approach of processing and ressources
- Definition of a normalized software between platform and library
- Constitution of a software library
- Redaction of a technical documentation

II/FLUORESCENT IN SITU HYBRIDIZATION AND CYTOGENETIC PRENATAL DIAGNOSIS

Detection of main aneuploidies

Using fluorescent in situ hybridization we are able to identify the main chromosome defects in amniotic fluid, chorionic villi and foetal blood (13, 18 and 21 trisomies, gonosomal aberrations) (Figs. 8, 9).

Sex determination

The biotin labelled DNA probes gives significant hybridization signals on chromosomes and interphase nuclei with all immunological used systems. This has been found with phase contrast and fluorescence microscopy. In all experiments we are able to demonstrate the hybridization signal on the Y chromosome and the results are obtained within two days. With the digoxigenin and biotin system it is possible to visualise the two probes together in different colors.

The non radioactive in situ hybridization technique with chromosome specific DNA probes appear to be a good additional method in classic human cytogenetic to estimate aneuploidy in metaphase spreads or interphase nuclei. Chromosomal in situ suppression (CISS) hybridization of human chromosome libraries provides a new tool for translocation detection and also comparative chromosome mapping with animal species. In situ hybridization of pools of DNA sequences established from specific chromosomal subregions or CISS hybridization of DNA clones established from the chromosome region of interest, such as YAC clones or cosmid clones will allow direct band-to-band comparisons between species at the DNA level. This can also be achieved by using probes from microdissected chromosome bands.

Fig. 8. Normal metaphase of amniotic fluid Chromosome labelling with a 13-21 specific probe

Fig. 9. Metaphase chromosomes of amniotic fluid: trisomy 18

III/GAMETES AND HUMAN EMBRYOS

1/ovocytes

A karyotype is obtained from 341 (73.30%) of 465 oocytes classified according to a cytological criterion of maturity (presence or absence of the first polar body)

a/ The 39 oocytes displaying no polar body are judged immature. Chromosome analysis shows 8 oocytes in diakinesis, 31 metaphase II diploid oocytes, including 10 with prematurely condensed paternal chromosomes in the form of single chromatids (PCC).

b/ Among the 302 oocytes having extruded the first polar body and presumably in metaphase II, 75 (24.8%) are aneuploid. One case of structural anomaly (deletion of a short arm of chromosome 5) is observed. Primarily small chromosomes in single chromatid are present in 5 (28.1%) of the 302 oocytes

2/Spermatozoa (Fig. 10)

120 human sperm metaphases are studied. Sixteen (13.3%) karyotypes show chromosome abnormalities, eight (6.6%) hyperploidy, five (4.1%) hypohaploidy and three (2.6%) structural aberrations. The gonosome ratio among spermatozoa is 51.7% X and 48.3% Y. The rate of abnormalities in sperm cells corresponds to the data currently available. In most cases karyotyping by image processing is feasible in gametes and embryos

3/Embryos

Among 47 embryos from 34 patients, we observe 2 haploid sets (23 chromosomes) with two blastomeres (probably related with a parthenogenesis phenomenon), 29 diploid, 6 hyperdiploid,

Fig. 10. Chromosome labelling in human spermatozoa (centromeric probe)

2 hypodiploid and 5 polyploid embryos.

The chromosome aberration ratio is 1/3. In vitro 33% is the estimated figure. We don't find any correlation between the type of cytological damage and the chromosome aberration ratio. Chromosome study should be of definite interest to detect the aberrations before implantation. However ethical problems still remain regarding the damages due to sampling at an early stage of embryogenesis.

The biotin labelled DNA probes give significant hybridization signals on chromosomes and interphase nuclei. The technique appears to be reproducible; it could be used in the future for preimplantation diagnosis in order to determine embryo's sex and to detect numerical chromosome aberrations.

IV/CANCER CELLS

Sixty metaphases were analysed in primary and subcultures. This tumor cell line was characterized by an hyperdiploid karyotype with a modal number of 48. During the passaging period no obvious alteration in the modal chromosome number was observed. Clonal numerical changes, involving chromosomes 7 (trisomy) 8 (trisomy) 9 (trisomy) and 19 (nullisomy) were observed in all classified metaphases. Clonal structural abnormalities were also always found in all examined cells, including several chromosomes. The long arm of chromosome 1 was involved in a balanced translocation with long arm of chromosome 5, t (1;5) (q12;q12). The karyotype contained also an isochromosome of the long arm of chromosome 3 i (3q). The supernumerary chromosome 8 showed on its short arms a translocated segment whose the banding

pattern may be originated from a part (band q23→qter) of chromosome 2. A second marker was also systematically noted. The banding pattern and absence of the two chromosome 19 suggested that one of these may be implicated in marker formation, with chromosomal segments translocated on the short and long arms. Breakpoints on chromosome 19 were located in telomeric regions at band p13 and q13. Finally a 14p+ was present. All cells presented therefore the karyotype:

48, XX, +7, +9, -19, -19, t(1;5) (q12;q12),
i(3q), +der(8), t(?;8)(?;p11), +der(19)t(?;19;?), 14p+

Repeated cytogenetic examination, at different passages, of the cell line showed the persistence of identical chromosomal aberrations. Fluorescent in situ hybridization has been applied to this case. Our studies have already shown the accuracy of the 8 trisomy and the 1;5 translocation labelling (Fig. 11 et 12).

Due to the frequency of complex karyotypes a specific protocol has to be suggested in cancer cells; it should include the following steps: automated search of metaphases; determination of the modal number; classification of normal chromosomes; identification of markers by the standard techniques; in situ hybridization.

Automatization and quantification of F.I.S.H. will be one of the main tasks of the next years for the cytogeneticist. The results will be a better knowledge of chromosome topography and routine applications in the field of constitutional and cancer cytogenetics.

Fig. 11. Bladder tumor cell line: 1; 5 translocation (painting)

Fig. 12. Bladder tumor cell line: Trisomy 8 (painting)

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