Non-Fluorescent Centromere/Chromosome Painting for Dicentric Analysis: Potential for Automation

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Abstract

Automation of metaphase-dicentric chromosome aberration analysis in peripheral blood lymphocytes has been viewed as a means of overcoming some of the shortcomings associated with this biodosimetry technique. Accurate identification of dicentrics is possible by centromeric painting, which may significantly enhance automated detection by image analysis.

Human lymphocytes are exposed *in vitro* to doses ranging from 0 to 4 Gy of 250-kVp X-rays. Metaphase spreads are prepared 48 h after phytohemagglutinin stimulation by colcemid arrest. Dicentric analysis is done in Giemsa-stained and centromere-painted metaphase-chromosome spreads. Results are compared, and the benefit of centromere painting in chromosome aberration analysis is discussed.

Use of a customized metaphase finding system, including efforts to develop software algorithm for dicentric aberration detection based on the permanent centromere painting, is presented.

Introduction

Exposure estimates in radiation accidents require rapid and reliable measurements of bioindicators. A drawback to the dicentric assay (Bender, 1964; Bender et al., 1988) is that it requires the analysis of a large number of suitably prepared metaphase spreads to address practical questions of partial-body and low-dose exposures.

There have been significant efforts to use automated metaphase finders alone, and coupled with image analysis systems to detect and score dicentrics in metaphases prepared by conventional methodology (Lloyd, 1984). In these instances, image recognition of dicentric chromosome aberration, was based on morphological criteria.

Here, we describe an alternative strategy to recognize dicentric chromosome aberrations. It uses an immunoenzymatic protocol to detect pancentromeric regions in human chromosomes and color-video image analysis.

Goals

Applications of the dicentric assay to support radiation dosimetry requires automation; to provide required throughput and to be capable of rapid and easy operation by non-specialist field personnel. The automation hardware will be developed to provide extension to routine analysis requiring minimum care, maintenance and training and be self-checking. Forward fielding also requires provision of specifically designed and "hardened" hardware.

Initial Task - Performance Requirements

The specific performance problems addressed relate to the development and validation of computer programs to enhance the throughput of and reduce the manpower costs for the dicentric biodosimetry assays.

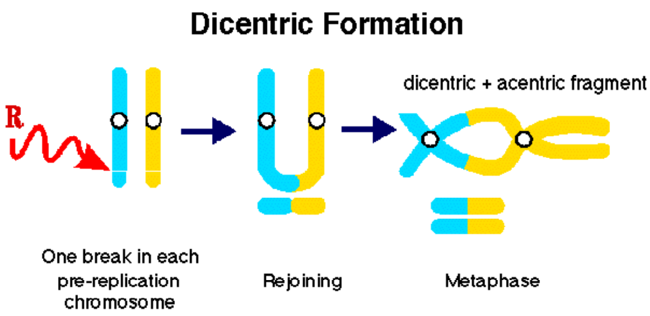
To accomplish the general program requirements, the AFRRI project was first recast into a set of required near-term goals.

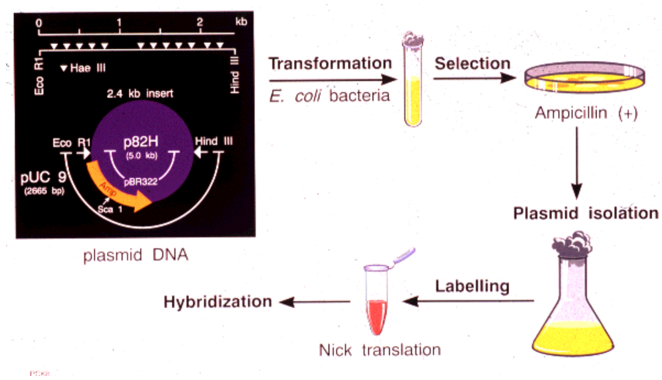
1. **Metaphase spread finding** - required to rapidly accommodate the key cytogenetic bioassay variants
2. Discrimination of chromosome /chromosome fragments
3. Discrimination of centromeres from chromosome background
4. Quantification of number of monocentrics and dicentrics
5. Assisted dicentric manual counting / record keeping - uses elements from the automated dicentric assay
6. **Resolution of chromosome overlaps** - common requirement of cytogenetic assays.

Materials and Methods

Lymphocyte Isolation

Whole blood from healthy human donors was collected into vacutainers containing ethylenediamineteraacetic acid (EDTA; Becton-Dickinson, Rutherford, NJ). The informed consent form used in this study was approved by the Uniformed Services University of the Health Sciences’ Human Use Committee, Bethesda, MD. Lymphocytes were isolated from whole peripheral blood on a density gradient (Histopaque; Sigma Chem. Co., St. Louis, MO). Cells for irradiation were suspended in tissue culture medium (Karyomax; Life Technologies, Gathersburg, MD) at an approximate concentration of 1.5 x 10^6/ml.





X-Ray Irradiation

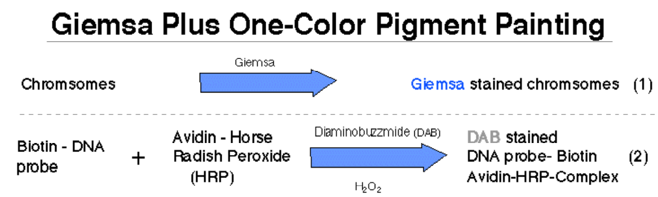
Cells suspended in 25-cm^2 tissue culture flasks were placed on a rotating Plexiglas holder for irradiation. Cells were exposed to x-rays generated by an industrial x-ray machine (Philips, Hamburg, Germany) at room temperature at a dose rate of 1 Gy/min with an effective energy of 83 keV (SSD = 55.2 cm, 250 kVp at 12.5mA, 0.20-mm Cu, and 1.0-mm Al filtration; Holahan et al., 1987). Dosimetry was confirmed by ion chambers placed in tissue culture flasks filled with tissue-equivalent plastic (ICRO, 1973).

Metaphase Spread Preparation

Lymphocytes were stimulated to divide by the addition of phytohemagglutinin (0.5 mg/ml, Murex Diagnostics Ltd., Dartford, England) and incubated at 37°C. After 44 h of stimulation, 1 µg/ml of colcemid was added to the culture and incubated for an additional 4 h to arrest cells in metaphase. Following hypotonic treatment in 1% sodium citrate solution, the cells were fixed in 1:3 acetic methanol fixative. Metaphase chromosome spreads were prepared on acid-cleaned microscope slides by the standard method (Priest, 1977). Slides were stored in 70% ethanol at 4°C and were used within 1 month for *in situ* hybridization studies.

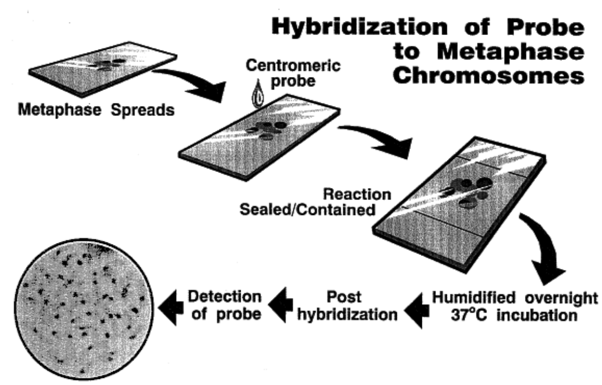
In Situ Hybridization

Slides were denatured in 70% formamide, 2X SSC buffer at 70°C, immedicately dehydrated in 70%, 90%, and 100% ethanol for 2 min each and then air dried. Purified p82H PCR-synthesized pancentromeric probe (Blakely, *et al*., 1993) was added to the probe denaturing solution (50% formamide, 2X SSC, 10% dextran sulfate, 1 µg/ml of salmon sperm DNA, 20 ng of p82H pancentromeric probe, pH 7.0), incubated at 70°C for 5 min, immediately applied to each slide, and sealed with glass coverslips with rubber cement. The samples were then incubated at 37°C overnight in a humidified chamber. Slides were washed in 50% formamide, 2X SSC, pH 7.0 at 37°C for 15 min, followed by 2X SSC at 37°C for 10 min. Slides were then transferred to PN (phosphate-nonidet)buffer (0.1 M sodium phosphate, 0.1% nonidet P-40, pH 8.0) for 5 min and then stored in this solution until detection of the biotinylated probe by immunostaining.



Immunoenzymatic Staining

Forty µl of PPN buffer containing 1% nonfat dry milk was added to the slides to block nonspecific binding. The reaction solution was sealed on the slide with a coverslip, and the slide was incubated for 30 min at 37°C in a humidified chamber. The reaction mixture was removed from the slides and replaced with a solution of PN buffer containing a 1:1000 dilution of avidin-horse radish peroxidase (Sigma, St. Louis, MO). The reaction mixture was incubated for 30 min at 37°C in humidified chamber after reattachment of coverslips to slides. The slides were then washed in PN buffer 3X for 5 min each. The peroxidase label was developed by incubating the metaphase spread preparation in a solution of 0.05 M Tris (pH 7.6), 0.05 g of 3’3’-diaminobenzidine (DAB; Sigma) and 0.01% hydrogen peroxide for approximately 10 to 20 min. The slides were then washed for 5 min in phosphate-buffered saline (PBS) and counterstained with 4% Giemsa in phosphate buffer.



Automated Finding and Scoring of Metaphase Spreads

Automated Metaphase Finder

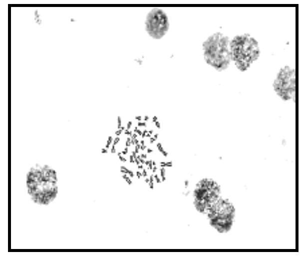
LAI Metaphase Finding and Scoring System

Special Features of the METAFIND system leading to Automation:

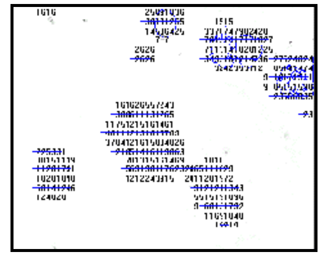
- Increased slide capacity - METAFIND can handle large data sets without human supervision or intervention.
- 100x objective image-based focus algorithm - required to allow chromosome aberration detection.
- "Bootstrap" Image Parameter cell/spread discrimination - provide direct extension to other assay scoring - PCC.
- For a new prep, an investigator selects good spreads, and "not good" spreads, and the computer program processes and generates optimal image parameter data.
- Color-based target discrimination - allows the color discrimination of pigmented or fluorescent chromosome elements.
- Servo-system (closed loop) cell/spread location - facilitates spread location at 100x and permits the transfer of locations to low-cost analysis station.

Spread Localization/Quality Technique

The METAFIND system automatically searches for spreads on slides using a low magnification objective. The classification parameters for locating spreads are based on the size, contrast, and texture of the spread, as well as number of objects (chromosomes) found within a spread. These parameters, quite stable with a stable prep, are interactively set by the operator, and easily optimized for differing spread characteristics and slide preps.



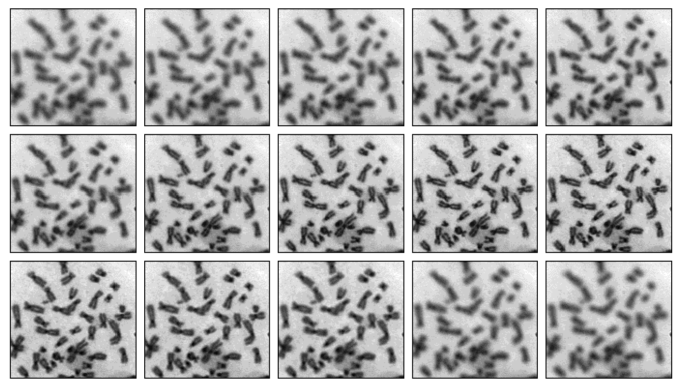
Field of View



Objects Classified

Best Focus Image Reconstruction

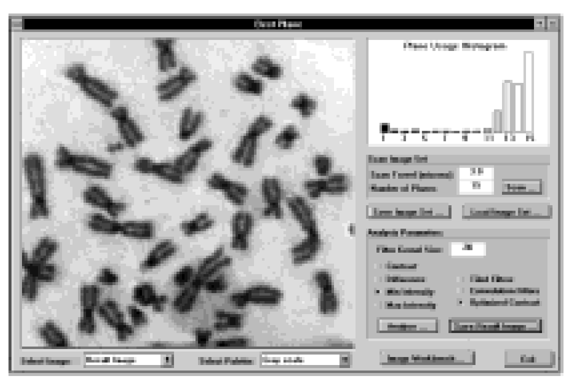
During the development of the METAPHASE scoring process we became aware that in a manual mode at the 100x (oil) magnification used for analysis, the scorer often had to adjust the z axis over a range of adjacent focal planes to get the whole chromosome in focus.



Focal Planes over 3.0 micron range

Particularly for the more complex images with overlaps and touching chromosomes, the scorer had to view different z-axis planes in order to make complete spread determinations.

A computerized version of the sequential viewing process accommodates this; in which sequential planes adjacent to the selected plane of focus best capturing the image were acquired. In the image, 15 planes spanning a total z-axis range of 3.0 µ (1/5 µ per plane) were acquired. Note that more than one plane is necessary to capture the image.



Best Plane Focus Utility

A graphical program based on a moving 3 x 3 pixel kernel was developed to investigate the image location producing the best information. “Best information” was based on a selectable set of criteria: minimum intensity, maximum intensity, contrast, intensity difference.

Automated Centromere Location / Dicentric Scoring

The dicentric score is accomplished in a 2 stage process

1. the chromosomes are isolated using an automated edge finding routine.
2. inside each chromosome edge is the number of centromeres, determined by a similar edge finding routine.

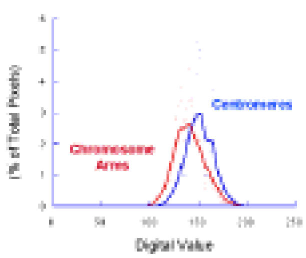
The specific detailed scoring of each spread can later be reviewed from the saved images. Total data sets can also be saved to CD-ROM and copied for external review.

This automated scoring process is now undergoing testing at AFRRI. The computer assisted manual program is used to implement the rapid comparison between manual trials and automated trials. This process allows performance analysis on a chromosome by chromosome basis.

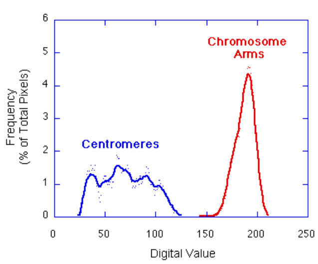
The use of the computerized manual scoring allows us to test both the validity of automated scoring and to rapidly and systematically isolate any elements requiring further enhancement or improvement.

Centromere Discrimination

Automated dicentric scoring uses a modification and extension of techniques shown. The benefit of centromeric painting process is illustrated in the graphs that compare centromere discrimination with or without centromere painting. The use of color-pigmented centromeres leads to easy ID vis-à-vis the chromosome background.



Non-Color Pigmented



Color Pigmented

Computer Assisted Dicentric Assay

Based on the ongoing development of a fully automated dicentric scoring program at LAI, we used the process techniques being developed for the automated dicentric scoring to produce a new computer assisted manual scoring program.

Figure a

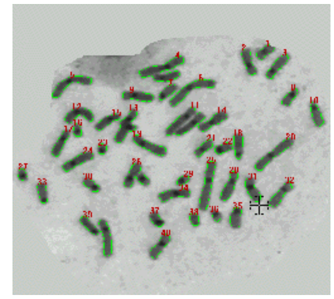


Figure b

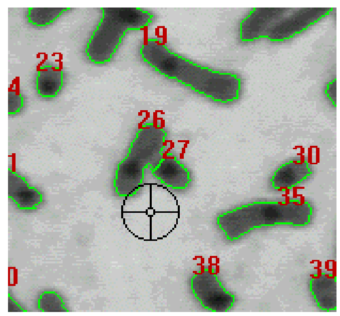


Figure c

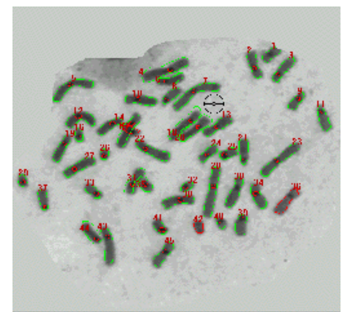
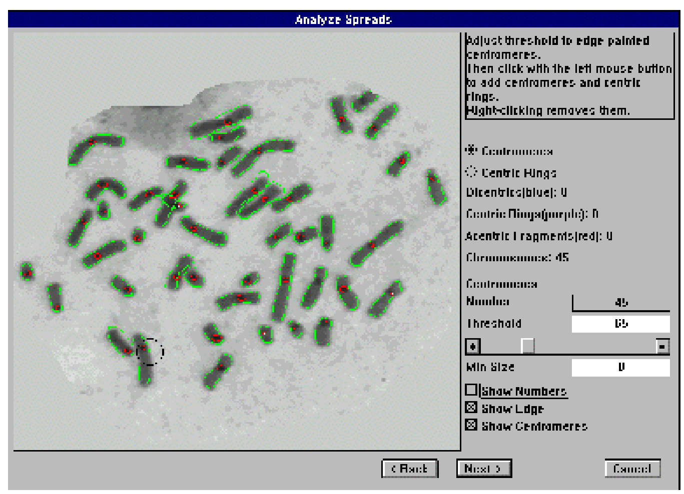


Figure d

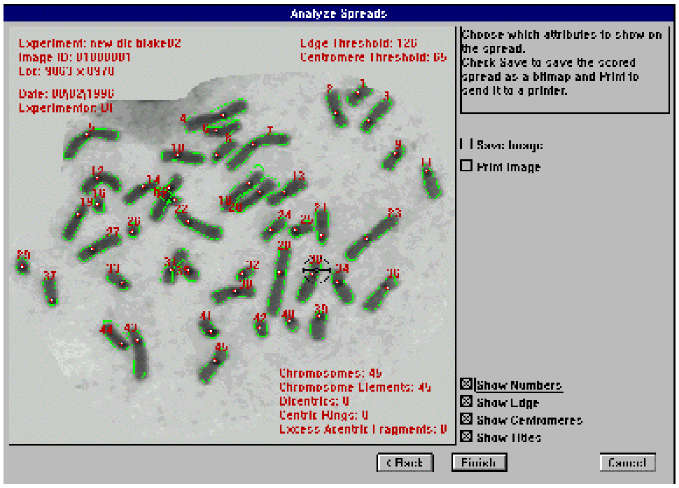
In Figure a, the pre-located spread (METAFIND) is isolated from any non-target cells or debris, by a mouse-controlled ROI determination. The computer then counts and numbers the discrete objects (Figure b). In Figure c, the scorer identifies overlaps by mouse point and click. In Figure d, the centromeres are identified by threshold and chromosomes are classified automatically as normal, dicentric or acentric.



Rings are identified by point and click.

Completed Spread Analysis

After the previous processes are complete (5-20 seconds), the completed spread count is displayed. ID information is automatically inscribed on the image. The resulting image can be saved as a digital image with inscribed data, or provided as a printed record. Results are immediately sent to a spreadsheet for statistical calculation.



Final Image with Annotations

The imaging printing process can be accomplished off-line in batch mode. Images can also be stored as a set on CD-ROM. One CD-ROM disk holds ~ 2400 spreads.

Results

Current Dicentric Assay Performance

- Location of metaphase spread (10x)
  ~ 17 seconds / spread
- Spread relocation (100x)
  3 – 5 seconds / spread

	Dicentric Scoring (Seconds/Spread)		
	Manual	**Assisted**	**Automated**
Location / identification	120	22	17
Printout count sheet	30	NA*	NA
Count / analyze	300	30	<15*>
Print documentation	60	5**	5**
Data transcription	120	NA	NA
Total	630	57	22

NA = not applicable
* = parallel operations
** = save to disk
*** = includes relocation

LAI/AFRRI CRADA Accomplishments

- Developed the technique for repeatable / transferable (100x) spread location
- Developed low-cost satellite dicentric scoring station
- Metaphase spread parameter development
- Developed computer program for semi-automated dicentric scoring
- Automated the discrimination of centromeres
- Developed the technique for composite multi-focus metaphase spread image formation

Acknowledgments

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