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SCGE-PRO: A FLUORESCENCE BASED DIGITAL IMAGING SYSTEM DEVELOPED FOR MEASURING DNA DAMAGE USING SINGLE CELL GEL ELECTROPHORESIS (COMET ASSAY)

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Introduction

PC based Digital Imaging Systems have gained wider attention since 1985 with the development of PCI bus and Pentium chip, which together have contributed to high speed computation and data transfer to PC's central processing unit (CPU). Similarly, with the availability of a wide variety of imaging devices such as high-resolution digital video, 3-CCD (R-G-B) and cooled-CCD cameras have also contributed significantly to the progress in this area. During recent years a number of digital imaging techniques and software packages have been developed for

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various applications in bio-medical research. Earlier we have reported the development of a semi-automatic Digital Imaging System: *Cyto-Pro* for various cytogenetic applications. (Chaubey, R.C. *et al.* 1999). Here we report the development of a *Fluorescence based Digital Imaging System and Application Software SCGE-Pro,* for the first time in India, to measure DNA damage using Single Cell Gel Electrophoresis (SCGE).

SCGE or Comet assay is a sensitive, versatile technique and can be used in diverse areas of bio-medical research, e.g., genetic toxicology, radiation biology, ageing, clinical and molecular epidemiological studies, predictive assays in cancer radio-therapy and biological dosimetry in case of radiation exposure (Singh *et al.* 1996; Olive *et al.* 1993; Olive & Banath 1995; Olive *et al.* 1998). The technique is capable of detecting a wide variety of DNA damage and lesions such as DNA single strand breaks, double strand breaks, cross-linking agents, base damage, including DNA repair (Pouget *et al.*1999; Sauvaigo *et al.* 1998; Nocentini, 1995; Tice *et al.* 1990).

Systems Description

The system consists of a Carl Zeiss Axioplan microscope with bright field, phase contrast and epi-fluorescence facility (HBO 50 high pressure mercury lamp), 0.5x camera adapter lens, high performance colour camera with 750 lines horizontal resolution (KY-F55BE 3CCD, JVC, Japan). The Integral Flashpoint Intrigue frame grabber, used in this system, is a PC based card and it accepts colour composite video output of the camera. It digitizes each of RGB planes at a tonal resolution of 24 bits per pixel. It has spatial resolution of 768 x 576 per frame. A Pentium P-II computer with super VGA colour monitor, CD-ROM drive, 4.3 GB Hard Disk and Win 95 or Win NT operating system is required for image acquisition and processing. A complete color image requires 640 X 480 X 24 bits (921600 bytes) of data space. Thus complex operations on large images require large storage space and a fast computer. The system also has a CD writer for image storage, and HP Desk Jet printer. Fig.1A shows the monogram of the software and 1B shows the photograph of the *Digital Imaging System: SCGE-Pro.*

Fig.1A shows the monogram of the software and 1B shows the photograph of the Digital Imaging System SCGE-Pro.

Materials and Methods

Irradiation

Heparinised whole blood from human samples was

irradiated at 0°C to different doses of gamma rays, e.g., 2, 4 and 8 Gy, using a ^{60}Cobalt Teletherapy machine at a dose rate of 0.668 Gy/min.

Alkaline single cell gel electrophoresis for detecting DNA single strand breaks

- 100 µl of heparinised whole blood was mixed with 1.5 ml of 0.8% agarose solution at 42°C and poured on fully frosted slides uniformly.
- After polymerization, the slides were kept in 50 ml lysing buffer, (2.5 M NaCl, 100 mM Na_2-EDTA with freshly added 1% Triton X-100 and 10% DMSO), for 1h at 4°C.
- The slides were removed from the lysing solution, washed with alkaline electrophoresis buffer 3 times, and then placed on horizontal electrophoresis tank filled with freshly prepared alkaline buffer (300mM NaOH, 1mM Na_2-EDTA, pH 13.0) at 4°C.
- The slides were kept in the same buffer (pH 13) for 20 min to allow DNA unwinding and expression of alkali-labile sites.
- Electrophoresis was carried out for 20 min at 1.1 V/cm (25 Volts, 300 mA) using a compact power supply. After electrophoresis, the slides were washed gently to remove the alkali and detergents by placing them horizontally and flooding them slowly with 0.4 M Tris buffer at pH 7.5.
- The slides were stained in propidium iodide (5µg/ml) for 1 h. After staining the slides were rinsed with distilled water and kept on wet paper in a closed box at 4°C.
- Stained slides were observed under fluorescence microscope at 40x magnification using SCGE-Pro System.

Neutral single cell gel electrophoresis for detecting DNA double strand breaks

- 100 µl of heparinised whole blood was mixed with 1.5 ml of 0.8% agarose solution at 42°C and is poured on fully frosted slides uniformly.
- After polymerization, the slides were kept in 50 ml lysing buffer, (30 mM EDTA; 0.5% SDS pH 8.3 and 0.5 mg/ml Proteinase-K) at 50°C for 3 h.
- The slides were kept in 1xTBE (45 mM Tris-borate, 2mM EDTA) buffer over night.
- Next day the slides were again washed in 1xTBE buffer for 5-10 min.
- Electrophoresis was carried out in 1xTBE buffer at 0.9 V/cm. (20 volts, 20 mA) for 25 min.
- Slides were stained in propidium iodide (5µg/ml) for 1 h and observed under Carl Zeiss Axioplan microscope at 40x magnification using SCGE-Pro System.

Steps involved in measurement of DNA damage using the SCGE-Pro system

Carl Zeiss Axioplan microscope with epifluorescence, using Filter 15 (BP546/12, FT580, LP590), was used for observation and measurement. The images of the individual comets are captured using a 3-CCD camera and stored in separate files. The acquired images are pre-processed to remove acquired artifacts. The signal to noise ratio is improved by frame averaging technique. The total propidium iodide fluorescence intensity is taken as total DNA content in the comet. The software allows quantitative measurements of total fluorescence of the comet, fluorescence of the tail, length of migrated DNA fragments and finally calculates the tail moment (product of fraction of

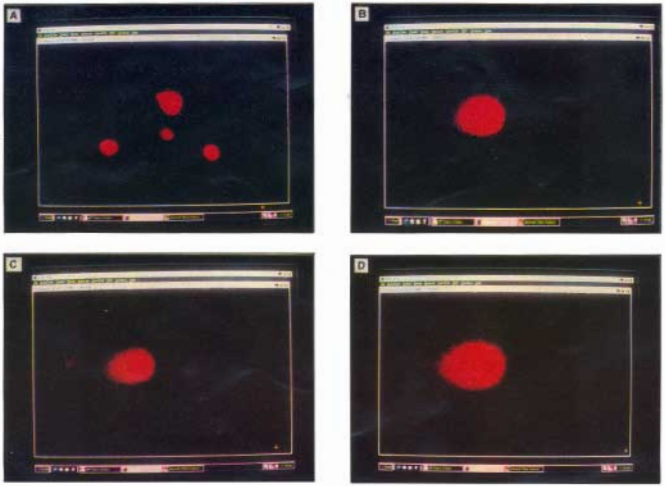

Fig.2 Nuclei of human leucocytes exposed to different doses of gamma rays. Fig.2A shows undamaged and damaged nuclei (25x magnification). Fig. 2B, 2C and 2D show damaged nuclei to different extent, as evident from the increase in the tail length of the comets (40x magnification).

DNA in the tail and tail length), an internationally most accepted parameter for comparing the DNA damage.

Fig. 2 shows nuclei of human leucocytes exposed to different doses of gamma rays. Fig.2A shows undamaged and damaged nuclei (25x magnification) and Fig. 2B-D shows nuclei damaged to different extents, as evident from the increase in the tail length of the comets (40x magnification). Fig. 3 shows the various steps involved in measurement of tail moment. Fig. 3A shows the enhanced image of the comet. The system has to be calibrated at 25x, 40x or 100x before making any measurement, depending on the magnification used during image acquisition. Fig. 3B shows provision for selecting area of interest (AOI), dialogue boxes for intensity and length measurement, gray level setting, both lower threshold (LT) and upper threshold (UT) limits, and the result window. The DNA content or the fluorescence intensity of the entire comet can be measured by pressing a single button. Fig. 3C shows the selection of fresh AOI, restricted only to the tail region of the comet. For measuring the DNA content in the tail region, both LT and UT have to be set to the same level as it is in the case of total DNA measurement in the comet. Fig. 3D shows the measurement of the length of the migrated DNA fragments (μm) by selecting the line mode. Fig. 4 shows an alternate mode for measuring DNA content in comet, tail, tail length and tail moment.

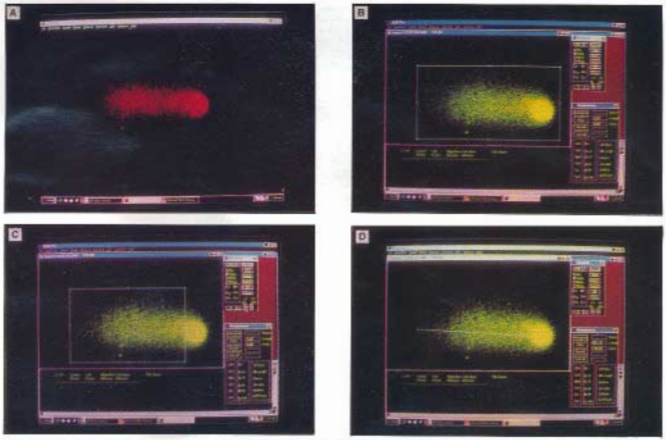

Fig.3 shows the various steps involved in measurement of tail length and tail moment (product of DNA content in the tail and length of migrated DNA fragments in μm) by using the software SCGE-Pro (For details see text).

Here, again the basic steps involved in the measurement of DNA content in the comet or in the tail region is essentially the same as described in Fig. 3, but this mode allows more distinct differentiation between the head (nucleus) and tail of the comet. This is a unique feature, which allows more accurate measurements. As we can see, while setting the gray levels (both LT and UT), the head of the comet or the nucleus is seen very clearly with the original color of the dye used (propidium iodide, red fluorescence), while the tail region is showing the pseudo color. Fig. 5A-C shows the measurement of tail length and tail moment from the control nucleus. The data is automatically stored in the application specific format in the result file, which can be imported to Microcal Origin ver. 5 for various statistical calculations and graphical representations.

Results

Heparinised whole blood from human samples was exposed to different doses, e.g., 2, 4 and 8 Gy, of gamma rays, embedded in agarose, lysed and electrophoresed under different conditions, e.g. alkaline condition for measuring DNA single strand breaks (ssb) and neutral condition for double strand breaks (dsb). The broken DNA fragments migrate towards anode during electrophoresis depending on the total charge and fragment size. After staining with propidium iodide, the nuclei were observed under fluorescence microscope using SCGE-Pro system. Each cell appears as a comet with brightly fluorescing head and with diminishing fluorescence intensity in the tail. Fig.6 shows the relationship between DNA damage (tail moment) produced by different doses of gamma radiation. Cells were analyzed for DNA single strand breaks using

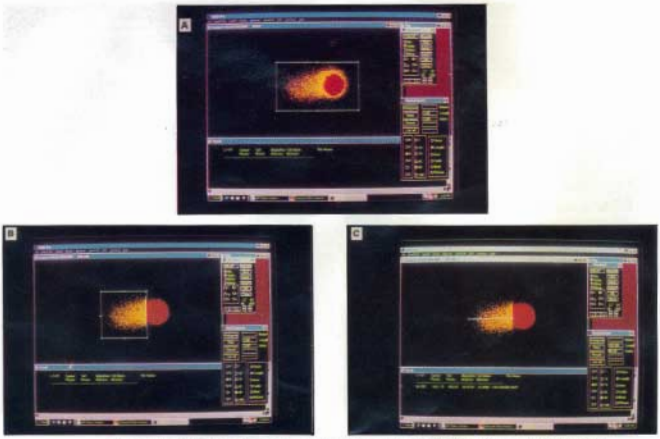

Fig.4 An alternate mode for measurement of tail length and tail moment (For details see text).

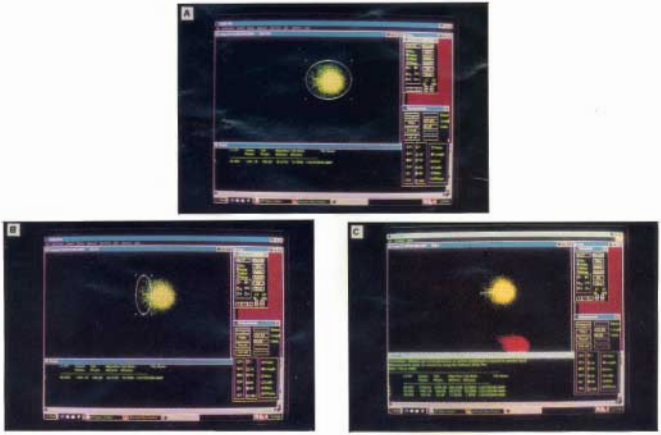

Fig.5 Measurement of tail length and tail moment from the control nucleus.

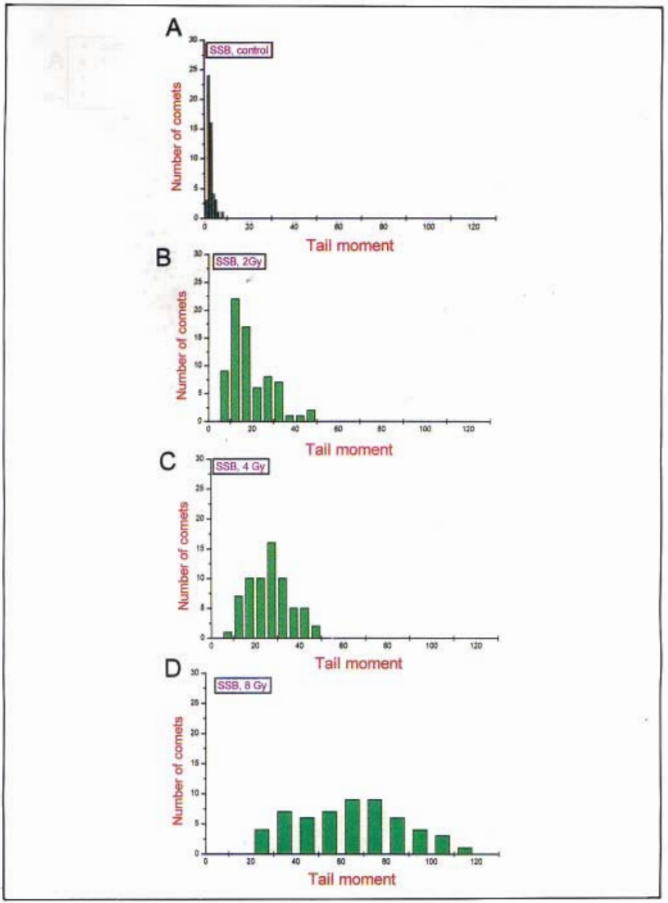

Fig.6 DNA single strand breaks: Distribution of comets with different tail moments, Panel A: control, Panel B: 2 Gy, Panel C: 4 Gy and Panel D: 8 Gy gamma irradiated human leucocytes.

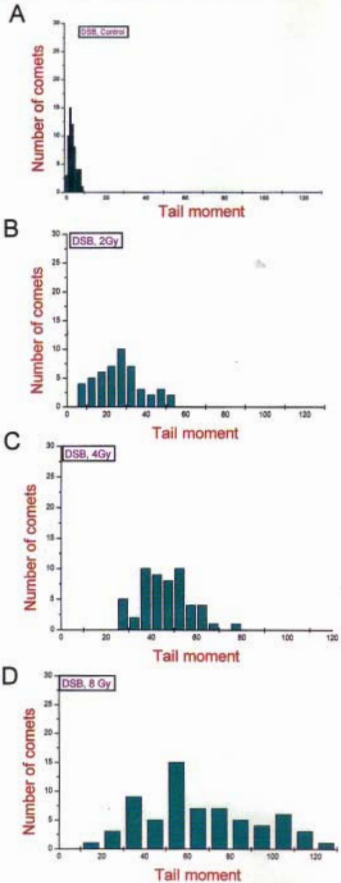

Fig.7 DNA double strand breaks: Distribution of comets with different tail moments, Panel A: control, Panel B: 2 Gy, Panel C: 4 Gy and Panel D: 8 Gy gamma irradiated human leucocytes.

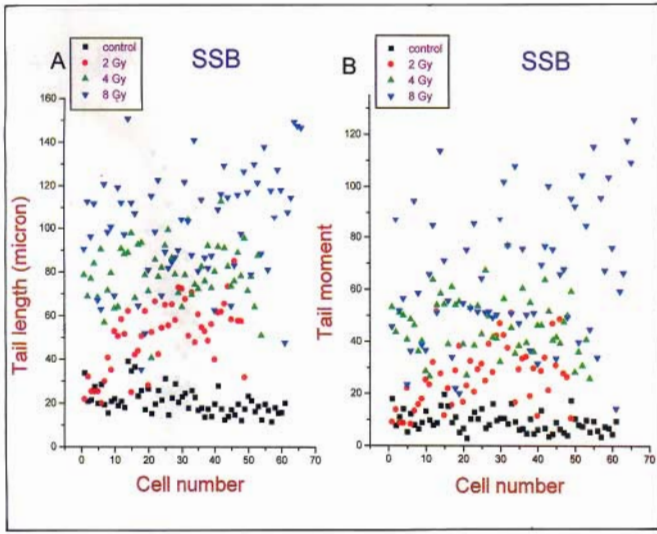

Fig.8 DNA single strand breaks: Distribution of individual cells based on tail length (Panel A) and tail moment (Panel B) in control and gamma irradiated human leucocytes.

alkaline gel electrophoresis. The plot shows frequency distribution of comets with different tail moments in control and radiation exposed cells. As we can see from the data, there is a clear-cut difference in the median value of tail moment in control and irradiated samples, the values being 1.89, 16.57, 25.92 and 64.63 in control and 2, 4 and 8 Gy samples respectively. Fig. 7 shows data on gamma ray induced DNA double strand breaks in human leucocytes. Fig.7A to D shows distribution of comets with different tail moments in control (Fig. 7A) and gamma irradiated (Fig.7B to D) human leucocytes. Here also a clear-cut difference in the median tail moment was observed between the control and treated groups. This data also shows heterogeneity in tail moments in different irradiated groups similar to the data obtained on single strand breaks. Fig.8 shows results of DNA single strand breaks, the plot shows distribution of individual cells based on tail length (Panel A) and tail moment (Panel B) in control and gamma irradiated human leucocytes. A distinct difference in distribution pattern was observed in control and different treated groups. Fig. 9 shows results of DNA double strand breaks. The plot shows distribution of individual cells based on tail length (Panel A) and tail moment (Panel B) in control and gamma irradiated human blood cells. A heterogeneous pattern of DNA migration was observed in individual cells exposed to different doses of gamma radiation.

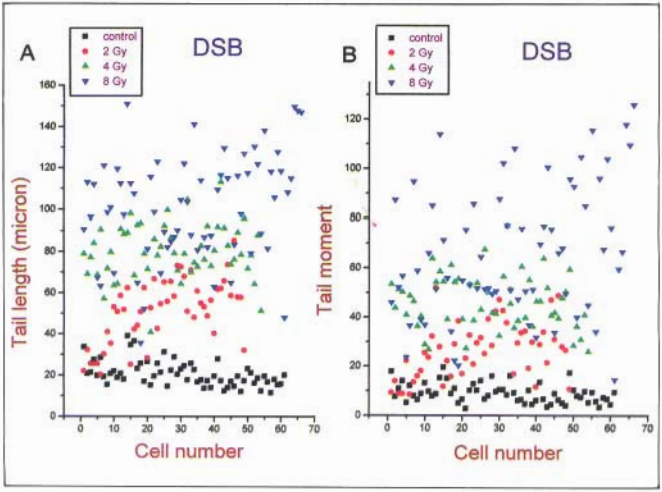

Fig.9 DNA double strand breaks: Distribution of individual cells based on tail length (Panel A) and tail moment (Panel B) in control and gamma irradiated human leucocytes.

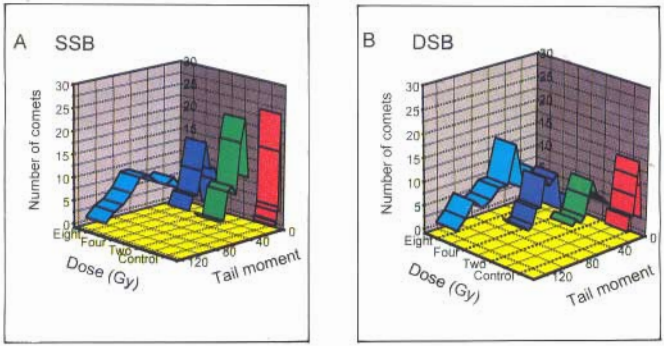

Fig.10 3 D Ribbon Plot : Effect of gamma radiation on DNA single strand breaks (Panel A) and double strand breaks (Panel B) in human leucocytes : Dose response.

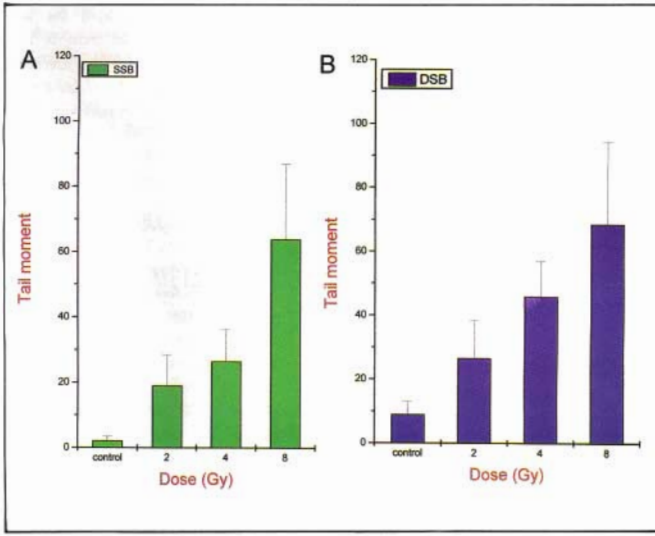

Fig.11 Effect of gamma irradiation on DNA single strand breaks (Panel A) and double strand breaks (Panel B) : Dose response. Values are mean tail moment ± standard deviation. All the values from irradiated samples are statistically significant from the control (One way ANOVA).

Fig. 10 shows 3 D ribbon plot of data on dose response effect of gamma radiation on DNA single strand breaks (Panel A) and double strand breaks (Panel B) in human leucocytes. Plot on distribution of comet length or tail moment is very important and should be presented for each experiment, because it may reflect evidence for any heterogeneity among different cell populations. Fig.11 shows the histogram of data obtained on DNA single strand breaks (Panel A) and double strand breaks (Panel B) of human leucocytes. Fifty to seventy cells were measured for each dose point. Values are mean of tail moment ± standard deviation. One-way ANOVA was applied to test the statistical significance of the data. Tail moment of cells measured for DNA single strand breaks showed statistically significant ($p<0.001$) difference with increasing doses of gamma rays. Similarly, data on DNA double strand breaks also showed statistically significant difference in tail moment with the increasing doses of gamma radiation. Data on tail length of comets also showed dose dependent increase ($p<0.001$) for both ssb and dsb in human leucocytes.

There are a number of factors which may affect the sensitivity and resolving power of the SCGE technique, e.g., concentration of low-melting agarose, composition of the lysing solution,

composition and pH of the electrophoresis buffer, electrophoretic conditions such as voltage, amperage, duration and gel size. The sensitivity of this technique also depends upon the type of DNA-specific dye used for visualization. The nuclei or the DNA fragments can be stained with a variety of dyes such as ethidium bromide, propidium iodide, etc. However, currently more sensitive fluorochromes such as Sybr green, YOYO-1 (benzoxazolium-4-quinolinium dimer), etc. are available commercially which can increase the sensitivity of detection. Image acquisition through cooled-CCD camera will further increase the sensitivity of this technique.

Conclusions

SCGE is a sensitive technique and can be used for detecting DNA damage under *in vivo* and *in vitro* conditions from human tissues exposed to ionizing radiations. The technique is best suited for monitoring DNA damage in human population exposed occupationally, clinically or environmentally to any physical or chemical agent, because very small amount of blood or few thousand cells are enough for measurement. The indigenously developed *Fluorescence based Digital Imaging System SCGE-Pro* was found to be very sensitive, accurate, fast and reproducible means to measure and analyze the cells or comets with DNA single strand and double strand breaks. The software has the advantage that the data once stored in the result file (which could be more than 1000 cells per study), can be imported to different software for various statistical calculations and graphics, as can be seen from various analysis done in this study.

Acknowledgement

Authors wish to express their sincere gratitude to Dr. Anil Kakodkar, Director, Bhabha Atomic Research Centre, for encouraging this Inter-Divisional Collaborative Project under IX Plan, between Cell Biology Division and Electronics Systems Division to develop and validate a digital imaging system and software for various cytogenetic applications.

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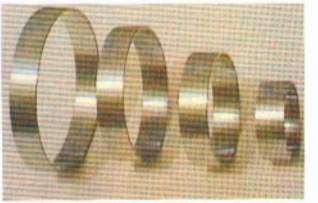
Ni-Ti SHAPE MEMORY ALLOY HEAT SHRINKABLE SLEEVES

Heat shrinkable sleeves of a Ni-Ti-Fe shape memory alloy have been successfully developed by the Materials Science Division (MSD), BARC, for application in an insulator assembly of an advanced aircraft under development. Research on shape memory alloys has been going on in the Materials Science Division for over a decade. A number of important scientific contributions on shape memory phenomena have originated from MSD. These include, the determination and rationalization of reversion stress, the role of self accommodation of martensite crystals in the shape memory effect and identification of the criteria to be fulfilled by an alloy to exhibit shape memory. In a parallel effort, the development of the melting and fabrication flow sheet for shape memory alloys has been pursued and several Ni-Ti, Cu-Zn-Al and Fe-based alloys have been successfully produced in small quantities.

Few demonstration items have also been fabricated, viz., a solid-state heat engine, a heat shrinkable pipe coupling and a few thermally activated devices. The present achievement in developing the heat shrinkable sleeves for the aircraft application is a culmination of these research and development



Ni-Ti shape memory alloy heat shrinkable sleeves

efforts. The entire flow sheet involving alloy preparation, fabrication and machining was scaled up from a laboratory scale to about 20 kg ingot size. While the making and hot rolling of these alloys have been carried out at the Atomic Fuels Division, the required thermo-mechanical treatments and the detailed characterization have been performed in MSD.

The Ni-Ti-Fe sleeves are utilized to clasp polymer insulators onto metallic conduit pipes. During the assembly of this component, the sleeves are expanded in a liquid nitrogen bath and applied to the insulator-conduit pipe joint. In these alloys, any shape change imparted by deformation of the

martensite phase is annulled and the original dimensions recovered in the course of the reverse transformation to the austenite phase on heating the material to room temperature. The dimensions of the assembly and the sleeves are so chosen that the complete shape recovery is not possible and there is a residual strain in the sleeve. The sleeves generate a large stress under the constrained condition of shape recovery and tightly clasp the insulators onto the conduit pipes. The tools for expanding the sleeves have also been developed at BARC and are supplied along with the sleeves.

These components successfully gone through rigorous tests for airworthiness and have already been certified for application in combat aircraft. The components are tested for leakage, sealing, ultimate pressure, thermal shock, vibration, pull out and endurance upto 1,40,000 cycles of pressurization under the combined action of shear and bending forces. These tests ensure that the sleeves apply sufficient grip over the temperature range (-50°C to 150°C) encountered in service. At this point in time, the development phase of this project has been accomplished.

BARC TRANSFERS TECHNOLOGY OF LASCAN DIA GAUGE

BARC has transferred the technology of Lascan Dia Gauge developed by Laser & Plasma Technology Division to M/s Jasch Industries Ltd., New Delhi. The technology transfer agreement was signed on March 14, 2000. Lascan Dia Gauge is a laser based non-contact diameter/linear dimension measuring (1 to 25 mm) instrument, ideally suitable for dimensional measurement of high temperature, toxic, radioactive or corrosive products. It can also be used for on-line measurement and for process monitoring and control. It works on the principle of laser beam scanning.

Dr R.B. Grover, Head, TTCD, BARC, and Mr V.A. Likhate of M/s Jasch Industries Ltd., New Delhi, greet each other after signing the agreement.

A fine beam of visible light from a laser diode is reflected by a high speed rotating mirror on to a lens to produce parallel scanning beams. These parallel beams after interacting with the object are focussed by receiver optics on to a photodiode. Any object kept in the measuring plane will obstruct the scanning beam for a period proportional to its dimension. The parallel beams are then focussed by optical means on to a photodiode to generate a shadow pulse of the object, which is electronically processed to give the dimension of the object.

Mr V.A. Likhate from M/s Jasch Industries Ltd., New Delhi, Dr N. Venkatramani, Head, Laser & Plasma Technology Division (L&PTD); Dr R.B. Grover, Head, Technology Transfer & Collaboration Division (TT&CD); Mr M.K. Makker, Mr A.S. Rawat and Mr U.C. Bhartiya from L&PTD; Dr A.K. Kohli and Mr S. Nawathe from TT&CD participated in the technology transfer programme.

RETROFITTING OF CNC DECKEL MILLING MACHINE

The first Computerized Numerical Control Machine of BARC was installed in Central Workshops (CWS) about eighteen years ago. The deteriorated Grundig Control System was recently replaced with indigenous control system at a cost of Rs 5 lakhs. The necessary control logic was developed in-house at CWS and the machine was restored to the original condition. Commercially available equivalent system (including commissioning) would have cost about Rs 12 lakhs. Thus, in-house development has resulted in self-reliance and a substantial saving of around Rs 7 lakhs. With the experience gained, it is now proposed to convert the present conventional DIXI Jig-boring Machine into CNC Machine at a saving of around Rs 15 lakhs.

BARC GETS AERB INDUSTRIAL SAFETY AWARDS

BARC was the receipient of Atomic Energy Regulatory Board (AERB) Industrial Safety Award for the best safety performance for the year 1999, under the category of R&D units and also for the category R&D (Industrial), the latter being won by the Ore Dressing Section, Hyderabad. The shields are instituted by AERB to promote industrial safety among the different constituent Units of DAE. The shields were given by Prof. Sukhatme, Chairman, AERB, on March 6, 2000.

Prof. S.P. Sukhatme, Chairman, AERB, presenting the AERB Industrial Safety Award (R&D category) for year 1999 to Dr M.C. Abani, Head, Radiation Safety Systems Division (RSSD), BARC. In the photograph from left to right are: Mr S. Narayan, Head, Industrial Hygiene & Safety Section (IHSS), Dr M.C. Abani, Head, RSSD, Prof. S.P. Sukhatme, Chairman, AERB, and Mr P.G.G. Menon, IHSS

BARC SCIENTIST DEVELOPS A NEW COMPUTER SOFTWARE

Mr R.P. Hans of Division of Remote Handling and Robotics, BARC, has recently developed a computer software which enables the user to type in "Type as you pronounce" mode in Hindi, Marathi or Sanskrit languages. This software follows the "phonetic conventions" and is available for typing in "Typewriter" mode as well as in Hindi. The software, named as "Dropadi Shabd", can also be used in

many packages under Windows, like MS Word, Corel Draw, MS Paint, Excel, etc. It can be used for creating HTML files and for sending e-mails in Indian languages. Bilingual Hindi font has been included in the software for typing both in Devnagari and Roman scripts.

A workshop was held in the Hindi Cell of BARC during January 12-14, 2000 to give training to the staff for working in Hindi using computers. In the workshop, the "Dropadi Shabd" software was introduced and about 20 participants took part. Mr Hans was given a Special Award of Rs. 1,000/- for the development of the software.

BARC SCIENTISTS HONOURED

• Dr Hirendra Nath Ghosh of Radiation Chemistry & Chemical Dynamics Division, BARC, has been selected for the prestigious Anil Kumar Bose Memorial Award (2000) by the Indian National Science Academy, New Delhi. The award carries a bronze medal and cash prize of Rs 1,000/-. The award will be presented during the Annual General Meeting of the Academy scheduled at Delhi during October 9 to 11, 2000. Dr Ghosh had won the prestigious INSA Young Scientist Award (1998). The present award is given to an INSA Young Scientist awardee for best research output during the year after the INSA Young Scientist Award.

• Dr B.K. Jain, Head, Nuclear Physics Division, BARC, was recently appointed the Chairman of the Review Panel constituted by the Department of Arts, Culture, Science and Technology, Govt. of South Africa to review in depth the research reactor, SAFARI, of their Nuclear Energy Corporation (NECSA). Other members of the panel were from Atomic Energy, U.K., IAEA and South African educational and medical institutes. The review was a part of the ongoing South African AEC review. The panel submitted its report following deliberations during April 6-11, 2000.

• Dr K.G. Raghavan of Radiation Biology Division, BARC, was nominated by the Director and faculty members of Dr A.L.M.P.G. Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai, to deliver the Dr P. Kutumbaiah Memorial (Medicine) Endowment Lecture for the year 1999-2000 on February 28, 2000. The topic of the lecture was "Urolithiasis: Yesteryears - Today - Tomorrow."

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