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# Cytogenetic Analysis for Radiation Dose Assessment

## A Manual



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CYTOGENETIC ANALYSIS FOR  
RADIATION DOSE ASSESSMENT

A Manual

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## FOREWORD

Chromosome aberration analysis is recognized as a valuable dose assessment method which fills a gap in dosimetric technology, particularly when there are difficulties in interpreting the data, in cases where there is reason to believe that persons not wearing dosimeters have been exposed to radiation, in cases of claims for compensation for radiation injuries that are not supported by unequivocal dosimetric evidence, or in cases of exposure over an individual's working lifetime.

The IAEA has maintained a long standing involvement in biological dosimetry commencing in 1978. This has been via a sequence of Co-ordinated Research Programmes (CRPs), the running of Regional Training Courses, the sponsorship of individual training fellowships and the provision of necessary equipment to laboratories in developing Member States.

The CRP on the "Use of Chromosome Aberration Analysis in Radiation Protection" was initiated by IAEA in 1982. It ended with the publication of the IAEA Technical Report Series No. 260, titled "Biological Dosimetry: Chromosomal Aberration Analysis for Dose Assessment", in 1986. The overall objective of the CRP (1998–2000) on "Radiation Dosimetry through Biological Indicators" is to review and standardize the available methods and amend the above mentioned previous IAEA publication with current techniques on cytogenetic bioindicators which may be of practical use in biological dosimetry worldwide. An additional objective is to identify promising cytogenetic techniques to provide Member States with up to date and generally agreed advice regarding the best focus for research and suggestions for the most suitable techniques for near future practice in biodosimetry. This activity is in accordance with the International Basic Safety Standards (BSS) published in 1996.

To pursue this task the IAEA has conducted a Research Co-ordination Meeting (Budapest, Hungary, June 1998) with the participation of senior scientists of 24 biodosimetry laboratories to discuss the methods and their modifications used for biological dose assessment worldwide, as well as the need and the possible ways of standardization of the available and promising cytogenetic techniques to evaluate the dose of accidental acute or cumulative chronic exposure to ionizing radiation. The contribution of the chief scientific investigators of the IAEA CRP on "Radiation Dosimetry through Biological Indicators" is sincerely acknowledged (the list of the participating biodosimetry laboratories is given at the end of the Manual). Four Consultancy Services Meetings were held in 1999–2000 to prepare this Manual on standardized conventional methods and amend them with newly available, proven techniques such as fluorescence in situ hybridization (FISH), premature chromosomal condensation (PCC) and study of micronuclei (MN) frequency.

The IAEA wishes to express its thanks to all authors and reviewers of this Manual as listed at the end of the book. Primary contributions of D.C. Lloyd (United

Kingdom), F. Darroudi (Netherlands), M. Fenech (Australia) and G.J. Köteles (Hungary) are especially acknowledged.

I. Turai of the Division of Radiation and Waste Safety was the IAEA Scientific Secretary responsible for the preparation of this Manual and for the CRP on “Radiation Dosimetry through Biological Indicators” in 1998–2000.

#### *EDITORIAL NOTE*

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## 1. INTRODUCTION

Biological dosimetry, based on the analysis of solid stained dicentric chromosomes, has been used since the mid 1960s. The intervening years have seen great improvements bringing the technique to a point where dicentric analysis has become a routine component of the radiological protection programmes of many Member States [1]. Experience of its application in thousands of cases of actual or suspected overexposures has proved the worth of the method and also helped to define its limitations.

It should be emphasized that chromosomal aberrations are used as a dosimeter and provide one input, frequently a very important one, into the compendium of information that needs to be collected and considered when a radiological accident is investigated [2].

In the investigation of radiation accidents it is important to estimate the dose to exposed persons for several reasons. In the case of high exposures (>1 Gy acute), information on doses assists in the planning of therapy and in alerting physicians to likely deterministic health consequences that could arise in the following weeks and months.

For exposures below the level where treatment is needed, dosimetric information is important for the physician in counselling irradiated persons on the risk of their developing late stochastic diseases — i.e. cancer. For persons whose exposure is very low (<50 mGy), the knowledge that no significant elevation in chromosomal damage could be found is frequently very reassuring. This is particularly the case where details of events are poorly known and no physical dose measurements or calculations are available. Then biological dosimetry may be the only means of quantifying dose, although, as will be discussed, there are quantification problems associated with factors such as non-uniform exposures, intake of radionuclides and delayed blood sampling.

Biological dosimetry also has a valuable role to contribute in the early period after an accident where many persons may have been exposed. At this time, triage of casualties using biological and clinical endpoints that initially and rapidly can give just an approximate estimate of dose is needed.

The primary objective of this Manual is to provide the reader with technical information for selecting and implementing, in a standardized manner, the appropriate cytogenetic technique to ensure comparable dose assessment following accidental exposure to ionizing radiation. The Manual describes all four possible cytogenetic methods currently available for biodosimetry. It is appropriate to have all these techniques readily available in main geographical regions, but, given a degree of international co-operation, it is not necessary to have all of them available in each national biodosimetry laboratory.

This Manual is arranged as follows:

Following the Introduction (Section 1), in Section 2 consideration is given to what is meant by ‘dose’ as determined from chromosome damage and how this relates to the values of personal dose derived by physical methods and the concept of equivalent dose as defined by the International Commission of Radiological Protection (ICRP). In Section 3 some biophysical and microdosimetric background to the induction of chromosomal damage is described. This is followed, in Section 4, by a brief description of the human lymphocytes from which the T types are the cells used for biological dosimetry. In Section 5 the chromosomal structure is outlined. In Section 6 the types of DNA lesion induced by interactions with ionizing radiation, together with a description and classification of those chromosomal alterations that can be observed in lymphocytes after irradiation of G0/G1 cells, are discussed. In Section 7 the requirements of blood sampling are described. Sections 8, 9, 10 and 11 then describe the techniques for performing biological dosimetry with, respectively, the four cytogenetic endpoints of dicentrics, fluorescence in situ hybridization (FISH) based translocations, prematurely condensed chromosomal breaks and micronuclei. Sections 12 and 13 provide brief information on automatic analysis of chromosomal alterations and on laboratory safety for staff carrying out cytogenetic analysis, respectively. The comprehensive up to date reference list is followed by four annexes describing reproducible working protocols for dicentric, FISH based translocation, PCC and MN assays. The Manual concludes with a list of abbreviations used, an index of the most important terms, a listing of 24 participating biosimetry laboratories and the chief scientific investigators of the IAEA Co-ordinated Research Programme (CRP) on Radiation Dosimetry through Biological Indicators, 1998–2000 [2], and finally a list of contributors to drafting and peer review.

The IAEA publication Technical Report Series No. 260. Biological Dosimetry: Chromosomal Aberration Analysis for Dose Assessment [1] concentrated exclusively on the dicentric assay. That timely publication provided a valuable and frequently cited landmark in biological dosimetry. It was written to be read at two levels. First, it was to serve as a laboratory manual, providing a convenient and comprehensive source of information at the technical level. In addition, it was intended to provide a concise summary of the technical background of the subject, for use in educational establishments, having a curriculum in radiation biology.

Now, 15 years later, this present book comprises a greatly extended revision of TRS-260, which is hereby superseded. Much of the original text concerning the dicentric assay is still valid and has been retained although, where appropriate, updated. The major extensions have been to include FISH chromosome painting, premature chromosomal condensation (PCC) and micronuclei (MN) assays.

In 1986 the MN assay already existed but was not widely applied. The applications of FISH chromosome painting and PCC to biological dosimetry are more recent

developments which are proving their worth in overcoming some of the limitations of the dicentric assay; it is hoped that the present Manual will demonstrate this fact.

## 2. APPLICATION OF DOSE CONCEPTS IN BIOLOGICAL DOSIMETRY

This section provides brief information on dosimetric terms, on the physical meaning of absorbed dose and on its interpretation for biological (cytogenetic) assessment of the dose from accidental exposure to different types of ionizing radiation.

Chromosome aberrations in lymphocytes are used to estimate absorbed dose to overexposed persons. Absorbed dose is defined by the Basic Safety Standards (BSS) as  $D = d\epsilon / dm$ , where  $d\epsilon$  is the mean energy imparted by ionizing radiation to matter in a volume element and  $dm$  is the mass of matter in the volume element. The SI (Système international d'unités) unit of absorbed dose is the joule per kilogram, termed the gray (Gy) [3]; 1 Gy = 1 J/kg (1 Gy = 100 rad).

The aberrations scored in the lymphocytes are interpreted in terms of absorbed dose by reference to a dose response calibration curve. This curve will have been produced by exposure of blood *in vitro* to doses of the appropriate quality of radiation. The doses given to the specimens should be traceable via a physical instrument such as an ionization chamber, to a primary or secondary standard.

Physical devices that measure photons are usually calibrated in terms of air kerma, and therefore when considering doses delivered to tissue (or blood specimens) correction factors need to be applied. These are derived from the ratio of mass energy absorption coefficients, and the values to be used may be obtained from standard tables [4]. For neutrons, instruments may be made of tissue equivalent material and thus indicate dose to tissue. Alternatively, some primary or secondary dosimetry laboratories calibrate in terms of neutron fluence, which may be converted to dose to tissue.

As the biological endpoint being scored is chromosomal aberrations, strictly speaking these reflect dose to the cells' nuclei. For photons and neutrons, dose to soft tissue is a very good approximation to the dose to the nucleus. This is because the lymphocyte nucleus diameter is small, ~6  $\mu\text{m}$ , compared with the ranges of secondary particles produced by both photons and neutrons. Thus the Bragg-Gray cavity theory can be applied [5].

There are, however, a few exceptions. For example, with exposure to tritiated water, the distances travelled by the beta particles lie in the range 0–7  $\mu\text{m}$ . Therefore, most of the dose to a cell nucleus is due to emissions from tritium contained within that nucleus. In this case, the dose to the lymphocyte nucleus forms the basis of calibration, and this depends on the water content of the nucleus, with respect to that of blood. Another example could be exposure to low energy neutrons of less than about

100 keV, where the recoil protons have a range of less than 2  $\mu\text{m}$ . In this case the dose to the lymphocyte nucleus would relate to its hydrogen content. It is, however, unlikely that an accident would involve exposure to neutrons predominantly in this energy range.

The dose value obtained by referring a measured yield of aberrations, such as dicentric, to a calibration curve represents an averaged absorbed dose to the lymphocytes. This would approximate to an averaged out whole body dose because lymphocytes are widely distributed around the body and are mobile. By methods to be described later in this Manual, it is sometimes possible to refine the whole body dose estimate for situations where non-uniform or part body irradiations have occurred. For retrospective biological dosimetry, a decade or more after exposure, where translocations are measured by the FISH method, the dose estimate represents average dose to the active bone marrow. This is because the original exposure was to the stem cell precursors of the lymphocytes that are scored. For shorter times the translocations will be observed in a mixture of long lived lymphocytes and descendants of irradiated stem cells.

Often it is the result of a routine measurement of dose recorded by an individual dosimeter that triggers an investigation. Individual dosimeters are normally calibrated to measure the personal dose equivalent at a specified depth. This operational quantity provides a reasonable estimate of effective or equivalent dose in most radiation fields encountered in practice. Effective dose and equivalent dose are the protection quantities in which the dose limits given in the BSS are expressed. The unit is the joule per kilogram, with the specified name of sievert (Sv). Both quantities are intended for use in radiological protection, including the assessment of risks in general terms. They are not suitable for determining the effects of high absorbed doses.

It is therefore recommended that laboratories undertaking biological dosimetry should calibrate their procedures in terms of absorbed dose specifying, where appropriate, the radiation type involved [6–9].

### **3. BIOPHYSICAL BACKGROUND TO CHROMOSOME DAMAGE**

This section provides information that is intended to aid in understanding and interpreting the principles that underlie the methodology presented in the later sections. References [10–12] should be consulted for additional information.

When ionizing radiation passes through an object, it ejects electrons from the atoms through which it travels, leaving positively charged ions. The distribution of primary events, ionizations and excitations along the track of an ionizing particle will vary according to the type of radiation. The average separation of these primary



events decreases with increasing charge and mass of particles (neutrons or alpha particles). As will be discussed below, it is necessary to define a particular radiation in terms of the amount of energy deposited per unit of track length, because this characteristic alters the effectiveness of the particular radiation type in inducing various biological endpoints.

A useful comparative term to describe the deposition of energy by different types of radiation is linear energy transfer (LET). This linear energy transfer of charged particles in a medium is defined by the International Commission on Radiation Units and Measurements (ICRU) as the quotient of  $d\epsilon/dl$ , where  $d\epsilon$  is the average energy locally imparted to the medium by a charged particle of specific energy in traversing a distance of  $dl$  [13]. For radiations with a wide range of LET, e.g. neutrons, an average LET may be derived. This may be obtained by weighting each LET interval according to the energy imparted (or dose) or according to the length of the track travelled. These give, respectively, dose average and track average LET. Track average appears to be the better quantity to describe the RBE variations for chromosomal damage [14]. The track average LET for 250 kVp (kilovolts peak) X rays is about 2 keV/ $\mu\text{m}$ , as compared with heavy charged particles that have track average LET values of 100–2000 keV/ $\mu\text{m}$  or greater. The important point to consider is that various types of radiation can differ considerably in the quantity of energy deposited per micrometre of track, and this can clearly alter the biological effectiveness of different types of radiation.

One consequence of the distribution of ionization for radiation of different linear energy transfers is in the frequency distribution of chromosome aberrations between cells. With low LET, or sparsely ionizing radiation, the ionization at any particular dose will be randomly distributed between cells, particularly since there will be a very large number of tracks. The DNA damage will also be randomly distributed between cells and, on the assumption that there is an equal probability that any damage can potentially be converted into an aberration, therefore, the aberrations will be also randomly distributed between cells. This has been shown to be the case following X or  $\gamma$  irradiation, where the induced chromosome aberrations fit a Poisson distribution. With high LET, or densely ionizing radiation, the ionization tracks will be non-randomly distributed between cells, with the energy being deposited in more ‘discrete packets’. The number of tracks will be much lower than with low LET radiation at equivalent doses. The result, making the same assumptions as for low LET radiation, is that the induced aberrations will be non-randomly distributed between cells. At any observed mean aberration frequency, there will be more cells with multiple aberrations and with zero aberrations than expected from a Poisson distribution. These features can be of use in biological dosimetry, as will be discussed in Section 8.8.4.4, particularly with regard to non-uniform or partial body exposures.

The effectiveness of different types of radiation for inducing a particular biological endpoint is commonly represented by the term ‘relative biological

effectiveness' (RBE). The RBE is defined as the ratio of the dose of the reference radiation (usually, orthovoltage X rays) to the dose of the particular radiation being studied that produces the same biological effect. That is,

$$\text{RBE} = \frac{\text{dose of 200–250 kVp X rays producing effect } Z}{\text{dose of test radiation producing effect } Z}$$

Figure 1 shows the typically shaped linear and linear quadratic dicentric dose response curves obtained with, respectively, high and low LET radiations. The reasons for the shapes are discussed later in this section. The RBE at a high dicentric yield that would be associated with high doses is illustrated by the upper horizontal dashed line which intercepts the two curves at 1.0 and 3.5 Gy. The RBE is the ratio of the two doses which is  $3.5/1.0 = 3.5$ . The lower horizontal dashed line intercepts at 0.1 and 1.0 Gy, resulting in a higher RBE:  $1.0/0.1 = 10$ . The maximum RBE, usually designated as  $\text{RBE}_m$ , would be the ratio of the  $\alpha$  coefficients of the two curves' yield equations.

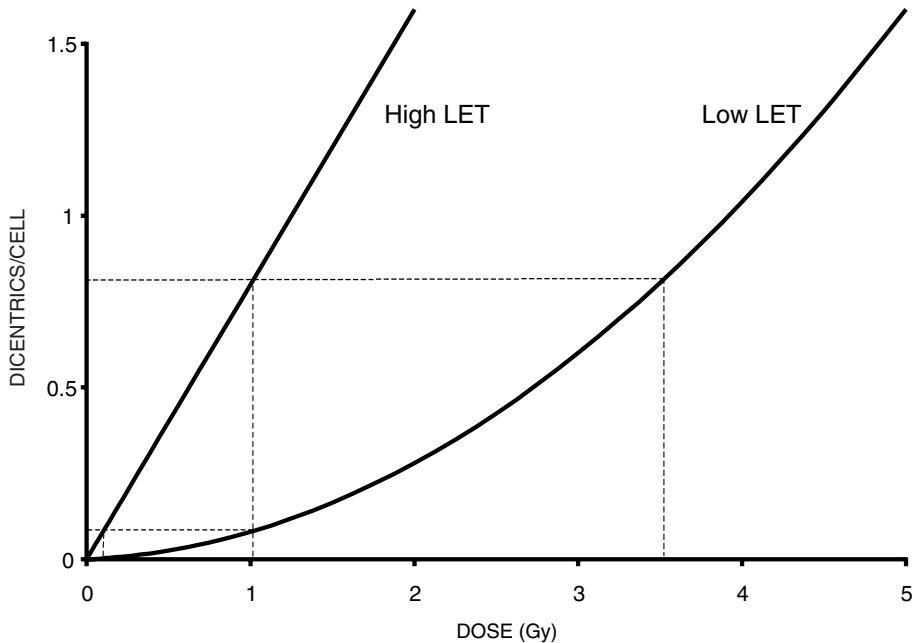


FIG. 1. Typical linear and linear quadratic dose response curves, showing how RBE changes with yield.

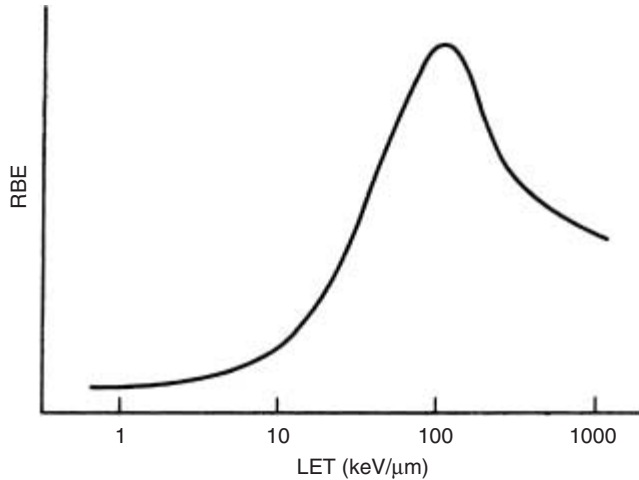


FIG. 2. Generalized relationship between RBE and LET.

It has been shown for many endpoints (including mutations, cell killing and chromosome aberrations) that the RBE varies with LET such that a hump shaped response curve is obtained (Fig. 2). This curve shows that the RBE increases up to an optimum value of about 100 keV/ $\mu\text{m}$  and then decreases at higher values of LET. The interpretation of the curve is best considered here for the induction of chromosome aberrations. For illustrative purposes, the dicentric aberration is used as an example, partly because it clearly involves an interaction (or exchange) between two chromosomes, and also because it is the aberration type that is most frequently used in biological dosimetry.

In order to produce a dicentric aberration, DNA damage must be induced in the two unreplicated chromosomes involved such that the damaged chromosomes can undergo exchange. This exchange can occur either as a result of the misrepair of DNA strand breaks induced directly by the radiation, or as a result of misrepair during the excision repair of base damage. Thus it can be seen that the lesions in the two chromosomes must be close together, within what is called 'rejoining distance', for misrepair to be able to take place. This defined region can be considered as the target. Two lesions, one in the DNA double helix of each unreplicated chromosome, need to be produced within this target. This target, or zone of interaction, is small, generally considered to be less than 1.0  $\mu\text{m}$  diameter. X rays have low LET, with low frequencies of ionization per unit track length. Thus there is a low probability that two ionizing events from a single track will occur within the target. Two ionizations, at a minimum, are necessary to produce damage in the two chromosomes involved in a dicentric. There

is a much higher probability that the two lesions will be produced by ionization from two independent tracks. Dicentric produced by one track will have a frequency that is proportional to a linear function of dose, whereas dicentric induced by two tracks will have a frequency proportional to the square of the dose. At doses below 0.5 Gy, the probability of two tracks traversing a target is sufficiently low that dicentric will be produced almost exclusively by one track and at a low frequency. As the dose increases, the contribution of two track induced dicentric will also increase. Thus the dose–response curve for low LET induced dicentric (Fig. 1) will be a combination of one- and two-track events, with the former being more frequent at low and the latter much more frequent at high doses. The dose–response curve is generally assumed to fit the equation

$$Y = A + \alpha D + \beta D^2$$

where  $Y$  is the yield of dicentric,  $D$  is the dose,  $A$  is the control (background frequency),  $\alpha$  is the linear coefficient and  $\beta$  is the dose squared coefficient. The ratio of  $\alpha/\beta$  of the two coefficients is equal to the dose at which the linear and the quadratic components contribute equally to the formation of dicentric.

As the LET of the radiation increases, there is a greater probability that two lesions within the target will be induced by two ionizing events along the same track, resulting in two consequences. The dose–response curve at linear energy transfers above approximately 20 keV/μm will be linear (Fig. 1). Also, the efficiency, or RBE, of the higher LET radiation for inducing dicentric increases with increasing LET as a result of the increasing probability that the two lesions will be produced by one track. Producing the two required lesions by one track is much more efficient than the random process of producing a lesion by a second track close to a lesion already produced by another track, particularly at lower doses, where the track density is low. The maximum RBE will be at a LET value where ionization is optimally spaced to produce damage in each of the two DNA helices involved in the dicentric without ‘wasting’ energy, that is, depositing more ionization in the target than is needed. However, as LET increases above this optimum value, more energy will be deposited in the target than is necessary, and under these circumstances the RBE will decrease as LET increases, as shown in the plot of RBE versus LET in Fig. 2.

Summarizing this discussion, the dose–response curve (Fig. 1) for low LET radiation, high energy protons and fast neutrons will be non-linear and best fit a linear–quadratic model; the dose–response curve for high LET radiation (fission neutrons and  $\alpha$  particles) will be linear, or close to linear; the RBE increases with increasing LET to a maximum of 100 keV/μm and decreases at higher LET values (Fig. 2).

How does the dose rate of the radiation affect the yield of cytogenetic alterations? For the purpose of this discussion, it is easier to refer to dicentric although

the principles also apply to micronuclei and translocations. It is known that those lesions induced in the DNA that can be converted into dicentrics can be repaired — taking from a few minutes up to several hours, depending on the particular lesion. If the two lesions needed for inducing a dicentric are produced by separate tracks, and the dose rate is reduced, there is a probability that the lesion produced by the first track will be repaired before the target is traversed by a second track, forming the second lesion. Although two lesions have been produced within the target, they cannot interact to produce a dicentric. The probability of the two lesions being able to interact will decrease with decreasing dose rate — the lower the dose rate, the lower the frequency of ionization tracks per unit time and thus the longer the time available for repair of the first lesion before the second can be formed. Thus the situation for low LET radiation is the following: lowering the dose rate decreases the dicentric frequency per unit dose. The dose–response curve for dicentrics at very low dose rates, where the probability of two-track aberrations is essentially zero, will be linear, with a slope equal to that of the linear portion of the linear-quadratic curve for acute exposures. The same argument holds true for fractionated or split doses. If two or more doses are received, lesions from the first can interact with lesions produced by the second, or subsequent, dose, provided that the time interval between the first dose and the subsequent dose fraction is not longer than the time it takes to repair the lesions induced by the first or previous dose. Thus, if doses are separated by times longer than the repair time, the frequency of dicentrics produced by the total dose (the sum of the fractions) will be less than that from the total dose delivered at one time.

The situation is different with regard to high LET radiation, since both lesions involved in the induction of dicentrics are produced by a single track. Thus, lowering the dose rate does not alter the frequency of dicentrics, because repair of the lesions during longer exposures will not be an influencing factor. The same argument applies to fractionated exposures — the repair of lesions between the fractions does not have much influence since both are produced coincidentally by a single track.

The points discussed in this section indicate factors that should be considered in the practice of biological dosimetry. The shape of the dose–response curve is influenced by the radiation quality (LET). Therefore, when attempting to make estimates of dose, the standard curve to be used should be that of a radiation quality which is the same as, or very similar to, that of the particular type of radiation under study. For low LET radiation, decreasing the dose rate also decreases the dicentric frequency per unit dose, such that at very low dose rates the curve is linear and is the same as the linear component of the dose–response curve for acute exposure. A linear curve can be produced from a standard acute curve for X and/or  $\gamma$  rays and could possibly be used as a standard curve for chronic exposures, with appropriate corrections for the duration of the exposure and the lifetimes of

lymphocytes. With high LET radiation, changes in dose rate do not affect dicentric frequency, and so the curve obtained for acute exposures can be used for chronic or fractionated exposures, again taking into consideration the duration of exposure and the lifetimes of peripheral lymphocytes.

#### 4. HUMAN LYMPHOCYTES

Human peripheral lymphocytes represent a cell population which is predominantly in a DNA presynthetic stage of the cell cycle (i.e. the  $G_0$  phase). Only 0.2% or less of the peripheral lymphocytes are in the autosynthetic cell cycle, and these probably come from the pool of large lymphoid cells representing stimulated lymphocytes or immature plasma cells. Cells from this group may give rise to the rare mitoses found occasionally in peripheral blood.

Nowell [15] was the first to show that peripheral ‘human leukocytes’ can be stimulated by phytohaemagglutinin (PHA) to undergo *in vitro* mitoses, while Carstairs [16] showed that ‘small lymphocytes’ are the target cells for mitogenic initiation by PHA.

Peripheral small lymphocytes have dense nuclei which have little cytoplasm surrounding them. They have a diameter of around  $6\ \mu\text{m}$ , and the volume is estimated to be around  $110\ \mu\text{m}^3$ .

Two main types of lymphocytes can be distinguished, i.e. T and B cells. Both types originate from immunologically incompetent stem cells in the yolk sac and eventually settle in the bone marrow. These undifferentiated stem cells migrate into the thymus and other primary lymphoid organs, multiply there and, probably by somatic mutations, give rise to a pool of long lived lymphocytes that circulate. On the basis of their surface markers, T and B cells comprise a mixture of naïve and memory cells with differing life spans and differing roles in the immunological processes [17]. It is the T cells, mostly of the CD4 and CD8 subtypes, that are stimulated *in vitro* by phytohaemagglutinin and are used for biological dosimetry.

Lymphocyte concentrations in the peripheral blood are variable. For example, for a healthy adult the normal range is  $1300\text{--}4800/\text{mm}^3$  [18]. However, in the case of irradiation to high doses of a few Gy to much of the body, one of the early deterministic reactions is a rapid fall in the peripheral blood lymphocyte counts. This should be borne in mind for early blood sampling of radiation casualties for biodosimetry.

The total number of lymphocytes in a healthy young adult has been estimated to be approximately  $500 \times 10^9$ . Only about 2% ( $10 \times 10^9$ ) of these are present in the peripheral blood, the others being located generally throughout other tissues, with particular concentrations in the thymus, lymph nodes, tonsils, the lymphatic tissues of

the intestines, the spleen and in bone marrow. The lifetimes of lymphocytes are variable and the definition of lifespan can mean either that the cell dies or that it divides. T cells of the CD4 and CD8 subtypes can be further divided into subsets based on the expression of different isoforms of the CD45 antigen. At birth, >90% of T cells express CD45RA isoform, and these have been called unprimed or naïve cells. By adulthood, this falls to about 50% by conversion to a CD45RO subset of primed or memory cells.

Chromosomal damage induced by radiotherapy has been studied in PHA stimulated T cells of both RA and RO forms [13]. The persistence of unstable damage has shown that the naïve RA cells divide on average once every 3.5 years, whilst the memory RO cells divide more frequently, on average every 22 weeks. Memory cells may also revert to the naïve phenotype but only, on average, after about 3.5 years in the memory class. The memory cell is eight times more likely to divide than to revert to the naïve class.

Lymphocytes with stable damage decline very slowly, which leads to low estimates of their death rates. The average time to death of a T cell is estimated to be around 20 years, and this value applies to both naïve and memory cells.

For interpreting *in vivo* induced chromosome aberrations in humans, it is of great importance that the bulk of the peripheral lymphocytes belongs to the 'redistributive pool'. That is, the lymphocytes should be able to leave the peripheral blood, pass through the spleen, the lymph nodes and other tissues, and re-enter the circulation. The mean time that a given lymphocyte of the redistributive pool is present in the peripheral blood is about 30 min. It has been estimated that about 80%, that is,  $400 \times 10^9$  lymphocytes, belong to the redistributive pool and that the overall recirculation time is about 12 h. This means that lymphocytes with chromosome aberrations that have been induced anywhere in the body will eventually be present in the peripheral blood. Thus, with the human lymphocyte test system, not only can chromosome aberrations that have been induced in lymphocytes in the peripheral blood itself be detected, but also those that have been induced in lymphocytes distributed in different organs throughout the body [20].

Most of the peripheral lymphocytes are in a 'resting' stage of the cell cycle ( $G_0$ ) and have a diploid DNA content of about 5.6 pg. These cells can be initiated to undergo *in vitro* mitotic divisions by the introduction of PHA, a protein derived from the bean plant *Phaseolus vulgaris* [15]. PHA is an extremely comprehensive mitogen that stimulates a broad spectrum of T cells. Under the influence of PHA, the lymphocytes are transformed into blastoid cells, and the volumes of the nucleus and of the whole cells increase. Peripheral lymphocytes 48 h after stimulation have a cell volume of about  $500 \mu\text{m}^3$ , as compared with  $\sim 110 \mu\text{m}^3$  before stimulation. The cytoplasmic volumes are  $\sim 50 \mu\text{m}^3$  before and  $\sim 350 \mu\text{m}^3$  after stimulation. Nuclear volume increases from about  $\sim 50 \mu\text{m}^3$  to  $170 \mu\text{m}^3$  following stimulation. During this 48 h period, the amount of heterochromatin decreases from 70 to 13%.

The cell cycle progression of lymphocytes following stimulation with PHA can be quite different depending on the culture conditions using different culture media such as Ham's F-10, RPMI (Roswell Park Memorial Institute, RPMI-1640), medium TC-199, or minimum essential medium (MEM). For example, in Ham's F-10 medium, the DNA synthesis starts about 26 h after culture initiation and the first mitoses are found after about 10 h. There are two peaks of DNA synthesis measured by tritiated thymidine treatment, one at 34 h and a second at 40 h, and two peaks of mitotic activity, one at around 44 h and a second at around 49 h. This may represent two subpopulations of cells which show different stimulation patterns in a culture set up with Ham's F-10 and PHA [21]. However, in lymphocytes grown in TC-199 medium, the tritium labelled interphases, as well as the mitotic indices, follow an irregular pattern, thus making it difficult to draw any conclusions about the subpopulations.

## 5. CHROMOSOMAL STRUCTURE

### 5.1. CHROMATIN PACKING

The association of DNA and histones in a nucleosome structure has been demonstrated in considerable detail, although the association of the non-histone proteins with the nucleosome assembly is not yet fully understood. In addition, it is clear that DNA is external to the histone core of the nucleosome. Some studies support the existence of an axial core structure formed by non-histone proteins or a non-histone protein scaffold [22, 23] in a metaphase chromosome. The involvement of such core structures in the formation of chromosome aberrations has not yet been elucidated. Core structures can also be demonstrated in a light microscope as silver stainable regions in the chromosome of different mitotic stages. Although the existence of an organized nuclear protein matrix in interphase is well documented, the existence of a scaffold in metaphase chromosomes is probably an artifact. A simple model of the organization of a metaphase chromosome is shown in Fig. 3.

### 5.2. HUMAN KARYOTYPE

The human male karyotype (using G banding) is shown in Fig. 4. These are normally combined into five groups of autosomes (A to E) and the pair of sex chromosomes. The chromosomal groups are: A: 1–3, B: 4 and 5, C: 6–12, D: 13–15, E:16–18, F: 19 and 20, G: 21 and 22.



### 5.3. DNA CONTENTS OF CHROMOSOMES

The relative DNA contents of the human chromosomes for either gender are shown in Tables I and II. These data have been calculated from Morton 1991 [24].

### 5.4. CELL CYCLE

Important information on the clastogenic effects of physical or chemical agents on interphase cells can be obtained by examining the chromosomes at the point of ensuing cell division which for somatic cells is a mitosis. The somatic cells of interest to biological dosimetry are the peripheral lymphocytes and haemopoietic stem

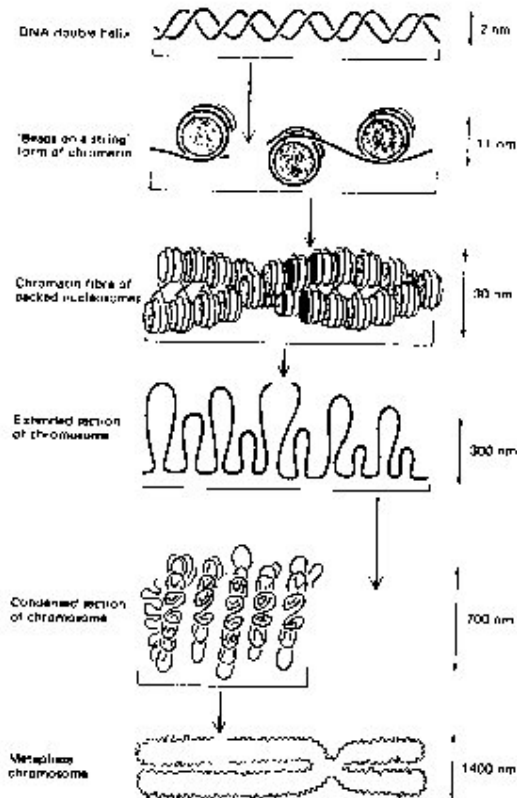


FIG. 3. Schematic illustration of the many different orders of chromatin packing postulated to give rise to the highly condensed metaphase chromosome.

cells. These processes have stages which can be distinguished by their appearance and function. During mitosis, stages such as prophase, metaphase, anaphase and telophase are recognized. During the interphase, the chromosome material (that is, DNA and associated proteins) duplicates. This is called the 'S' (synthetic) period and is preceded by a period called G1 (a presynthetic gap) and is followed by G2 (a postsynthetic gap) within the interphase. In cells which are not cycling, for example peripheral lymphocytes, the cell remains in G<sub>0</sub>. Figure 5 shows the stages of the cell cycle and the approximate timings for a PHA stimulated human lymphocyte.

For cycling cells, interphase is metabolically the most active part of the cell cycle, and most of the energy requiring reactions in the nucleus take place at this stage. The duration of each stage in the cell varies with the type of cell and the conditions of growth. One can determine the length of the stages by using radiolabelled DNA precursors such as tritiated thymidine. In lymphocytes, the first cell cycle following stimulation is nearly synchronized, and these cells are especially convenient for radiobiological studies. Cycling mammalian cells in cultures are, of course, not synchronized, but can be brought to synchrony by several techniques. Different stages in the cell cycle vary in their sensitivity to the action of chemicals or radiation, and the types of chromosomal aberration produced vary depending on the cell stage that was treated [25]. Thus, it is important in such studies to work with a synchronized population, or at least to have an estimate of the proportions of cells at the different stages that are present at the time of treatment.

## 6. RADIATION INDUCED CHROMOSOMAL ALTERATIONS

### 6.1. INTRODUCTION

The first reported evidence that X rays could induce chromosomal aberrations came from the genetic studies by Müller [26] of *Drosophila*. This was confirmed by the cytological studies of Painter and Müller [27]. Sax [28] later developed his 'breakage first' hypothesis on the origin of X ray induced chromosome aberrations, followed by Revell [29] who proposed the alternative exchange hypothesis. In essence, Sax [28] proposed that damaged regions of separate chromosomes come into contact after complete breaks have been induced and the ends move about and eventually combine to form exchanges. Alternatively, Revell [29] envisaged that the points of damage are not complete severances but are unstable sites which can interact with similar sites to form pairwise exchanges. There is a third possibility, introduced later by Chadwick and Leenhouts [30], of a lesion/non-lesion interaction whereby a



FIG. 4. The normal human male karyotype.

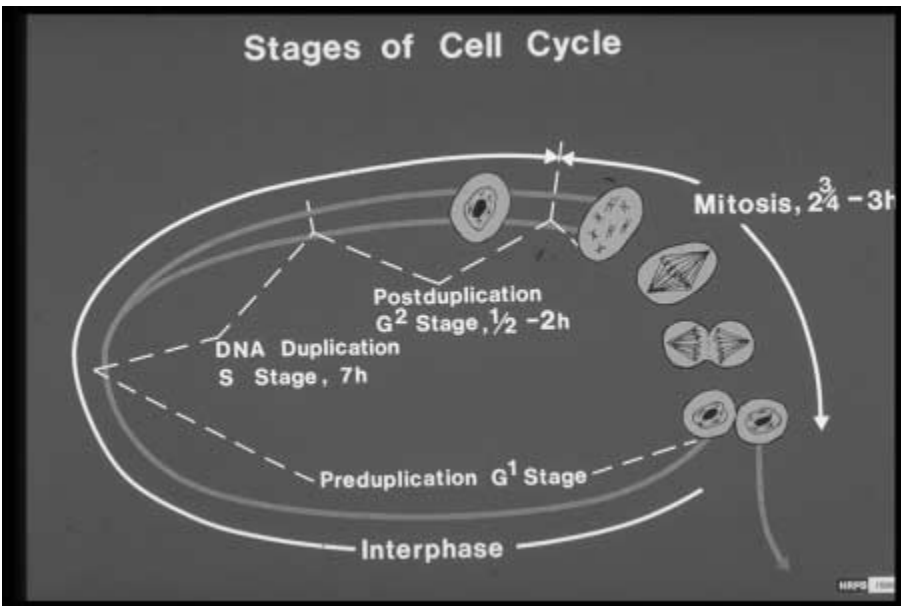


FIG. 5. The cell cycle.

TABLE I. PER CENT DNA CONTENT OF THE HUMAN MALE GENOME OCCUPIED BY EACH PAIR OF AUTOSOMES AND EACH SEX CHROMOSOME

Chromosome No.	p arm	q arm	Both arms	Chromosome No.	p arm	q arm	Both arms
1	4.03	4.25	8.28	13	0.50	3.09	3.59
2	3.12	4.92	8.04	14	0.50	2.93	3.43
3	3.12	3.62	6.74	15	0.54	2.80	3.34
4	1.76	4.63	6.39	16	1.23	1.86	3.09
5	1.64	4.47	6.11	17	0.88	2.02	2.90
6	2.05	3.72	5.77	18	0.63	2.05	2.68
7	2.05	3.34	5.39	19	0.94	1.17	2.11
8	1.57	3.31	4.88	20	0.98	1.29	2.27
9	1.61	2.96	4.57	21	0.35	1.23	1.58
10	1.38	3.15	4.53	22	0.41	1.35	1.76
11	1.83	2.71	4.54	X	0.97	1.61	2.58
12	1.23	3.27	4.50	Y	0.20	0.73	0.93
						Total	100

TABLE II. PER CENT DNA CONTENT OF THE HUMAN FEMALE GENOME OCCUPIED BY EACH PAIR OF CHROMOSOMES

Chromosome No.	p arm	q arm	Both arms	Chromosome No.	p arm	q arm	Both arms
1	3.97	4.18	8.15	13	0.49	3.04	3.53
2	3.07	4.83	7.90	14	0.50	2.88	3.38
3	3.07	3.56	6.63	15	0.53	2.76	3.29
4	1.74	4.55	6.29	16	1.21	1.83	3.04
5	1.61	4.40	6.01	17	0.87	1.98	2.85
6	2.02	3.66	5.68	18	0.62	2.01	2.63
7	2.01	3.29	5.30	19	0.93	1.15	2.08
8	1.55	3.25	4.80	20	0.96	1.27	2.23
9	1.58	2.91	4.49	21	0.34	1.21	1.55
10	1.36	3.10	4.46	22	0.40	1.34	1.74
11	1.80	2.66	4.46	X	1.92	3.16	<u>5.08</u>
12	1.21	3.22	4.43			Total	100

damaged site, in the Revell sense, may interact with an undamaged chromosome to form an exchange.

## 6.2. RADIATION INDUCED DNA LESIONS

Many different primary lesions are induced in cellular DNA by physical and chemical mutagens. Those attributable to ionizing radiation are single and double strand breaks (SSBs and DSBs), base damage and DNA protein cross-links as illustrated schematically in Fig. 6.

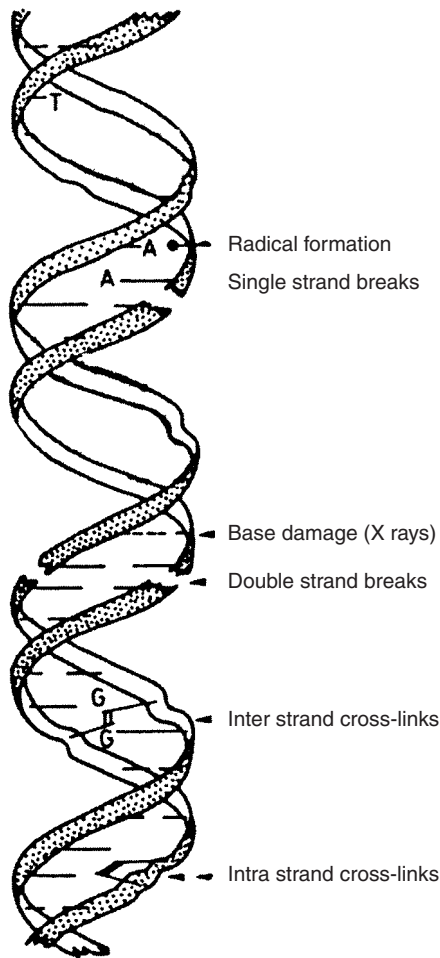


FIG. 6. Radiation induced DNA lesions.

DNA damage can be repaired to give an apparently normal chromosome. Alternatively, it can be misrepaired to form an exchange or remain unrepaired. The DSB is the lesion responsible for most visible chromosomal aberrations observed at metaphase after irradiation of normal cells [31, 32].

### 6.3. CHROMOSOME TYPE ABERRATIONS

Schemes for the classification of chromosomal aberrations have been presented [33, 34]. For the purposes of this Manual, only those chromosome type aberrations observed in metaphase after irradiation of cells in  $G_0$  or  $G_1$  are described.

The peripheral lymphocyte population that is mitogenically stimulated is normally non-cycling and resides in the  $G_0$  (non-cycling  $G_1$ ) stage of the cell cycle. The chromosome aberrations induced by radiation will consequently be of the chromosome type, i.e. they involve both chromatids of a chromosome. It is well known that ionizing radiation is an S independent clastogen, unlike UV radiation and chemical mutagens, which are S dependent agents. Therefore, with ionizing radiation, chromosome and chromatid type aberrations are induced following treatment of  $G_0/G_1$  and  $G_2/S$  cells, respectively. However, UV and chemicals induce only chromatid type aberrations at all stages of the cell cycle. If chromatid type aberrations are observed in  $G_0/G_1$  cells that have been exposed to ionizing radiation, it can be assumed that these are either not radiation induced or have already passed through a second in vitro cell cycle.

#### 6.3.1. Unstable aberrations

##### *Dicentrics*

The dicentric (Fig. 7) is the main aberration used for biodosimetry. It is an exchange between the centromeric pieces of two broken chromosomes which in its complete form is accompanied by a fragment composed of the acentric pieces of these chromosomes. Particularly after high doses, multicentric configurations can be formed. Tricentrics are accompanied by two fragments, quadricentrics by three fragments, etc. The dicentric assay is covered in detail in Section 8.

##### *Centric rings*

In human lymphocytes, centric rings are much rarer than the dicentrics. Some researchers combine them with dicentrics while others choose to ignore them for dose estimation. The ring chromosome is an exchange between two breaks on separate arms of the same chromosome and is also accompanied by an acentric fragment (Fig. 7).



*FIG. 7. Two dicentric chromosomes and one centric ring with their associated fragments (Giemsa staining).*

### *Acentrics*

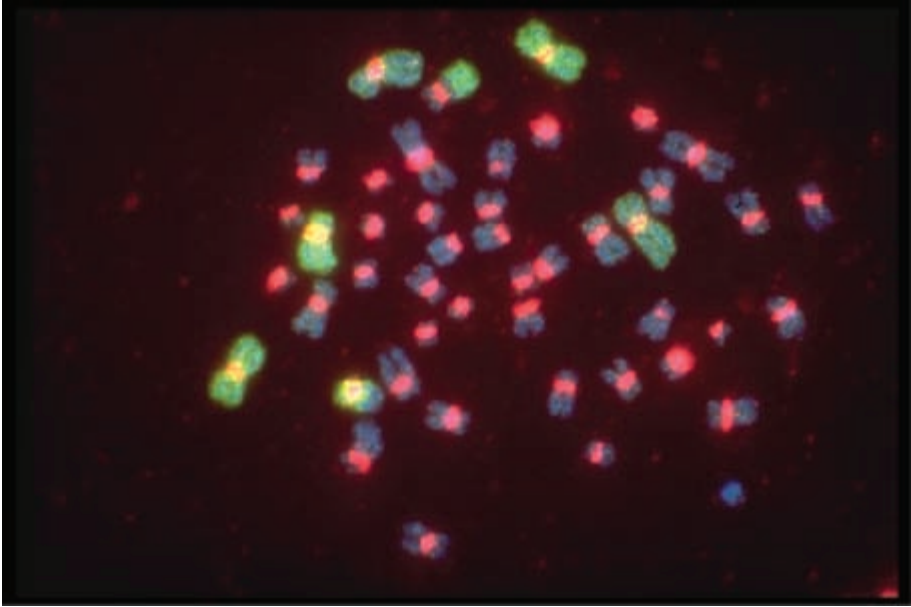
Acentric aberrations can be formed independently of the exchanges described above and as such are usually referred to as excess acentrics. They can be terminal or interstitial deletions of varying sizes but it is not always possible to determine their origin and so they are combined. Minutes which appear as double dots and acentric rings where clear spaces may be seen within the dots are normally considered to be interstitial deletions; this has been confirmed by telomere probing [35].

### **6.3.2. Stable aberrations**

#### *Reciprocal translocations*

The reciprocal (or complete or two-way) translocation is the exchange of terminal portions of two separate chromosomes. The various types of translocation were originally described by using the G banding technique and karyotyping, but this



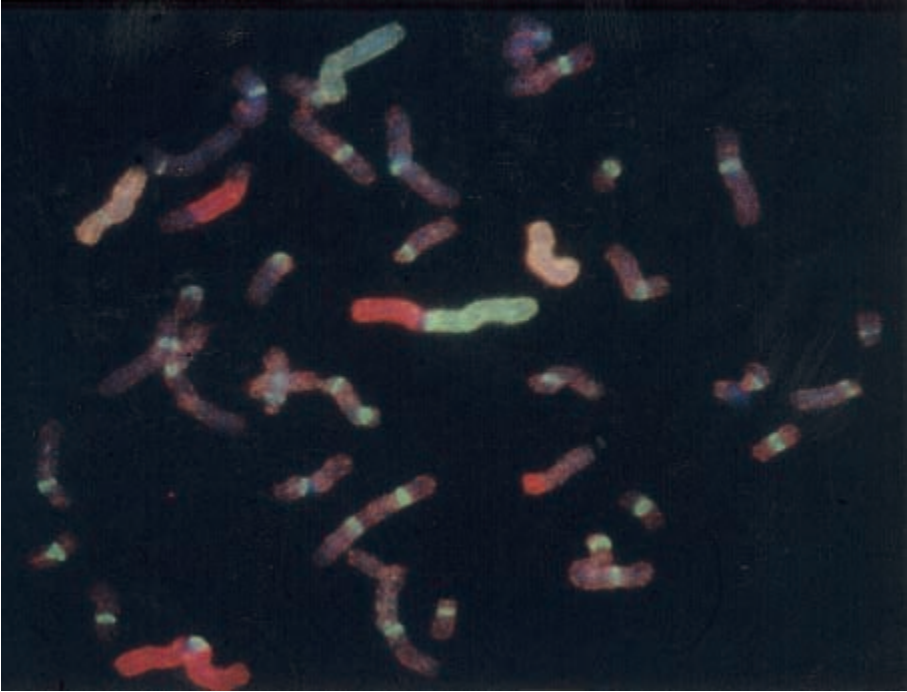


*FIG. 8. Human metaphase with chromosomes #2, 3 and 5 single coloured, painted with FITC, centromeres painted with Texas red and counterstaining with DAPI. Note the bicoloured reciprocal translocation  $-t(Ab); t(Ba)$  –involving one of the #5 chromosomes and a counter-stained chromosome.*

procedure is too laborious for routine biodosimetry. With solid Giemsa staining, translocations are not observed reliably. Their application to dosimetry is now possible with the FISH method (fluorescence in situ hybridization); see Section 9. By the FISH methods these are visualized as bicoloured monocentric chromosomes (Fig. 8).

#### *Non-reciprocal translocations*

When only one bicoloured chromosome can be seen, this has often been called a terminal, or incomplete, or one-way translocation. However, using a combination of chromosome painting, centromeres and telomere probes, a number of translocations designated as terminal or incomplete was found to be in reality reciprocal. It is very likely that the signal of the missing counterpart is below the limit of visual resolution, and it has therefore been suggested to designate such patterns as one-way exchanges or translocations. The current view is that true terminal translocations do exist but they form a small percentage of the total, e.g. at 4 Gy they are about 5% [35].



*FIG. 9. Human metaphase with triple coloured painted chromosomes #1 (red), #4 (green) and #8 (yellow), centromeres painted with FITC and counterstaining with DAPI. A bicoloured dicentric involving chromosomes #1 and #4, insertion of part of chromosome #1, and a one-way translocation involving chromosome #1 and a counterstained chromosome are presented.*

#### *Interstitial translocations (insertions)*

This is a bicoloured chromosome where an acentric piece of one chromosome has been inserted within an arm of another chromosome (Fig. 9). Detailed information on translocation analysis is given in Section 9.

#### 6.4. PREMATURE CHROMOSOME CONDENSATION (PCC)

When cycling cells enter mitosis, the chromatin condenses into the familiar shaped chromosomes. Techniques have been developed to cause chromatin also to condense when it is not in mitosis and this is termed premature chromosome condensation (PCC). Premature condensation can be induced by fusing interphase cells



*FIG. 10. PCC of human  $G_0$  lymphocytes exposed to 5 Gy X rays, fused with CHO mitotic cells and detected by FPG staining.*

such as human  $G_0$  blood lymphocytes to mitotic Chinese hamster ovary (CHO) or HeLa cells using Sendai virus or polyethylene glycol as fusing agent [36, 37].

Chemical methods of inducing PCC, using inhibitors of DNA phosphorylation such as okadaic acid or calyculin A, have also been developed. Most of these methods require the cells to be cycling in culture [38, 39].

Figure 10 shows an example of PCC produced by the CHO mitotic fusion method. Detailed information on PCC analysis is given in Section 10.

The PCC technique enables direct observation of radiation induced chromosome damage in non-stimulated interphase human peripheral blood lymphocytes [40, 41]. The PCC method is proving to be a very useful research tool to probe the immediate post-irradiation processes and kinetics of chromosomal break restitution and/or misrepair to form aberrations (i.e. dicentric and translocations) [40, 42, 43]. These studies demonstrate that the dicentric, complete and incomplete translocations and acentric fragments that one sees eventually at metaphase are formed in  $G_0$  at differing times that are dependent on the dose. In human lymphocytes, at low doses of X rays (1–2 Gy), both dicentric and translocations are formed rapidly. However, at higher doses of 4 and 6 Gy, the frequencies of chromosome exchanges increase proportionally to the restitution of chromosome breaks (repair).

Chemically induced PCC in resting or non-cycling human peripheral blood lymphocytes permits the rapid identification of interphase cells with radiation induced chromosomal alterations. Inhibitors of DNA phosphorylation, such as okadaic acid or calyculin A, have recently been used on proliferating tumour cell lines, and when combined with whole-chromosome specific hybridization probes permit the identification of discrete chromosome domains or spots in interphase cells [44]. Radiation causes a dose dependent increase in interphase tumour cells with more than two spots. Recently, specific inhibitors of protein phosphatase (e.g. okadaic acid) were used in combination with a protein kinase (e.g. p34<sup>cdc2</sup>/cyclin B kinase) to induce PCC in resting lymphocytes [45].

## 6.5. MICRONUCLEI (MN)

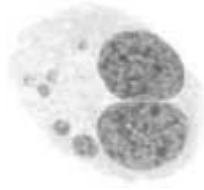
Micronuclei are formed from chromosomal fragments or lagging chromosomes at an anaphase (due to mitotic spindle damage) which are not included in the nuclei of the daughter cells. They are therefore seen as distinctly separate objects within the cytoplasm of the daughter cells [46].

In the mid-1980s a major technical innovation was introduced. This was the method for blocking cytokinesis in cultured lymphocytes by adding cytochalasin B to the medium without inhibiting nuclear division. The so-called cytokinesis blocked (CB) cells [47, 48] thus produce binucleate cells rather than permitting the two daughter cells to separate. With this protocol it is possible to distinguish between proliferating (following the first mitosis) and non-proliferating cells, and MN should be scored only in binucleate cells with preserved cytoplasm (Fig. 11). Detailed information on MN analysis will be given in Section 11.

# 7. BLOOD SAMPLING

## 7.1. TIMING

A venipuncture blood sample, ideally 10 mL, should be taken as soon as possible after radiation exposure. In the case of a partial-body or non-uniform exposure the lymphocytes in the circulating and extravascular pools will not have reached equilibrium until about 24 h [49]. This could result in an unrepresentative proportion of irradiated cells in the specimen. An effort should be made to ensure that the sample is obtained before about four weeks have elapsed, since after this time aberration yields begin to fall, causing greater uncertainty in any estimates of the radiation dose [50].



*FIG. 11. Irradiated CB binucleate human lymphocyte with six MN.*

In the event of a serious overexposure, where there is the likelihood of severe depletion of the white cell count, a 'time window' of possibly only a few hours or days exists before the lymphocyte count drops to a level where insufficient cells can be obtained for cytogenetic analysis. In such cases, medical treatment may include whole-blood or blood fraction transfusions, and it is important to obtain a specimen of the patient's blood before this treatment commences. For purposes of scientific interest, the laboratory should endeavour to obtain a sequence of blood samples at frequent intervals. This should be ethically acceptable as such samples would be taken to monitor changes in the differential white cell count.

It may not always be possible to culture cells promptly if, for example, sampling occurs in a remote region with poor communications. Blood samples may be kept refrigerated but loss of lymphocyte viability soon becomes a major problem [51]. The problem is overcome by stimulating the lymphocytes with phytohaemagglutinin (PHA) immediately after venipuncture and keeping them cold (below 20°C) so that the lymphocytes do not transform and progress through the cell cycle until the cells are warmed up to 37°C. The following method devised by M.S. Sasaki (personal communication) has enabled cells to be cultured up to two weeks later:

- (1) Prepare in advance 10 mL sterile tubes containing 5 mL of Leibovitz's L-15 medium with 20% fetal bovine serum and 4% dehydrated PHA.
- (2) Take a blood sample into a conventional heparinized tube (see Section 7.3).
- (3) Put 5 mL of heparinized blood into the tube with L-15 medium and mix.
- (4) Keep the tubes cool (<20°C); in this condition they may be stored or despatched to the laboratory without a significant reduction in viability.
- (5) Their processing upon arrival is described in the following Section 8.1.

## 7.2. ANTICOAGULANT

Preservative free lithium heparin is the best anticoagulant for lymphocyte cultures. It is even superior to sodium heparin. Other common anticoagulants, e.g.

ethylenediaminetetraacetic acid (EDTA), often result in poor cell growth and should not be used. A high mitotic index is needed for biological dosimetry, which is particularly the case for the FISH method.

### 7.3. CONTAINERS

Disposable glass or plastic specimen tubes containing the correct amounts of lithium heparin are available from several manufacturers. Ideally, these should be sterile, and most manufacturers will, as an optional extra, arrange to have a batch gamma sterilized. Evacuated type tubes are also suitable. Tubes containing glass or plastic beads or gels should be avoided. If dried heparin is used, it is important that the blood be properly mixed by inverting the tube several times. It is preferable if the cytogenetics laboratory can supply the specimen tube from its own stock. This, incidentally, provides an opportunity to include a detailed set of instructions for the doctor and a correctly addressed package for the return of the sample.

Tubes containing dry anticoagulant have a very long shelf-life. Therefore, it is worth considering having stocks of tubes and instructions already available with suitable people, such as medical officers at nuclear establishments and specialists to whom irradiated casualties are likely to be sent. This would reduce delays while empty tubes are sent out from the laboratory.

### 7.4. TRANSPORT

There is no need for the patient to travel to the laboratory. Blood specimens should be transported with sufficient packaging to prevent breakage. The optimum temperature is in the range from 18 to 24°C, and if excessive temperatures (>38°C) are likely to be experienced, such as in tropical regions, the provision of coolant packs is advisable. A fast postal service is often satisfactory, and transit times of two or three days can be tolerated; however, blood samples need special provision if long delays are expected, such as around national holidays.

For international transit, specimens may be sent by air cargo. The laboratory should be advised of the flight number and airway bill number, the latter enabling the specimen to be traced through cargo handling and customs. It is often convenient to use an international courier company that provides a rapid 'door to door' service and deals with all customs paperwork, etc. During air transport the blood should not be X rayed in security checks. If this is likely, a piece of X ray film could be included in the package.

The International Air Transport Association, many airlines and courier companies stipulate packaging for blood samples that conforms to United Nations

Regulations for transporting dangerous goods class 6.2; infectious substances. In brief, standard glass or plastic lithium heparin tubes can be used. They should be placed in a rigid, crushproof and watertight secondary container. This container should also contain cushioning material and sufficient absorbent material to be able to absorb the entire contents, but it must not contain cooling packs. The secondary container should then be placed in rigid outer packaging, e.g. a sturdy cardboard box, with suitable labelling. The labelling should include the name and telephone number of a responsible person and have a Regulation Class 6.2 Hazard Label. If it is felt that cooling packs are needed they should be outside the secondary container, and the outer packaging should be of thermal insulation material such as an expanded polystyrene box. Packaging materials that conform to the regulations are commercially available.

## **8. DICENTRIC ANALYSIS**

### **8.1. CULTURING**

On receipt of a blood specimen several replicate cultures should be set up.

#### **8.1.1. Choice of culture medium**

There are several defined culture media which may be employed. All are commercially available and have been shown to be suitable for lymphocyte culture. Media formulated without folic acid, in order to detect inherited fragile sites on chromosomes, should not be used. Certain media (F-10 and RPMI-1640) appear to encourage faster growth than, for example, MEM and TC-199 [52]. Although the numbers of second in vitro metaphase (M2) cells can be determined by fluorescence plus Giemsa (FPG) staining, it is a good policy to use routinely a culture procedure which generally gives a minimal number of M2 cells at 48 h.

Medium should be supplemented with L-glutamine (200 mM), heparin (13 U/mL) and antibiotics. Penicillin (100 IU/mL) and streptomycin (100 µg/mL) are commonly used. Depending on the manufacturer, many media already containing these antibiotics are supplied. However, antibiotics may need to be added when diluting the medium to working strength, especially if concentrated or powdered media are purchased. Some laboratories prefer to use media without antibiotics, in which case aseptic working procedures, including the use of gamma irradiated blood specimen tubes, are essential.

### **8.1.2. Choice of serum**

Foetal calf or human AB serum should be used. As there may be considerable variations between batches of sera, new consignments should be quality tested for their ability to support cell growth. The serum should be heat inactivated at 56°C for 0.5–1 h in a water bath as this helps to reduce batch variability. It is possible also to grow lymphocytes in serum free medium, and such media are commercially available.

### **8.1.3. Bromodeoxyuridine**

Bromodeoxyuridine (BrdU) should be included in the cultures in order to permit fluorescence plus Giemsa (FPG) staining [53]. This thymidine analogue is taken up preferentially into replicating DNA. When one chromatid is bifilarly and the other one unifilarly substituted, FPG staining produces a 'harlequin' effect in the metaphase chromosome of cells which are in their second or later post-substitution division. There is no universally established concentration of BrdU that can be used. The optimum will vary depending on such factors as the thymidine concentration in the particular culture medium employed. A laboratory should experiment for itself to determine a satisfactory level. It is customary to add the BrdU to the culture medium at a concentration such that the concentration in the final culture mixture does not exceed about 50  $\mu\text{M}$  (15.4  $\mu\text{g}/\text{mL}$ ). Above this level there is the possibility of BrdU causing excessive mitotic delay [54]. With fresh (<24 h) blood specimens, a final culture concentration of about 15  $\mu\text{M}$  is often satisfactory. If blood specimens are delayed in transit so that they are more than 24 h old, the BrdU concentration may have to be increased to, say, 40  $\mu\text{M}$  in order to achieve reliable FPG staining [55]. It should be noted that BrdU is light sensitive, and therefore the cultures should be prepared in subdued lighting (e.g. a yellow safe light) and then incubated in the dark. It can be helpful to wrap the culture vessels in aluminium foil.

### **8.1.4. Mitogens**

Several mitogens, mostly plant lectins, are commercially available. In most cases the particular populations of lymphocytes which they stimulate have not been precisely defined. It is recommended that phytohaemagglutinin (PHA), which is the most widely used mitogen, should be employed. Several manufacturers market two versions of PHA, often called types M and P or HA15 (9 mg/mL) and HA16 (1 mg/mL), respectively. The more expensive and highly purified material (HA16) is not necessary for routine whole-blood cultures; it is, however, advisable to use it for culturing isolated lymphocytes.

There are other mitogens available, e.g. Concanavalin A or pokeweed mitogen, which stimulate particular subsets of lymphocytes. These have applications in certain



experimental systems and with non-human cells. None are as broadly acting as PHA, and for biological dosimetry they should not be used.

### 8.1.5. The cultures

Autoclavable glass or sterile, disposable plastic containers that can be sealed should be used. The size of the container is important; for a 5 mL culture the head space should be at least 10 mL. The culture should be kept in an incubator at 37°C with 5% CO<sub>2</sub>.

Cells should be incubated at 37 ± 0.5°C. The thermostability of the incubator is important, and it is advisable to monitor its performance with, for example, a thermocouple and a chart recorder. Too low a temperature will result in a poor yield, if any, of metaphases after 48 h. If the temperature is high (38°C, or above), cells will progress more quickly through the cycle so that unacceptably high numbers of second-division metaphases may be present by 48 h [56].

The culturing methods are based, with modifications, on the techniques originally published by Moorhead et al. [57] and Hungerford [58]. In brief, one may set up cultures with whole blood or with separated lymphocytes. The advantages and disadvantages of the techniques concern the volumes of blood sample supplied, the time taken in setting up a culture and the number of scorable metaphases (higher mitotic index) which result.

#### 8.1.5.1. Whole blood

This method can be used with smaller blood samples (1–2 mL) and, if necessary, can be performed with blood collected from a finger prick. A further advantage is the speed and ease with which cultures can be set up. However, the number of resultant metaphases per microscope slide is generally smaller than with the other methods. The procedure is to add 0.3 mL of whole blood and 0.1 mL of PHA working solution to a vessel containing 4 mL of medium and 1 mL of serum and then to incubate in the dark.

#### 8.1.5.2. Separated lymphocytes

In this method, an enriched inoculum of lymphocytes is added to the medium. It is suitable for cases where a blood sample greater than 4 mL is available. There are two techniques for producing enriched inocula:

- (a) Firstly, 0.15 mL of PHA is added to 2 mL of blood, and the mixture is then gently agitated. Blood will agglutinate on the walls of the vessel. Then, 2 mL of serum is added, gently mixed and centrifuged for one minute at 50 g. The

supernatant of about 3 mL, comprising serum, plasma and buffy coat, is removed with a syringe, leaving behind most of the agglutinated red cells. It helps to disturb the buffy coat with the tip of the needle while drawing up the supernatant. Use a widebore needle to minimize sheering stress on the cells. The 3 mL of fluid is sufficient to make two cultures and is divided equally into two vessels, each containing 4 mL of medium.

- (b) Secondly, lymphocytes may be separated from whole blood by layering onto a sterile Ficoll Hypaque column. Ready to use tubes for such lymphocyte separation are commercially available. The tubes are centrifuged and the lymphocyte rich layer is removed. This is washed in phosphate buffered saline and, if necessary, an optimum number of cells ( $2 \times 10^6/\text{mL}$ ) is placed in culture. The concentration of viable cells can be established by dye exclusion of a small aliquot counted in a haemocytometer chamber. A detailed protocol for this has been given by McFee et al. (1997) [59], who point out that the method is particularly suitable for producing clean preparations with a lot of metaphases. This is especially useful for FISH analysis. It is probably unnecessarily complicated for conventional Giemsa staining, where method (a) above, or whole-blood cultures, are sufficient.

#### **8.1.6. Mitotic arrest**

Colchicine or demecolcine (Colcemid) can be used, with Colcemid being the arresting agent preferred by most workers. A suitable stock solution will contain 25  $\mu\text{g}/\text{mL}$  of Colcemid in physiological saline and, if prepared aseptically and stored at 4°C, will keep for six months. Adding 0.1 mL of this solution to each culture of 5.0 mL should provide a sufficient number of metaphases while avoiding problems of cell toxicity which occur with higher concentrations. Colcemid is usually added 2 or 3 h before terminating the cultures. A few researchers [60] prefer to add the Colcemid midway through the culture period, i.e. after about 24 h. This should prevent cells from progressing beyond the first metaphase and is thus an alternative means of avoiding the analysis of M2 cells. It should be noted that early addition of Colcemid could produce excessive contraction of the chromosomes, making analysis more difficult particularly for FISH.

### **8.2. FIXATION PROCEDURE**

Lymphocyte cultures are conventionally incubated for 48 h, although the exact time may vary between laboratories from 46 to 52 h. Laboratories should establish the optimum time that normally produces good yields of M1 metaphases with their routine procedure. It is also advisable to fix only some of the replicate cultures at the

routine time, leaving the remainder in the incubator. This allows for the possibility of cells from some donors taking longer to reach metaphase, and also offers the opportunity for scoring later cells if a high dose may have caused mitotic delay.

On terminating the cultures it is no longer necessary to observe aseptic procedures, and further processing may be carried out at room temperature. The cultures should be centrifuged and the supernatant removed and replaced by a hypotonic solution (5 mL) of 0.075 M potassium chloride. If the supernatant is to be removed by suction, the centrifuge speed should be 200 g for 10 min. If, however, the supernatant is to be tipped off, a firmer pellet is required (600 g for 3 min), though this can lead to more broken cells. The tubes should be left to stand for 15–20 min at 37°C; when isolated lymphocytes are used, 3–5 min are enough. The tubes should then be spun again, the hypotonic solution removed and the cell pellet resuspended in 5–10 mL of freshly prepared fixative (3:1 methanol/acetic acid). The fixative should be added slowly, but at a constant rate, while the tube is agitated with, for example, a vortex mixer. This is important since it ensures that the cells are dispersed into a uniform suspension. The cells should then be spun down again and resuspended in three changes of fixative. The cells may, if required, be stored long term in fixative, ideally in a –20°C freezer. Alternatively, slides can be prepared either immediately or the next day, and for short term storage the cell suspension can be kept at 4°C.

The final wash of fixative should be removed, leaving a sufficient quantity of it (0.25 mL) to give a suitable volume of suspension for dispensing onto slides. However, the final volume depends on the cell density and can be diluted with more fixative solution if found necessary. Clean and grease free slides should be used. While some manufacturers claim that the slides that they supply are sufficiently clean, many laboratories prefer to make doubly certain and store the slides in a degreasing fluid. This can be a 1:1 mixture of acetone and methanol or a 1:1 mixture of ether and ethanol, or 1% concentrated hydrochloric acid in methanol. When needed the slides can be dried and polished with clean tissue paper. One should note that better quality paper handkerchiefs are not suitable because they have lanoline added to make them soft. Separation of the chromosomes is improved if the slides are cold and wet. This can be achieved by storing the slides in a freezer, taking them out just prior to use and melting the frost with one's breath a few moments before dispensing the cells. Alternatively, the slides can be dipped for a few seconds into a beaker of distilled water and ice cubes. Improved wetting of the slides is obtained if some methanol is poured on top of the iced water, but not stirred in. Surface liquid should be shaken from the slide a moment before the cells are dispensed.

The cells should be thoroughly suspended in the remaining fixative by bubbling with a Pasteur pipette and then drawn up into the pipette, with two or three drops being allowed to fall from a height of at least 10 cm onto the slide. The cells from one culture should be dispensed onto at least two slides and many workers prefer to produce up to ten slides from a culture. Before dispensing all the cells from a culture onto

slides, it is a good policy to place one drop of the suspension on a test slide. This enables the concentration of metaphases to be judged, and, if necessary, the remaining suspension can be further concentrated or diluted with fixative. If the appearance of the metaphases on the test slide is poor, i.e. badly spread clusters of chromosomes and an excessive amount of debris, it often helps to add one more wash of fixative, stopper and store the tubes overnight in a refrigerator and then spin down and dispense the cells on the following day. The slides should be allowed to air dry, and this can be speeded up by gentle heating over a hot plate, by placing them in a gentle draught of warmed or ambient air from a fan, or by waving them through a spirit lamp (avoid igniting the fixative).

### 8.3. STAINING

Fluorescence plus Giemsa (FPG) staining is recommended as this permits the analysis to be confined to the first in vitro division metaphases (M1) [61]. However, this method has certain drawbacks which can be overcome by using conventional Giemsa staining, as well as FPG, or by the early addition of Colcemid. Many workers have noted considerable variation in the quality of FPG staining between replicate slides and also between different patches of the same slide. The FPG technique is most successful if delayed until a few days (up to five) after the slides are made. The rest of the slides can be put in a box and kept at  $-20^{\circ}\text{C}$  before use. The quality is poorer if fresher slides are used and also if the slides are more than two or three weeks old. Storage of FPG stained slides for more than a few weeks before scoring can result in their deterioration. Thus, there is the risk that the images of FPG stained metaphases may not be clear enough for accurate discernment of all aberrations. However, the quality is usually sufficient to determine the relative proportions of M1 cells, which are not differentially stained, and M2 cells which display the harlequin effect. As a positive control that the staining has worked, the batch of slides should also include a few slides prepared from longer (72 h) cultures known to contain M2 cells.

Thus, the recommended protocol is to FPG stain one or more replicate slides from each culture. If the staining is good these may be used for scoring aberrations in the M1/M2 cells. If not, the slides should be used to check the M1/M2 ratio, and aberration analysis should be done with replicate slides from the same culture which have been stained with Giemsa. The (replicate) slides can also be stained only with Giemsa solution provided that the level of M2 cells is less than 5%, as assessed by FPG, because their inferred presence on the conventionally stained replicates may be ignored. If the level is higher, this may require an arithmetic adjustment of the aberration yield which could introduce some extra error. This would require certain assumptions regarding, for example, the proportion of dicentrics in M2 cells which are still accompanied by an acentric fragment. As stressed earlier, it is a better policy

to adopt a culture method which usually results in few M2 cells although of course this cannot be predicted for any individual because donors behave differently in their stimulation and proliferation capacity [62].

### **8.3.1. Pretreatment**

A pretreatment of slides with RNase A, prior to staining, can remove residual stainable cytoplasmic material [63]. This is an optional procedure that can provide much clearer images of the chromosomes for scoring block stained, harlequin stained or banded preparations. Additionally, it has proved useful for slides assessed with automatic image analysis systems.

The experimental protocol is as follows: A stock solution of 10 mg/mL RNase A in Tris EDTA buffer is heated for 10 min at 70°C and then allowed to cool slowly. Aliquots may be stored for several years at -20°C. Slides are rinsed in distilled water and placed in 0.5 mg/mL RNase A solution (stock solution:distilled water 1:20) for 10 min at 37°C. This may be done either in a prewarmed staining jar or, to be more economical, a smaller volume of the diluted stock solution can be placed on the slide beneath a coverslip. The slides are then washed in distilled water, placed in 3:1 methanol:acetic acid fixative for 2 min, dried and stained as described below. The RNase A cleaning procedure can also be used after destaining old slides or on micronucleus preparations. For these applications, concentrations and times may vary [63].

### **8.3.2. Fluorescence plus Giemsa (FPG) staining**

This method is derived from that published by Perry and Wolff [53] with some modifications. About ten drops of Hoechst 33258 stain (0.5 µg/mL in pH 6.8 phosphate buffer) should be placed on the slide beneath a coverslip, ensuring that no air bubbles are trapped. At this point workers with a fluorescence microscope can, if they wish, make a quick check of the M1/M2 ratio using Latt's method [64], which produces a harlequin effect, but which fades very rapidly. Otherwise the slides can be illuminated under a 20 W UV lamp (>310 nm) for 0.5 h or, alternatively, a 30 W fluorescent strip lamp for about 1.5 h. After careful removal of the coverslips, the slides should be washed well with pH 6.8 phosphate buffer. At this point some workers put the slides into 2 × SSC (0.3M sodium chloride and 0.03M trisodium citrate) at 60°C for about 20–30 min.

Experience has shown that this stage can be omitted if it results in an undesirable swelling of the chromatids which makes microscope analysis more difficult. The use of 2 × SSC, however, removes some cellular debris and so leads to cleaner preparations. The slides are then washed in distilled water, followed by immersion in Giemsa stain (5–10% in pH 6.8 buffer, Gurr R66) for 3 to 5 min. They are then rinsed in the buffer, then with distilled water and allowed to dry. The slides can

be examined at this stage under the microscope or cleared and mounted beneath a coverslip.

### 8.3.3. Conventional Giemsa staining

The slides should be immersed in 2% (Gurr R66 improved) Giemsa stain in pH 6.8 phosphate buffer for 5 min, washed in buffer, briefly rinsed in distilled water and allowed to dry, finally mounted with a cover glass using Depex.

## 8.4. ANALYSIS OF SLIDES

The slides should be coded to prevent bias in the scoring and should be scanned methodically so that the entire area is covered. The scanning should be done at low magnification (about  $\times 100$  to  $\times 200$ ). At this level, it is not possible to count whether all the chromosomes are present, nor is it possible to detect aberrations. However, with practice the scorer can identify those spreads which have about 40 or more pieces and an appearance which is likely at higher magnification to be of analysable quality. It is important that this initial scanning be done at a magnification low enough to prevent a bias towards selecting cells which contain aberrations. Having found a likely metaphase, the scorer should switch to high magnification (about  $\times 1000$  to  $\times 2000$ ), ignore, if possible, the presence of any aberrations and make a snap judgement on whether the chromosomes are of a quality suitable for scoring. This will be based on the sharpness of the images and the amount of twisting and overlapping of chromosomes. With FPG stained material the cell should be rejected if it displays the harlequin effect, indicating that it is not an M1 spread.

If the decision is taken to analyse the spread, then the number of individual chromosome pieces should be counted and the presence of aberrations noted (see Section 6). The cells should be scored by directly viewing down the microscope, although some workers routinely use a drawing attachment. It is recommended that only complete metaphases be recorded, i.e. those with 46 or more pieces. If the cell contains unstable aberrations, then it should balance. For example, a spread containing a dicentric should also have an acentric fragment, yet still count to 46 pieces. By contrast, a centric ring will also have an accompanying fragment, but the total number of objects in the cell will count to 47. Each excess acentric, i.e. one not associated with a dicentric or centric ring, will increase the count of pieces beyond 46. When recording the aberrations, the fragments associated with a dicentric or ring must not be included with the count of excess acentrics. When high radiation doses are involved there may be more than one aberration in the spread, but the pieces should

still balance. Tricentric aberrations are equivalent to two dicentrics and should have two accompanying fragments, while quadricentrics will have three fragments, and so on. All abnormalities in the cell should be recorded, although for dosimetry purposes only the data on dicentrics, or dicentrics plus rings will normally be used. The x and y stage co-ordinates of all complete cells analysed, including those free from aberrations, should be recorded for possible future reference.

Metaphase finding by automated pattern recognition systems is becoming increasingly popular. Some metaphase finders also include semi-automated analysis of digitized images that assist with locating aberrant chromosomes. However, no system is fully automatic; all incorporate steps where the operator's judgement and decision are required. Use of these instruments should be such that the same recommended criteria as outlined above are maintained, namely, selection of candidate metaphases for scoring should not introduce bias likely to distort aberration yields and only complete spreads of chromosomes should be scored.

## 8.5. RECORDING OF DATA

Good laboratory practice requires that a unique identifier code or labelling system be devised for specimens, slides and associated paperwork. The receipt and processing of specimens, whether for experiments or for overdose investigations, should be recorded in a laboratory diary. Electronic systems for data storage and handling are available. However, most researchers still work by recording their microscope observations onto a score sheet and most laboratories have evolved their own preferred way of recording the data. Any electronic system must have the facility for producing a paper printout, for example, for inclusion with documents submitted in evidence to an investigation or legal action arising from a radiation accident.

Table III illustrates a simple layout of a data sheet for recording aberrations. From the information on this sheet any cell can be identified for re-examination on a future occasion. Using the conventional Giemsa staining technique, data on unstable aberrations are most important for biological dosimetry, although no attempt has been made to list separately the minutes, fragments and acentric rings. This is because accurate discrimination between them is not always possible. However, if it is preferred, they could be recorded as M, F and AR, respectively, instead of numerals in the column headed 'Excess acentrics'. The Remarks column can be used to record other abnormalities, e.g. numerical aberrations, stable chromosome damage or chromatid aberrations. Any other numerical information which may be required, such as the percentage of cells with damage, or distributions of aberrations among the cells, can be easily extracted from the sheet.

TABLE III. LAYOUT OF A DATA SHEET FOR RECORDING ABERRATIONS

Slide No:

Scorer:

Microscope No.:

Date:

Cell No.	Stage co-ordinates		No. of chromosomal pieces	Dicentrics	Centric rings	Excess acentrics	Remarks
	x	y					
1	100.1	1.2	46				
2	103.4	1.5	47	1		1	
3	105.4	1.2	49	2	1	2	
4	112.4	1.6	—				Endoreduplication
5	112.7	1.8	48			2	
6	120.1	1.2	46	1			
7	122.7	1.5	47		1		
8	124.1	1.4	46				Chromatid exchange
9	126.8	1.7	46	2*			*= 1 tricentric
etc.							



## 8.6. STORAGE OF INFORMATION AND SLIDES

Clearly, research data have to be filed and stored for future reference; it is worth emphasizing that files relating to overdose cases may need to be re-examined long afterwards. In the event of a person developing a malignancy, perhaps decades later, the case may be reopened to resolve a claim for compensation.

Most laboratories would wish or are obliged to store the microscope slides as well, and this can create some problems. Conventionally Giemsa stained preparations have a tendency to fade and FPG stained material creates more difficulty as it frequently fades after several months. It is advisable to keep the stained slides in a box in a dry place at room temperature. However, faded slides can be retrieved by carefully soaking off the coverslip and restaining with conventional Giemsa. Attempts to restain with FPG will not succeed. Stored replicate slides, kept at  $-20^{\circ}\text{C}$ , that have never been stained can also be stained with conventional Giemsa many years later.

It is also good practice to store surplus fixed cells from overdose investigations. For ease of storage they can be concentrated down into small (2 mL) ampoules and kept at  $-20^{\circ}\text{C}$ . Slides made from this material can, years later if required, be stained conventionally, with FPG or FISH.

## 8.7. PRODUCTION OF AN IN VITRO DOSE-RESPONSE CURVE

Despite improvements in techniques and the adoption by different workers of more comparable statistical programs for data analysis, significant differences between laboratories still remain. The interpretation of dose using a calibration curve produced elsewhere may introduce substantial extra uncertainty, and therefore it must be recommended that any laboratory intending to carry out biological dosimetry should establish its own dose-response data.

Lymphocytes should be irradiated in vitro to approximate as closely as possible the in vivo situation and when this is done the same dose-response relationship is obtained [32, 65]. Freshly taken blood specimens in lithium heparin tubes should be used and irradiated as whole blood at  $37^{\circ}\text{C}$ . They should then be cultured by a standard method identical to that used for specimens from suspected overexposure patients.

### 8.7.1. Physical considerations

Most accidental overexposures are to X or  $\gamma$  radiation sources, and for a laboratory embarking upon a programme of biological dosimetry these are the qualities of radiation for which the dose response should be established first. The preparation of

a dose–response curve must be supported by reliable and accurate physical dosimetry, and there are a number of points requiring consideration. The blood needs to be positioned such that the dose can be easily inferred, and it should be exposed far enough away from the source so that the irradiation can be regarded as uniform. For example, if the sample is 1 cm thick, then it needs to be at least 1 m from the source for the difference in dose between front and back to be less than 2%. There must be sufficient material surrounding the blood for charged particle equilibrium to exist. For  $^{60}\text{Co}$   $\gamma$  rays, 4 mm of unit dense material is sufficient; for 250 kVp X rays, only 1 mm is necessary. For neutrons, 1 mm is usually also sufficient.

The surrounding materials should be reduced to a minimum to avoid the effects of scattered radiation. The materials should have atomic compositions similar to blood because the dose to blood close to the wall will be caused by electrons arising from interactions within the wall. A serious mismatch of atomic composition will result in a non-uniform irradiation of the cells. For X and  $\gamma$  rays, electron density is the main factor when considering mismatch, while for neutrons the atomic constituents are important because neutrons interact with the nuclei of the target atoms.

The ionization chamber should be surrounded by material equivalent to that which surrounds the blood. If possible the chamber should have similar dimensions to the blood sample so that it can replace the sample for dosimetry purposes. The chamber should be calibrated in air kerma with the unit of Gy. The conversion factor to Gy in soft tissue is the ratio of mass energy absorption coefficients. Numerically it is obtained by multiplying the air kerma value with a factor of 1.09 for 250 kVp X rays and 1.10 for  $^{60}\text{Co}$   $\gamma$  rays. The factor is therefore energy dependent and is lower at lower energies. There is also a difference between the conversion factors for soft tissue and for blood, but for low LET radiation this is small enough to be ignored. For neutrons it may approach 5%. The calibration factor includes any absorption by the wall of the ionization chamber, but it will often be necessary to correct the dose rate owing to self-absorption by the blood.

The usual method of determining doses is to convert the measured air kerma into absorbed dose in tissue or blood and then to convert as necessary for distance (the inverse square law), absorption and mismatch of material at the blood interface. The size and general geometry of the apparatus are a compromise between these factors because the smaller the blood specimen, the smaller the absorption correction and the larger the mismatch correction. Nevertheless, geometry and materials should be chosen to minimize the necessary corrections.

In order to produce an *in vitro* calibration curve applicable to cases of acute accidental exposure, the dose rate should be chosen such that all doses are given in less than 15 min. The differences in delivery times between the different doses are then sufficiently small that the  $\beta$  or dose squared coefficient of yield will be influenced by no more than about 4%.

### 8.7.2. Statistical considerations

Adequate curve fitting requires a sufficient number of degrees of freedom; therefore, ideally, about 10 doses should be used in the range 0.25–5.0 Gy. For low LET radiation it is not necessary to have data higher than at about 5.0 Gy and, indeed, beyond this dose there is evidence of saturation of the aberration yield which will lead to a distortion of the  $\beta$  coefficient [66]. For high LET radiation a maximum of 2 Gy would be reasonable. As most radiation accidents involve doses of less than 1.0 Gy, the lower end of the curve will be particularly important in estimating doses. An effort should therefore be made to reduce the statistical uncertainty associated with the  $\alpha$  coefficient of yield, and this requires that several doses, minimum four, should be in the range of 0.25–1.0 Gy. If the laboratory is capable of obtaining data at doses below 0.25 Gy, this is very desirable. At higher doses scoring should aim at about 100 dicentrics at each dose, but this cannot be achieved at the lower doses. Here, however, several thousand cells should be scored.

Opinions vary as to how to treat the background level of aberrations in fitting dose–response data. Some authors include a measured yield at zero dose and use this as one of the points in the curve fitting so that the background is a variable parameter. However, since the yield of dicentrics in unirradiated cells is usually low, often none are observed, and the measured zero dose yield is zero. Because of the generally poor statistics at low doses, the results of curve fitting sometimes lead to negative estimates of the background value and negative linear coefficients which obviously have no biological basis. Some investigators resolve this problem by ignoring zero dose data points and constraining the curve to pass through the origin. There are, however, sufficient data published from surveys of subjects exposed only to background radiation to show that there is a small positive background level of unstable aberrations. An alternative method adopted by some workers is therefore to use a small positive background value as a data point and to ascribe a large percentage of uncertainty to it. Ideally a laboratory should generate its own background data, although this requires the analysis of many thousands of cells. A consensus has emerged that the background level of dicentrics is 1 to 2 in 1000 cells [67].

There is very strong evidence that the yield of chromosome aberrations ( $Y$ ) is related to dose ( $D$ ) by the equation

$$Y = A + \alpha D + \beta D^2 \quad (\text{terms defined in Section 3})$$

The objective of curve fitting is to determine those values of the coefficients  $A$ ,  $\alpha$  and  $\beta$  which best fit the data points. For dicentrics, irradiation with  $X$  and  $\gamma$  rays produces a Poisson distribution, whereas neutrons and other types of high LET radiation produce distributions where the variance exceeds the mean. Whether the ratio of variance to mean is a function of dose is at present an open question. The technique

recommended for determining the best fit coefficients is that of maximum likelihood [68]. This can be done by maximizing the likelihood of the observations, assuming Poisson distributions, by the method of iteratively reweighted least squares. For overdispersed non-Poisson distributions, the weights must take into account the overdispersion. If one considers that the data show a trend of  $\sigma^2/y$  (variance to mean) with dose, then that trend should be used. Otherwise, one should divide all Poisson weights by an average value of  $\sigma^2/y$ . The standard errors of the coefficients and the fitted values should be based on the Poisson distribution, or overdispersed distribution, if applicable, but if there is evidence of a lack of fit, then the standard error should be increased by  $(\chi^2/\text{degree of freedom})^{1/2}$ .

The statistical computer program used for curve fitting should give sufficient information to enable variances and co-variances on all fitted coefficients to be obtained. These are needed when calculating the uncertainties on dose estimates (Section 8.8.4).

## 8.8. DOSE ASSESSMENT IN WHOLE BODY EXPOSURE

### 8.8.1. Choice of curve

The sources of radiation to which personnel are usually exposed are  $\gamma$ , X rays and, occasionally, degraded neutrons. It is commonly found that there is a difference between the yield curves of X and  $\gamma$  rays, particularly at low doses (<0.5 Gy). Therefore it is advisable to have a calibration curve for a suitable energy of X rays (e.g. 200–250 kVp) as well as for either  $^{60}\text{Co}$  or  $^{137}\text{Cs}$ . For neutrons a degraded energy spectrum is similar to a fission spectrum. Available evidence indicates that the dose response curves for fission spectrum neutrons are linear and do not alter much with neutron energy. Thus a calibration curve produced with a fission spectrum would suffice.

In industrial radiography  $^{192}\text{Ir}$  is commonly used and its mean gamma energy is about 400 keV. The calibration for this isotope should lie somewhere between the X and  $\gamma$  ray curves. However, it is generally considered to lie closer to the  $^{60}\text{Co}/^{137}\text{Cs}$  curve, and it is recommended that the gamma ray curve be used.

### 8.8.2. Number of cells to be analysed

In order to produce a dose estimate with a statistical uncertainty small enough to be of value, a large number of cells usually needs to be scored. The decision on how many to analyse is a compromise based on the importance of the case, the available labour and the quality of the preparations. For example, after a high dose of, for example, several Gy, the subject's lymphocyte count may be severely depleted and this will be reflected in a low number of metaphases on the slides. However, as the

number of aberrations per cell will be high, a reasonable estimate could be made from the analysis of just a few tens of cells.

For lower doses, where the number of available cells is not the limiting factor, a dose estimate could be based on about 200 cells. It is, however, advisable to increase the scoring to 500 cells. This may require 2–3 person-days at the microscope, although in an emergency several people can collaborate in scoring replicate slides. For a low or zero dicentric yield, the improved confidence limits resulting from 500 scored cells are worth while and are usually sufficient. The decision to extend scoring beyond 500 to 1000 or more cells depends on whether there is evidence of a serious overexposure, in which case there may be a scientific justification, or if the continued employment of a radiation worker is in jeopardy. Clearly, there is no single number of cells that can be recommended as being applicable in all cases. As a general rule it may be suggested that scoring should aim at 500 cells or 100 dicentrics.

The methods for calculating confidence limits on dose estimates are discussed below. Table IV shows the limits to be expected using calculation method C (simplified), detailed in Section 8.8.4, for several dose estimates up to 1.0 Gy. These are made from the gamma ray curve in Fig. 12 and are based on scoring 200, 500 or 1000 cells.

### **8.8.3. Uncertainty on dose estimates**

While there is no difficulty in deriving a dose from a measured yield of dicentrics, there is no generally accepted way of deriving its uncertainty. The aim is to express uncertainty in terms of a confidence interval, and quite often 95% is chosen as the limit. The 95% confidence limits define an interval that will encompass the true dose on at least 95% of occasions. The difficulty in the computation of confidence limits arises because there are two components to the uncertainty. One is from the Poisson nature of the yield of aberrations, seen in the sample from the overexposed subject, and the other from uncertainties associated with the calibration curve which are approximately normally distributed. The problem has recently been discussed by Savage et al. [69], who presented a basic solution to the problem (Approach A). Merkle [70] has presented another two solutions (Approaches B and C). All three solutions are approximations.

#### ***Approach A***

The procedure is as follows:

- (1) Express dose as a function of the fitted coefficients of the calibration curve and the measured aberration yield from the exposed person. The equation is:

TABLE IV. EFFECT OF INCREASING THE NUMBER OF CELLS EXAMINED ON THE LOWER AND UPPER 95% CONFIDENCE LIMITS FOR FOUR ESTIMATES OF ACUTE GAMMA RADIATION DOSE

Dose estimate (mGy)		Confidence limits		
		No. of cells examined		
		200	500	1000
100	Upper	—	340	250
	Lower	—	<5	<5
250	Upper	610	500	400
	Lower	30	100	120
500	Upper	870	710	630
	Lower	190	300	300
1000	Upper	1350	1210	1130
	Lower	690	810	850

$$D = \left[ -\alpha + \sqrt{\alpha^2 + 4\beta(Y - A)} \right] 2/\beta$$

where D, Y, A,  $\alpha$  and  $\beta$  are defined in Section 3.

- (2) By differentiation of the above equation, express the variance on D (varD) in terms of the variances and co-variances of A,  $\hat{\alpha}$ ,  $\hat{\beta}$  and Y. The formal equation is:

$$\begin{aligned} \text{varD} = & (\partial D/\partial A)^2 \text{varA} + (\partial D/\partial \alpha)^2 \text{var}\alpha + (\partial D/\partial \beta)^2 \text{var}\beta + (\partial D/\partial Y)^2 \text{varY} \\ & + 2(\partial D/\partial \alpha \partial D/\partial \beta) \text{covar}(\alpha, \beta) + 2(\partial D/\partial A \partial D/\partial \alpha) \text{covar}(A, \alpha) \\ & + 2(\partial D/\partial A \partial D/\partial \beta) \text{covar}(A, \beta) \end{aligned}$$

The variance and co-variances on A,  $\alpha$  and  $\beta$  are derived from the fitted calibration curve. The variance on Y is derived on the assumption of a Poisson distribution.

- (3) The standard error in D is  $\text{SE}(D) = \sqrt{\text{varD}}$  and the 95% confidence limits are  $D - 1.96\text{SE}(D)$  and  $D + 1.96\text{SE}(D)$ .

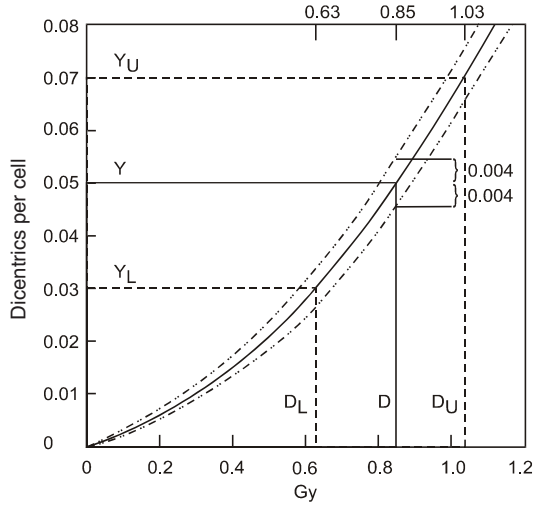


FIG. 12. A dose-response calibration curve with its 95% confidence limits used to estimate uncertainties on dose by approach B.

Savage et al. [69] simplified the above expression by defining the control parameter,  $A$ , as the observed control value in their calibration experiment. This effectively set to zero the last two co-variance terms and the first variance term.

*Example:* Consider an overexposure to gamma rays for which 500 cells are scored and 25 dicentrics observed, i.e. 0.05 dicentrics per cell. The parameters of the gamma ray calibration curve, which is drawn in Figs 12 and 13, are:

$A$	0.000476
$\alpha$	0.01645
$\beta$	0.04925
$\text{var}A$	$2.480 \times 10^{-7}$
$\text{var}\alpha$	$1.396 \times 10^{-5}$
$\text{var}\beta$	$8.189 \times 10^{-6}$
$\text{covar}(A, \alpha)$	$-7.679 \times 10^{-7}$
$\text{covar}(A, \beta)$	$2.569 \times 10^{-7}$
$\text{covar}(\alpha, \beta)$	$-7.228 \times 10^{-7}$

The standard error on 0.05 dicentrics per cell is 0.01.

The variance on the yield is therefore 0.0001.

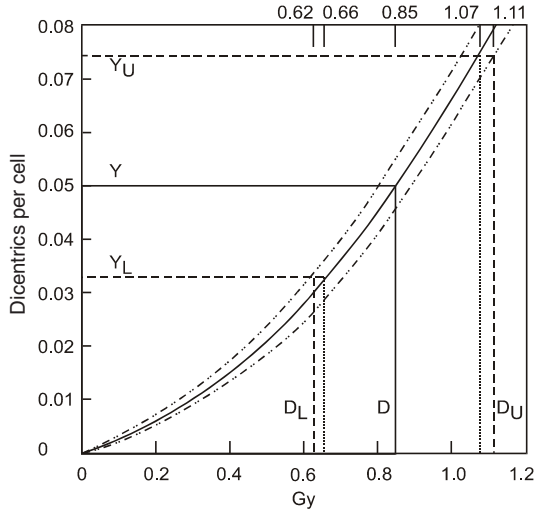


FIG. 13. A dose-response calibration curve with its 95% confidence limits, used to estimate uncertainties on dose by approach C.

For a yield of 0.05 dicentric per cell, the dose  $D$  is 0.85 Gy.  
 Evaluated at  $D = 0.85$

$$\left(\frac{\partial D}{\partial Y}\right) = 9.986 \quad \left(\frac{\partial D}{\partial \alpha}\right) = -8.484$$

$$\left(\frac{\partial D}{\partial A}\right) = -9.986 \quad \left(\frac{\partial D}{\partial \beta}\right) = -7.207$$

By substitution,  $\text{var}D = 0.01096$ . The standard error on  $D = 0.1047$ . The 95% confidence limits on  $D$  are 0.645 and 1.06 Gy.

### Approach B

This is similar to approach A but considers uncertainties in yields rather than in dose. The procedure is as follows:

- (1) From the observed yield estimate the dose.
- (2) From the dose-response curve, with its 95% confidence limits, estimate the standard error on the yield at that dose due to uncertainties in the curve. The SE is approximately half of the 95% confidence limit.



- (3) From the observed yield estimate its Poisson standard error.
- (4) Add the standard errors derived from items (2) and (3) above in quadrature to give the overall standard error.
- (5) By assuming a normal distribution, derive 95% confidence limits on the yield and convert these to doses.

*Example:* The same example of 25 dicentrics in 500 cells corresponding to 0.85 Gy is used and is illustrated in Fig. 12.

Going through the steps listed above,

- (1) 0.05 dicentrics per cell is equivalent to 0.85 Gy.
- (2) The 95% confidence limits on the curve intercept the dose of 0.85 Gy at 0.004 dicentrics/cell either side of the mean (0.05). The standard error is therefore approximately half of this value, i.e. 0.002 dicentrics/cell.
- (3) The Poisson standard error on the yield due to the observation is  $[25]^{1/2}/500$ , which is equal to 0.01 dicentrics per cell.
- (4) The total SE =  $[(0.002^2 + 0.01^2)]^{1/2} = 0.0102$ .
- (5) The 95% confidence limits in the observed yield are therefore  $0.05 + 1.96(0.0102)$ , i.e. 0.03 and 0.07 dicentrics per cell. In Fig. 12, these are labelled as  $Y_L$  and  $Y_U$  and correspond to 0.63 and 1.03 Gy.

### ***Approach C***

This approach, illustrated in Fig. 13, using the same calibration curve as in Fig. 12, involves the following steps:

- (1) Assuming the Poisson distribution, draw the yields corresponding to the lower and upper 95% confidence limits on the observed yield ( $Y_L$  and  $Y_U$ ).
- (2) Determine the dose at which  $Y_L$  crosses the upper curve. This is the lower confidence limit ( $D_L$ ).
- (3) Determine the dose at which  $Y_U$  crosses the lower curve. This is the upper confidence limit ( $D_U$ ).

*Example:* The same example is used, where a dose of 0.85 Gy is estimated from 25 dicentris observed in 500 cells. The steps listed above are thus as follows:

- (1) From standard statistical tables, the 95% lower and upper confidence limits on an observation of 25 are 16.18 and 36.9. Therefore values for  $Y_L$  and  $Y_U$  are  $16.18/500 = 0.032$  and  $36.9/500 = 0.074$  dicentrics per cell, respectively.
- (2)  $Y_L$  intersects the upper confidence curve at 0.62 Gy ( $D_L$ ).

- (3)  $Y_U$  intersects the lower confidence curve at 1.11 Gy ( $D_U$ ).
- (4) These are shown as the dashed vertical lines in Fig. 13.

#### *A simplified version of Approach C*

This is also illustrated in Fig. 13. With well established calibration curves based on a large amount of scoring, the variance due to the curve is small compared with the variance on the observed yield from the subject and can be ignored. A simpler approximate estimate of  $D_L$  and  $D_U$  may be obtained directly from the calibration curve, where  $Y_L$  and  $Y_U$  cross the solid line in Fig. 13, i.e. 0.66 and 1.07 Gy. These are shown as the dotted vertical lines.

#### *Conclusions*

Method A is superficially the most accurate approach. However, it involves the use of quite complicated equations. The apparent accuracy is misleading because the number of aberrations scored in the exposed person is usually small so that the assumption of a normal distribution is poor. This method may be reasonable when the number of aberrations scored is large as might happen following high doses. Approach B suffers from the same defect but the resulting equations are less complicated to use. Approach B is probably the best method to use in situations where the uncertainty on the measured yield is similar to that on the calibration curve. Approach C takes into account the Poisson nature of the yield in the exposed person but overestimates the effect of uncertainties in the calibration curve. The simplified approach C also takes into account the Poisson distribution in yield but ignores errors in the calibration curve. It thus has the great advantage of simplicity. Approach C and its simplified version are reasonable when the uncertainty on the measured yield dominates that on the calibration curve, which is usually the case at low doses.

#### **8.8.4. Actual examples of dose estimations**

In cases where an acute accidental overexposure to relatively low LET radiation is uniformly distributed over the whole body (and a blood sample is available promptly), the dicentric frequency per cell provides a reliable estimate of the average whole body absorbed dose. In practice, however, such ideal circumstances rarely occur and it is more usual for exposure to be non-uniform, perhaps involving only part of the body. A substantial time delay may also occur before a blood sample is taken for chromosome study. These factors will result in an inhomogeneous population of lymphocytes being sampled, and the resultant dicentric yield, when compared with a standard *in vitro* dose–response curve, will produce an unrealistic estimate of

dose. Inhomogeneity produces a yield of dicentrics which does not conform to a Poisson distribution, but is generally overdispersed. For a partial body exposure this obviously arises because those lymphocytes in tissues outside the radiation field will not be damaged. In cases of highly localized exposure, the smaller than expected number of cells that are damaged may each contain several aberrations. Even when the radiation dose is uniform at the skin, its monotonic reduction with depth in tissue will result in a variety of doses being received by lymphocytes. This effect will be especially marked with poorly penetrating radiation, but for more energetic radiation, such as 250 kVp X rays or gamma rays from  $^{60}\text{Co}$ ,  $^{192}\text{Ir}$  and  $^{137}\text{Cs}$  sources, the effect is sufficiently small for the dicentrics to have an approximately Poisson distribution.

Accidental exposure to high LET radiation such as neutrons will also produce an overdispersed distribution because of the manner in which the dose is deposited at the cellular level.

Delays in blood sampling will influence the aberration yield, as cells containing unstable aberrations are lost from the circulation and replaced by newly produced, undamaged cells.

In the following sections it is intended, with the aid of examples, to discuss how the yield of chromosome aberrations is influenced by inhomogeneity of exposure, by delayed sampling and by protracted exposure and how the data might nevertheless be used to provide a meaningful estimate of dose.

#### *8.8.4.1. Acute whole body exposure: An example*

Brewen et al. [50] and Preston et al. [71] have described an accident involving a  $^{60}\text{Co}$  source in which a high dose was received fairly homogeneously over the front of the body. The mean dose to the back was lower, but it too was exposed as the man turned and walked away from the source. The total exposure time was less than one minute. A number of blood samples were taken at intervals ranging from six hours to three years after the incident. The aberration yield remained fairly constant over the period of 6 hours to 32 days, during which time 7 blood samples were taken and 300 metaphases analysed from each. When the data for the 7 samples were combined, 478 dicentrics and rings were observed in 2100 cells. These workers used the in vitro, gamma ray dose–response curve, where the dose  $D$  was expressed in roentgen (R):

$$Y = 3.93 \times 10^{-4} D + 8.16 \times 10^{-6} D^2 \quad (1)$$

to estimate a mean whole body exposure of 144 R (1 R = 0.0095 Gy). This agreed well with the physical estimate of 127 R made from a thermoluminescence dosimeter that the man had worn and a reconstruction of the incident using a phantom. The general haematological changes noted were also consistent with an exposure of about 150 R.

#### 8.8.4.2. Criticality

In a criticality accident the body is irradiated by both neutrons and gamma rays. If the ratio of neutron to gamma ray doses is known, and this information is usually available from physical measurements, it is possible, by iteration, to estimate the neutron and gamma ray doses separately. The iteration proceeds as follows:

- (1) Assume that all the aberrations are attributable to neutrons, and from the measured yield of dicentric estimate a dose from the neutron curve;
- (2) Use the supplied neutron to gamma ray ratio to estimate the gamma ray dose;
- (3) Use the gamma ray dose to estimate the yield of dicentric due to gamma rays;
- (4) Subtract this calculated gamma ray yield of dicentric from the measured yield and assume that this is the yield due to neutrons;
- (5) Re-estimate the neutron dose and repeat stages 2 to 5 until self-consistent estimates are obtained.

*Example:* Consider a criticality accident in which 100 cells are scored and 120 dicentric observed, i.e. 1.2 dicentric per cell. The neutron to gamma ratio supplied from physical measurements is 2:3 in absorbed dose. Cytogenetic dose estimates are to be made using 0.7 MeV fission spectrum neutrons and  $^{60}\text{Co}$  gamma ray curves. The yield equations for these curves are:

$$\begin{aligned}\text{Neutrons: } Y &= 0.0005 + 83.2 \times 10^{-2} D \\ \text{Gamma rays: } Y &= 0.0005 + 1.64 \times 10^{-2} D + 4.92 \times 10^{-2} D^2\end{aligned}$$

Now, going through the steps listed above:

- (1) 1.2 dicentric per cell is equivalent to 1.44 Gy neutrons.
- (2)  $1.44 \times 3/2 = 2.16$  Gy gamma rays,
- (3) 2.16 Gy gamma rays are equivalent to 0.266 dicentric per cell,
- (4)  $1.2 - 0.266 = 0.934$ , which is the dicentric yield attributable to neutrons,
- (5) 0.934 dicentric per cell is equivalent to 1.122 Gy neutrons.

Then, going back to stage 2,  $1.122 \times 3/2 = 1.683$  Gy gamma rays, etc. After a few iterations, doses of 1.21 Gy neutrons and 1.82 Gy gamma rays are obtained. The complete sequence is laid out in Table V.

An in vitro validation of this approach has been described where very good estimates of actual neutron and gamma doses were obtained in an international exercise to compare criticality accident dosimetry [72].

TABLE V. SEQUENCE OF STEPS USED IN MAKING DOSE ESTIMATES FOR MIXED GAMMA AND NEUTRON IRRADIATION

Steps 1 and 5 Neutron dose (Gy)	Step 2 Gamma ray dose (Gy)	Step 3 Gamma ray yield of dicentrics per cell	Step 4 Neutron yield of dicentrics per cell
1.44	2.16	0.266	0.934
1.12	1.68	0.167	1.032
1.24	1.86	0.201	0.999
1.20	1.80	0.189	1.011
1.21	1.82	0.194	1.006

#### 8.8.4.3. Dealing with low dose overexposure cases

It is often stated that the lower limit of dose detection by dicentrics for low LET radiation is around 0.1–0.2 Gy. Sensitivity to low doses is a function of the background level of dicentrics (which of course is not known for an individual but is generically ~1/1000 cells) and the limit on the number of metaphases that can realistically be scored. Dose estimates at low doses therefore carry large statistical uncertainties. These come mainly from the error on, e.g., 1000 cells being scored but with some smaller contribution from the SE on the  $\alpha$  coefficient of the dose–response curve. For practical purposes the latter can be ignored unless the calibration data at low doses are sparse (Section 8.7.2).

Whilst 100–200 mGy is of minor concern when considering health consequences of exposure, in legal terms it is a high dose when compared with the ICRP recommended annual occupational dose limit of 20 mSv. There is often pressure on cytogenetics to try to resolve suspected low overdoses, perhaps pushing the method beyond its capabilities.

*Example:* An actual case (Lloyd, personal communication) can be cited to illustrate how one might deal with such a situation. A non-destructive testing radiographer, working with  $^{192}\text{Ir}$  sources, returned a monthly thermoluminescence dosimeter which recorded a penetrating radiation exposure of 250 mSv. No colleagues who regularly worked alongside him recorded exposures on their dosimeters. There was no evidence of any systems failure or any other explanation for the overdosed badge. The case was referred for cytogenetic analysis where 1000 metaphases were scored and all were undamaged. This was reported as the best estimate of dose being zero but, using the curve  $Y = 0.001 + 0.0164D + 0.0492D^2$  (Figs 12 and 13), zero carried an

TABLE VI. 95% UPPER AND LOWER CONFIDENCE LIMITS (UCL, LCL) ON VARIOUS LOW YIELDS OF DICENTRICS IN 1000 CELLS AND THE ODDS RATIOS SHOWING THE LIKELIHOODS OF ZERO DOSE OR 0.25 Gy

Observation (dicentrics)	Dose (Gy)			Odds ratio p(0 Gy):p(0.25 Gy)
	LCL	mean	UCL	
0	—	0	0.12	1300 : 1
1	0	0	0.18	160: 1
2	0	0.05	0.23	20 : 1
3	0	0.09	0.26	2.4 : 1
4	0.01	0.13	0.30	1 : 3.4
5	0.03	0.16	0.33	1 : 30
6	0.06	0.19	0.36	1 : 230
7	0.09	0.22	0.38	1 : 1900

upper 95% confidence limit of 0.12 Gy. Experience has shown that persons rarely understand the concept of upper confidence limits and so, in lay terms, which is not strictly statistically accurate, it is explained that there is only a 2.5% chance of the dose being greater than 120 mSv. Table VI indicates that the reporting of a dose significantly above zero, i.e. where the lower 95% confidence is a positive value, would have only been possible if four or more dicentrics had been observed in the 1000 cells.

Investigators were doubtful if the man had indeed been irradiated and so in this case it proved useful to present the results in a different way. If one considers just two possible scenarios; zero dose or 0.25 Gy, the odds in favour of zero come out at 1300:1. This is obtained from the relative probabilities of observing zero dicentrics given the two doses. If the dose was zero, then from the dose-response curve above, the background frequency of 1 dicentric in 1000 cells is expected. For a dose of 0.25 Gy a yield of 8.2/1000 is expected. The chances, derived from the Poisson distribution, of seeing no dicentrics when 1 and 8.2 are expected are, respectively, 0.368 and 0.00027, i.e.  $e^{-1}$  and  $e^{-8.2}$ ;  $0.368/0.00027 \cong 1300$ .

The values are shown in the top line of Table VI, with other values below that would have been quoted if different numbers of dicentrics had been seen in 1000 cells.

The reporting laboratory may use either or both approaches when presenting the results of the analysis, the decision depending on the particular circumstances of the case.

#### 8.8.4.4. Dose assessment in partial body exposure

There are basically two methods by which the observed yield of aberrations can be used to provide a more realistic dose estimate than simply quoting an average whole body dose.

##### **Method 1**

This was first proposed by Dolphin [73] and considers the overdispersed distribution of dicentric chromosomes among all the scored cells. The observed distribution is considered to be the sum of (a) a Poisson distribution which represents the irradiated fraction of the body and (b) the remaining unexposed fraction. Cells containing aberrations will obviously have been in the irradiated part of the body. Normal undamaged cells will comprise two subpopulations, those from the unexposed fraction and irradiated cells representing the first term ( $e^{-Y}$ ) of the Poisson series.

Maximum likelihood estimates of the fraction,  $f$ , of cells scored which were irradiated and the mean yield,  $Y$ , to this fraction may be made by using Eqs (2) and (3):

$$\frac{Y}{1 - e^{-Y}} = \frac{X}{N - n_0} \quad (2)$$

$$Yf = \frac{X}{N} \quad (3)$$

In these equations,  $N$  is the number of cells scored,  $X$  is the number of dicentric chromosomes observed and  $n_0$  is the number of cells free of dicentric chromosomes.

The mean dose to the irradiated fraction may then be obtained from  $Y$  by using a standard in vitro dose-effect curve. The size of the fraction of body irradiated may be derived from  $f$  after correction for the effects of interphase death and mitotic delay. These factors will cause irradiated cells, even if free from aberrations, to be less likely than unexposed cells to reach metaphase by 48 h in culture. If the fraction of irradiated cells which reach metaphase was  $p$ , the fraction of the body irradiated,  $F$ , is given by

$$F = \frac{f/p}{1 - f + f/p} \quad (4)$$

There are, however, a number of limitations to this approach:

- (1) The method assumes that the exposure to the irradiated fraction is homogeneous.
- (2) It derives the fraction of lymphocytes irradiated which can only be related to the fraction of body irradiated by making the simplifying assumption that lymphocytes are uniformly distributed throughout the body.
- (3) It requires a sufficiently high local dose so that there are a number of cells observed with two or more dicentrics. This is necessary for the best-fit calculation of the irradiated, but undamaged, cells.
- (4) The method assumes a minimal delay between irradiation and blood sampling, so that the dicentric yield is not significantly diluted by newly formed undamaged cells entering the circulation. Should dilution occur, then the fraction irradiated derived by this method is likely to be underestimated [74].

### ***Method 2***

This approach has been proposed by Sasaki and Miyata [75] and is termed the Qdr method. It considers the yield of dicentrics and rings only from those cells that contain unstable aberrations and assumes that these cells were present at the time of the accident. The method therefore circumvents problems of dilution by undamaged cells from an unexposed fraction of the body or post-irradiation replenishment from the stem cell pool. It also does not require the presence of heavily damaged cells containing two or more aberrations. Qdr is the expected yield of dicentrics and rings,  $X$ , among the damaged cells,  $N_u$ , and is given by

$$Qdr = \frac{X}{N_u} = \frac{Y}{1 - \exp(-Y_1 - Y_2)} \quad (5)$$

where  $Y_1$  and  $Y_2$  are yields of dicentrics plus rings and of excess acentrics, respectively. As  $Y_1$  and  $Y_2$  are known functions of the dose and are derivable from in vitro dose-response curves, Qdr is a function of dose alone and hence permits a dose estimate to be made for the irradiated part of the body.

There are several limitations also with this method:

- (1) It assumes, as does Method 1, that the exposure to the irradiated fraction is uniform, but according to Ref. [75] it provides no information on the size of this fraction. However, this can be derived, using essentially the same procedure as in Method 1, by converting dose to yield and then using Eqs (3) and (4).



- (2) It assumes that the excess acentric aberrations also have Poisson distributions, but this is not borne out by data from in vitro experiments. If this limitation is thought to be important, it could be avoided by considering the yield of dicentrics and rings in those damaged cells that contain just dicentrics and rings. Equation (5) would now reduce to

$$Q_{dr} = \frac{X}{N_u} = \frac{Y_1}{1 - \exp(-Y_1)} \quad (6)$$

which is identical with Eq. (2). This simplified form will produce a dose estimate identical with that obtained by Method 1 above.

- (3) The method assumes that all cells containing unstable aberrations were present at the time of irradiation and that there has been no recruitment of cells containing derived chromosome aberrations arising from chromatid damage in stem cells.

#### *Acute non-uniform exposure: An example*

An inhomogeneous irradiation, resulting in highly localized exposure sufficient to cause skin burns, occurred when a non-radiation worker picked up a 250 GBq (6.7 Ci)  $^{192}\text{Ir}$  source and placed it in his pocket [76]. Blood was sampled promptly and one thousand lymphocyte metaphases were examined; 99 of them contained the following unstable aberrations: 86 dicentrics, 2 centric rings and 60 excess acentrics. The distribution of dicentrics was:

Dicentrics per cell	0	1	2	3	4	5
No. of cells	932	56	9	1	1	1

The investigating laboratory's in vitro dose-response curves were:

$$Y_{(\text{dicentrics})} = 1.57 \times 10^{-2}D + 5.0 \times 10^{-2}D^2 \quad (7)$$

$$Y_{(\text{acentrics})} = 2.30 \times 10^{-2}D + 3.9 \times 10^{-2}D^2 \quad (8)$$

In using Method 1, the maximum likelihood estimate for the yield of dicentrics,  $Y$ , in the irradiated cells is given by substituting data from the example into Eq. (2). By iteration,  $Y = 0.489$  dicentrics per irradiated cell, which corresponds on the dose-response curve to 2.97 Gy.

The size of the irradiated fraction,  $f$ , is given by solving Eq. (3), which, in this example, gives  $f = 0.176$ . As this value represents the population of cells which was irradiated and survived, it needs to be adjusted, as described in Eq. (4), in order to

take account of selection against the irradiated cells by factors such as interphase death and mitotic delay. There is some experimental evidence [77] indicating that this selection is an exponential function of dose, with  $D_0 = 2.70$  Gy. In the present example, the dose estimate of about 3.0 Gy would imply that only about 0.33 —  $p$  in Eq. (4) — of the irradiated cells survived to be analysed. The fraction originally exposed,  $F$ , is equal to 0.393 and is obtained by solving Eq. (4). In round terms, therefore, the irradiated fraction of the body is about 40%, with an average dose of about 3.0 Gy.

In using Method 2, it should be noted that the investigating laboratory did not normally use the yield of dicentrics plus rings for dose estimation, but rather dicentrics alone. As rings are rarely observed aberrations, as compared with dicentrics and excess acentrics, this modification has only a trivial effect. Therefore, substituting values into Eq. (5) and omitting centric rings gives

$$Q_{dr} = \frac{86}{99} = \frac{1.57 \times 10^{-2}D + 5.0 \times 10^{-2}D^2}{1 - \exp(-3.87 \times 10^{-2}D - 8.9 \times 10^{-2}D^2)} \quad (9)$$

The equation can be solved for  $D$  by iteration and gives a dose estimate of 3.19 Gy. This is in good agreement with the value of 2.97 Gy derived from Method 1.

#### 8.8.4.5. *Dose assessment after delayed blood sampling*

It has been well documented that some lymphocytes containing aberrations continue to exist in the peripheral circulation for many years after an irradiation. However, a delay of more than a few weeks between irradiation and sampling has been shown to reduce the aberration yield. This is particularly so following large doses sufficiently high to cause early deterministic reactions such as the depression of white blood cell counts. For lower doses, below the threshold for deterministic injury, the potential for late recognition of an overdose is greater. Therefore, some adjustment needs to be made in order to produce a more realistic estimate of dose. Unfortunately, there are few data which enable a reliable correction factor to be applied. Indeed, since there is marked individual variation, depending on factors such as infections, the depression of aberration yield probably cannot be expressed simply as a function of time alone. Nevertheless, an exponential disappearance rate with a half-time of about three years has been suggested [67]. As a general approximation this seems suitable when the sampling delay is long, say five or more years. However, when brief accidental exposures are being investigated there are rarely delays of this length. Typically, they range from a few days to a few weeks. Delay of a few weeks

is more likely if the exposure is only appreciated when a routine personal dosimeter is processed, with irradiation having occurred early in its period of issue. At most one might encounter a sampling delay of up to one year, and over this time span an exponential disappearance half-time of about three years is inappropriate.

What is probably the most comprehensive body of data is that published by Buckton et al. [78–80] who, for over 30 years, repeatedly sampled a group of patients treated with fractionated X rays for ankylosing spondylitis. In these studies there was a long initial plateau in aberration yield, about 20 weeks, which was followed by a steep fall which persisted over four years. Over the first four years they calculated that the dicentric yield dropped at a rate of about 43% per year and thereafter the decline was about 14% per year. In view of the considerable variability in the limited data, no firm guidance can be given, especially for delays in excess of a few weeks. Uncorrected dicentric yields will, therefore, probably underestimate the dose, but the extent of the underestimate depends on generally unquantifiable factors particular to each individual.

It was noted in the discussion of partial body irradiation that the Qdr method considers the yield of dicentrics and rings only in damaged cells. Therefore, applying this approach to delayed blood sampling could also avoid the problem of dilution with time by undamaged cells entering the circulation, provided that sufficient numbers of cells containing unstable aberrations are still observed. This is obviously not feasible for very long delays. In such cases it may be possible, however, to consider the persistence of cells with stable aberrations. For many years this was only possible by karyotyping many block stained and later banded preparations. By these methods the study of ankylosing spondylitis revealed that the level of these cells remained more or less constant over the 30 years of follow-up. Awa [81] has also reported a good correlation between the frequency of stable aberrations and the DS86 estimates of dose in the atom bomb survivors. Dividing cells containing unstable aberrations are selectively eliminated by mitotic non-disjunction. The excess of stable aberrations with time is explained by assuming that cells with stable and unstable damage disappear at the same rate, but the loss of stable damage is offset by unimpeded divisions from the stem cell pool. Laborious banded karyotyping has now been replaced by FISH as the optimum method for screening large numbers of cells for the presence of rare, random, non-constitutional stable translocations for retrospective biological dosimetry. This is described in Section 9.

#### *Delayed blood sampling: Two examples*

#### *Adjusting the dicentric yield*

Stephan et al. [82] have reported an accident in which two men were fairly uniformly exposed for about five minutes to a  $^{60}\text{Co}$  gamma ray source. They wore film badges which indicated 470 and 170 mSv; and these values agreed very well with

physical calculations of the doses. Unfortunately, blood sampling was delayed by 215 days for the more highly exposed man and by 103 days for his colleague. About 1500 metaphases were examined from each man and almost identical yields of 0.47 and 0.46 dicentrics per 100 cells were obtained. These correspond to 0.13 Gy on the dose–response curve:

$$Y = 3.0 \times 10^{-4}D + 5.0 \times 10^{-6}D^2 \quad (10)$$

The authors chose to adjust the dicentric yields by  $\times 3$  and  $\times 2$ , respectively, to account for the delays. This decision was based on the data of Brewen et al. [50] and Preston et al. [71] from the accidental whole body irradiation described in Section 8.8.4.2. The adjusted dicentric yields produced dose estimates of 0.31 and 0.22 Gy. Although this brings the biological estimate of dose for the more highly exposed man closer to the physical estimate, there is still some discrepancy. Had the authors chosen to use the delay data from the study of ankylosing spondylitics [79], a correction of at most 1.4 might have applied, so that the discrepancy between biological and physical estimates for the first man would have been greater. In view of the spondylitics' effect lasting up to 20 weeks, the delay of 103 days would require no correction.

#### *Using the Qdr method*

Ishihara et al. [83] have described a serious incident in which an  $^{192}\text{Ir}$  industrial radiography source was taken into a dwelling, irradiating six people. The two most seriously overexposed subjects received partial body irradiation, which was evident from skin burns. This was further reflected in the aberration data, where doses estimated by the Qdr method were 1.95 and 1.50 Gy, substantially higher than the values of 1.52 and 0.54 Gy, respectively, from the yields of dicentrics and rings on a per cell basis. The values of dicentrics and rings per cell varied somewhat in the first two months after exposure, but then became more stable up to six months. By contrast, the Qdr values stayed relatively constant from the beginning up to 400 and 200 d, respectively, when further study ceased.

#### *8.8.4.6. Dose assessment after protracted and fractionated exposure*

Protraction or fractionation of the exposure may also produce a lower chromosome aberration yield than if the same dose is received acutely. For high LET radiation, where the dose–response relationship is close to linear, no dose rate or fractionation effect would be expected. For low LET radiation, however, the dose squared coefficient in the yield equation

$$Y = \alpha D + \beta D^2$$

is reduced. This term represents those aberrations, possibly of two track origin, which can be modified by repair mechanisms that have time to operate during the course of a protracted exposure or in the periods between intermittent acute exposures. A number of studies have shown that the decrease in the frequencies of aberrations appears to follow a single exponential function with a mean time of about two hours. The majority of lesions that are converted into chromosome aberrations will have been repaired or would become otherwise unavailable for interactions within about five to six hours after exposure.

A time dependent factor known as the G function was proposed by Lea and Catcheside [84] to enable modification of the dose squared coefficient and thus allow for the effects of dose protraction. Experimental evidence which supports the G function hypothesis has been presented by Bauchinger et al. [85] and Lloyd et al. [86]. The linear quadratic equation may be modified to

$$Y = \alpha D + \beta G(x) D^2 \quad (11)$$

where

$$G(x) = \frac{2}{x^2} [x - 1 + \exp(-x)] \quad (12)$$

and  $x = t/t_0$ , where  $t$  is the time over which the irradiation occurred and  $t_0$  is the mean lifetime ( $\sim 2$  h) of the breaks. Therefore, in the case of continuous irradiation, it is necessary to know the length of time for which the exposure has lasted and to make the simplifying assumption that the dose rate during the exposure remained more or less constant. It is only worth attempting this procedure if the total dose involved is sufficiently large and the duration of the exposure is a matter of hours, up to a few days. Obviously, for small exposures ( $< 0.3$  Gy) to low LET radiation, even delivered acutely, the majority of aberrations are produced by the passage of single ionizing tracks so that the yield approximates to  $Y = \alpha D$  anyway. When a dose is delivered over a long period,  $G(x)$  reduces virtually to zero. Therefore, even if a high dose ( $> 1.0$  Gy) is involved, the yield becomes, in effect,  $Y = \alpha D$ . For brief, intermittent exposures, where interfraction intervals of more than six hours are involved, the exposures may be considered as a number of isolated acute irradiations for each of which the induced aberration yields are additive. For shorter interfraction times,  $G$  in Eq. (11) can be replaced by  $\exp(-t_1/t_0)$ , where  $t_1$  is the time between fractions.

#### *Protracted exposure: An example*

In December 1998 a serious accident occurred in Istanbul, where a former radiotherapy  $^{60}\text{Co}$  source was broken open in a scrap metal yard [87]. Ten persons

were irradiated, mostly during one day, with exposure times ranging from 2 to 7 h [88]. One of the subjects is used here as an example. His exposure was for 7 h, and the dicentric frequency, from the pooled results of four laboratories, was 157 dicentrics in 688 cells =  $0.228 \pm 0.18$  dic/cell. From the acute dose–response curve

$$Y = 0.001 + 0.030D + 0.060D^2$$

the acute dose estimate ( $\pm$ SE) =  $1.7 \pm 0.1$  Gy. The uncertainty here is slightly simplified by ignoring any errors on the dose–response curve. Applying the G function, where

$$x = t/t_0 = 7/2 = 3.5$$

so that  $G(x) = 0.413$ , the dose–response curve now becomes

$$Y = 0.001 + 0.030D + 0.248D^2$$

The dicentric frequency now corresponds to a 7 h exposure of  $2.5 \pm 0.1$  Gy.

#### 8.8.4.7. *Internal incorporation of radionuclides*

This constitutes a particular type of protracted irradiation with the added complication that exposure of the body is usually very uneven. This is because the sites of deposition of a radionuclide and its retention time depend on a large number of factors. These include the route of entry into the body, the physico-chemical form, the quality of the radiation emitted, the metabolic pathways into which the nuclide may be incorporated and the subject's physiological status.

Chromosome aberrations in excess of background levels may be seen in lymphocytes taken from people who are internally contaminated. However, because of the many confounding factors, it is not possible to use the yield of aberrations to derive a meaningful estimate of radiation dose to the whole body or to specific organs. The aberration yield may be referred to a dose–response curve in which lymphocytes have been irradiated *in vitro* with the particular radionuclide, and this may enable an estimate to be made of the *in vivo* dose to the patient's circulating lymphocytes. An example of this has been presented by DuFrain et al. [89] for an accident in which a man received a massive contamination with  $^{241}\text{Am}$ . The dose to lymphocytes, however, particularly in the case of  $\alpha$  emitters, may grossly misrepresent the dose to other cells and tissues of the body.

Thus, in general, cytogenetic studies are of limited value in cases of internally incorporated radionuclides. Exceptions exist when radionuclides disperse fairly

uniformly around the body. Isotopes of caesium and tritiated water are two such examples. Caesium tends to concentrate in muscle which is rather ubiquitously distributed and has a biphasic clearance with 10% elimination with a half-time of 2 d and 90% with 100 d. Cs-137 was the nuclide released into the community in the Goiânia accident [90, 91] and was one of the major contributors to dose from environmental contamination at Chernobyl [92]. Tritium taken in as tritiated water or gas is incorporated into the water of the body and so produces a more or less uniform irradiation. Its biological half-life is about 10 d so that, as with caesium, the exposure could be considered as chronic and, in practice, a linear dose–response would be expected. In the absence of a specific in vitro dose–effect curve for tritium, an X ray curve around 200–300 kVp will suffice. Prosser et al. [93] have demonstrated an RBE of 1.13 at low doses or dose rates for tritium with respect to 250 kVp X rays.

#### *Overexposure to tritium: An example*

An accidental inhalation of about 35 GBq (~1 Ci) of tritiated water droplets by a factory worker is described by Lloyd et al. [94]. Removal of tritium from her body was speeded up by forced diuresis. A committed dose to soft tissue was obtained from measuring the concentration and rate of excretion of tritium in her urine. Dicentric yields were measured in blood samples taken at various times after the incident, and data from 40–50 d were used for biological dosimetry as by then all committed dose had been received. Dicentric yields were referred to a linear in vitro dose response calibration coefficient,  $5.37 \times 10^{-2} \text{D}$ , producing an estimate of average dose to lymphocytes of 0.58 Gy. This value needed further multiplication by a factor of 0.66. The derivation of this factor takes account of the differing water contents of the whole body, soft tissue and lymphocytes. Aberration yield is calibrated against dose to lymphocytes whereas tritiated water delivers dose principally to the soft tissues of the body. This correction produced a biological dose estimate of 0.38 Gy with 95% confidence limits of 0.48 and 0.28 Gy, and is a more realistic comparison with  $0.47 \text{ Gy} \pm 20\%$  obtained from the urine measurements. The conversion of tritium concentration in urine to dose to soft tissue also allowed for the water content of soft tissue [94].

## 9. TRANSLOCATION ANALYSIS

### 9.1. INTRODUCTION

A recognized drawback of the dicentric and MN assays is that the damage is unstable and therefore is eliminated from the peripheral blood lymphocyte pool at the rate that cell renewal occurs (see Section 8). It has long been recognized that analysis for more persistent types of damage, e.g. stable translocations, is needed to address biodosimetry for old or long term exposures. Translocations are detectable by karyotyping, which is, however, too laborious to be applied routinely in biodosimetry. The introduction of FISH [95] has opened the possibility to detect translocations by an alternative means.

The technique employs specific sequences of DNA which can be used as probes to particular part of the genome and then by attachment of various fluorochromes to highlight or 'paint' the regions in different colours. Translocations are seen as coloured rearrangements in a fluorescence microscope (Figs 7 and 8).

FISH has many applications in medicine and in fundamental cytogenetics. In this Manual, however, only its application to biological dosimetry will be addressed. For this purpose FISH is still in the process of validation, particularly regarding questions on the true persistence of translocations. It is nevertheless already clear that FISH is more effective in detecting old exposures than the dicentric or MN assays.

In this Manual it is intended to describe the development of the method so far and to indicate possible future directions. As with the other assays there are variations in technique which are discussed. Working protocols are included in Annex II.

### 9.2. CELL CULTURE AND FIXING PROCEDURES

The procedures for obtaining blood, culturing the lymphocytes and harvesting fixed cells are similar to those described for the dicentric assay (Section 8.1). Although translocations are stable through mitosis, it is still good practice to culture so that analysis is carried out on M1 metaphases. This is particularly so if the mitotic loss of cells containing unstable aberrations could distort the mean frequency of translocations. If preparations are to be stored before FISH analysis it is better to store them as refrigerated fixed cell suspensions. Cells dispensed onto microscope slides can be stored. They should be kept at  $-20^{\circ}\text{C}$  but, even so, deterioration has sometimes been noted. Fixed cell suspensions are more convenient for transporting to other laboratories.



Generally, for biological dosimetry, only a part of the genome (e.g. three pairs of chromosomes) is painted. This leads to the requirement to score more metaphases than would be scored with the dicentric assay. It is therefore helpful and more cost effective to produce slides, each with a large number of scorable quality metaphases. This can be achieved by using isolated lymphocytes and improved culture conditions as outlined in Section 8.1.5.2.

### 9.3. PAINTING THE CHROMOSOMES

#### 9.3.1. What should be painted

Generally, painting three of the larger chromosomes (i.e. #1 to #12 — see Fig. 4), representing about 20% of the genome (see Tables I and II), leads to about 33% efficiency in detecting translocations. The percentage of each cocktail relative to the total genome is estimated from the physical lengths of chromosomes [96]. The total genomic translocation frequencies may be estimated according to a standard formula proposed by Lucas et al. [97], which applies with the assumption of simple pairwise exchanges.

Application of a pan-centromeric probe [98] simultaneously with whole chromosome paints was found to be useful to distinguish between dicentrics and translocations more accurately. It is advisable not to include chromosomes 7 or 14 in probe combinations as translocations and other aberrations involving these chromosomes can arise *in vivo* during immunological development and may thus confound the quantification of a radiation effect [99, 100].

For retrospective biological dosimetry (past exposure longer than five years), a single colour FISH for a triple cocktail of target chromosomes appears to be sufficient. Multiple colour painting technique increases the detection efficiency; it was, however, found to be more useful for mechanistic studies and also, with respect to biological dosimetry, for detecting complex translocations that can be encountered following high dose recent exposures.

#### 9.3.2. Choice of chromosome specific DNA libraries

The technique of fluorescence in situ hybridization (FISH) allows the detection of DNA sequences in chromosome, cell and tissue preparations. Since the development of this technique it has found broad application in basic biological and medical sciences. Currently, it is possible to use either blue scribed plasmid or commercially available directly labelled probes. The latter are easier to use as fewer procedural steps are needed, but they are more expensive. Moreover, it is possible to use either single colour FISH for different chromosomes or multicolour FISH in which each

chromosome can be differentially stained and thereby individually distinguished. Working protocols of these two techniques are given in Annex II.

## 9.4. SCORING CRITERIA

### 9.4.1. Selection of scorable cells

There is no firm consensus on this. Some researchers consider that all the painted material should appear to be present although this involves a certain degree of judgement because the limits of resolution with current FISH technology are about 11–15 Mbp [101]. By contrast, other researchers will accept metaphases where it is clear that loss of painted material has occurred but provided that all centromeres from the painted pairs are present. Routinely, completeness of the counterstained chromosomes is not considered but most researchers would reject a metaphase if it is obviously missing several chromosomes, e.g. <40 objects.

### 9.4.2. Nomenclature

There are several ways of describing FISH aberrations. Two specific nomenclature systems have been developed independently, and descriptions based on the conventional terminology of routine cytogenetic scoring are also used [102–105]. These have been applied in parallel to a common data set by Knehr et al. [106]. The nomenclature systems were introduced because, with partial genome analysis, the conventional terminology proved inadequate as many patterns revealed by FISH appeared to be more complicated than expected.

- (i) PAINT is purely descriptive of each aberrant painted object in the metaphase [103]. Each is therefore described individually without cross-reference to other aberrant objects in the cell. Each colour is designated by a letter, starting alphabetically with the counterstain. A capital letter designates the component that bears a centromere. Thus, with single colour painting, t(Ab) is a bicoloured object consisting of a centromeric piece of a counterstained chromosome and a non-centromeric piece of a painted chromosome. Conversely, t(Ba) is an object where the centromere is on the painted component. Multiple coloured painting is accommodated by including further letters in the nomenclature. The reader is referred to Tucker et al. [103] for full descriptions of all the abbreviations used in the system. An additional suggestion made in that paper of counting colour junctions as an index of damage relating to dose has no practical application to retrospective dosimetry.

- (ii) Savage and Simpson (S&S) [104, 105] proposed a terminology comprising numerals and letters describing each exchange in its entirety. The numerals refer to the number of objects containing painted material, and the alphabetical ordering of letters reflects how common the patterns are expected to be. This so-called S&S system applies only to single paint patterns. However, it can be used with dual and triple paint patterns but each painted chromosome has to be scored in isolation irrespective of the colours of partners [69]. This nomenclature has considerable uses in mechanistic studies, particularly, for example, in understanding complex rearrangements.
- (iii) Conventional terminology names translocations as reciprocal, terminal or interstitial [107]. The first two have also been called complete or 2-way and incomplete or 1-way translocations, respectively. The third includes inversions and insertions. Complete/incomplete or reciprocal/terminal involve mechanistic concepts. For biological dosimetry purposes they are probably best referred to as 2- or 1-way, purely on the basis of their visual appearance and with no mechanistic implications. Indeed mechanistic studies have shown that 1-way patterns do not provide a reliable estimate of exchange incompleteness [108]. An insertion is one of many types of complex rearrangement which are formally defined as arising from three or more breaks on two or more chromosomes [105]. No conventional terminology exists to describe the variety of complex rearrangements.

### 9.4.3. Recording data

The nomenclature systems described above are not mutually exclusive but rather complementary. Generally, cells are scored for the presence of different types of translocations as well as dicentrics, rings and fragments (see Figs 8 and 9). The PAINT system is more widely used than S&S but in a slightly modified way. The abbreviations of the system are used but a note is made of the associations between objects in the metaphase, thereby incorporating aspects of the conventional terminology too.

For example, t(Ba) seen with t(Ab) is regarded as a 2-way translocation (2B in S&S nomenclature), and either pattern is regarded as a 1-way translocation when seen singly in a metaphase, sometimes with an associated painted acentric. Dicentrics, dic(AB), will be recorded with or without their associated painted fragments ace(ab) or ace(b).

Complexes are recorded as such and described either as insertions, e.g. ins(Aba), or as the more complicated rearrangements. For example, t(Ba) with ace(ab) has involved at least three breaks; one on a painted chromosome and two on counterstained chromosome(s). PAINT has proved to be a particularly useful means of reproducibly describing even the most extensively rearranged chromosomes. With

complex rearrangements some researchers also find it helpful to record aberrations with coloured diagrams.

## 9.5. DATA HANDLING

The genomic translocation frequency is usually calculated by using the formula for the painted fractions of the genome [24] as follows:

$$F_G = F_p / 2.05 f_p (1 - f_p)$$

where  $F_G$  is the full genome aberration frequency,  $F_p$  is the translocation frequency detected by FISH and  $f_p$  is the fraction of genome hybridized [97], taking into account the gender of the subjects. This is more fully elaborated in Section 9.5.1.

Control levels of translocations are higher than for dicentrics, and to some extent this is due to the former being a persisting type of aberration. It is therefore more important to take the translocation background into account, particularly after low doses, when attempting retrospective biodosimetry.

Of course, a pre-exposure control blood sample from the subject is unavailable, and therefore an assumed value based on generic survey data has to be used.

The most important confounding factor to take into account is the subject's age. A strong age correlation was first demonstrated by Ramsey et al. [109] but unfortunately their data are plotted as colour junctions rather than numbers of aberrations. Sorokine-Durm et al. [110] have summarized data from eight laboratories showing full genome equivalent yields of 2-way translocations ranging from 2 to 10 per 1000 cells over the age range 10 to 80 years. The values for 2- plus 1-way translocations combined ranged from 3 to 14 per 1000 cells. Lucas and Deng [111] have also published control data showing a clear correlation with age but presenting only 2-way translocations plus one type of 1-way;t(Ab). Bearing this in mind, the two data sets are quite similar.

Studies were made to consider the influence of smoking on the background incidence of translocations. Van Diemen et al. [112] concluded no significant effect when compared with a non-smoking group up to age 50 years. However, the situation is currently ambiguous as Tucker [113] concluded that matching for smoking is almost as important a life-style factor as age matching. It is strongly recommended that a laboratory should develop its own database of control frequencies of translocations.

The procedures for data handling described previously for the dicentric assay (Section 8) such as principles of curve fitting, distributions testing, derivation of uncertainties, also apply to translocations data generated by the FISH assay.

Statistical analysis using the frequency of chromosomal aberrations is generally based on the number of cells analysed, cocktails of chromosome used and post-exposure time. On the basis of genomic frequency of translocations, the exposure dose can be calculated from the in vitro dose–response curve, and the 95 confidence intervals will be determined on the basis of the statistical uncertainties.

The conversion of data to full genome equivalence is one method, employed with FISH, that has not been covered earlier in this Manual. This is a recommended procedure to use when data are to be combined or intercomparisons are to be made between results from various studies where different combinations of whole chromosome painting probes have been used. The assumption, sometimes referred to as the Lucas formula, is that the probability of the involvement of a particular painted chromosome in an aberration is proportional to its DNA content. In essence, the larger chromosomes present a larger target. The assumption has been questioned (see, e.g., Ref. [114]) although some earlier indications of exceptional overinvolvement of some chromosomes have not been sustained by later work. A tendency has been noted [115] for the smaller chromosomes generally to be more involved than would be expected based on their DNA contents. A consensus view has emerged that possible deviations from the Lucas formula do not introduce serious errors, so that for many practical purposes conversion of data to full genome equivalence is acceptable. The best data on relative DNA contents of the human chromosomes are given by Morton [24] and the values shown in Tables I and II have been calculated from data given in his Table 4, column 2.

Lucas et al. [97] derived the equations for calculating genome equivalence, and these have been further summarized by Lucas and Deng [111]. Their application is as follows.

### 9.5.1. Single colour painting

A fraction,  $f$ , of the genome is painted (green) and the remainder,  $1 - f$ , is counterstained (blue).

*Note:*  $f_p$  in the equation above in Section 9.5 has, for simplicity, here been shortened to  $f$ .

There will be	$f^2$	green–green exchanges
	$(1 - f)^2$	blue–blue exchanges
	$\frac{2f(1 - f)}{1.00}$	blue–green exchanges
Total	1.00	

However, this total includes exchanges within the same chromosome, e.g. inversions. The total number of interchromosomal exchanges is 0.974, using the same assumption of DNA proportionality (see calculations in Lucas et al. [97]). Hence, the fraction of all translocations that are blue–green translocations is given by

$$\frac{F_P}{F_G} = \frac{2f(1-f)}{0.974} = 2.05f(1-f)$$

where  $F_P$  and  $F_G$  are, respectively, the translocation frequency measured by FISH and the whole genome translocation frequency. The same formula would apply to blue-green dicentrics.

*Example*

Suppose that chromosome pairs 1, 2 and 4 are painted. Their respective DNA contents (male) from Table I are 0.0828, 0.0804 and 0.0639.

Therefore,  $f = 0.2271$ , so that  $F_P/F_G = 0.360$ .

This combination of chromosomes painted is 36% efficient in measuring bicoloured translocations. Therefore, to obtain the full genome translocation yield the observed yield is divided by 0.36.

**9.5.2. Two colour painting**

Suppose a fraction,  $f_1$ , is painted red, another fraction,  $f_2$ , is painted green and  $(1 - f_1 - f_2) = f_3$  is counterstained blue.

There will be	$f_1^2$	red-red exchanges
	$f_2^2$	green-green exchanges
	$(1 - f_1 - f_2)^2$	blue-blue exchanges
	$2f_1f_2$	red-green exchanges
	$2f_2f_3$	green-blue exchanges
	$2f_3f_1$	blue-red exchanges
Total	$1.00$	

Again, the total interchromosomal exchanges are 0.974, and hence the fraction of all bicoloured translocations is given by

$$\frac{2(f_1f_2 + f_2f_3 + f_3f_1)}{0.974} = 2.05[f_1(1 - f_1) + f_2(1 - f_2) - f_1f_2]$$

*Example*

Suppose that chromosome pairs 1, 2 and 4 are painted red; pairs 3, 5 and 6 are painted green and the rest is counterstained blue. The fractions from Table I are  $f_1 = 0.227$  and  $f_2 = 0.186$ :

$$F_P/F_G = 2.05(0.175 + 0.151 - 0.042) = 2.05 (0.284) = 0.582$$

This combination is 58% efficient in detecting translocations. It should be noted that where a 2-way exchange between two differently coloured painted chromosomes occurs it is still only counted as a single event.

### 9.5.3. More than two colours

The calculations can be extended to multicolour FISH painting. For many colours the equation becomes

$$F_P/F_G = 2.05[\sum f_i(1 - f_i) - \sum_{i < j} f_i f_j]$$

## 9.6. CONTEMPORARY AND RETROSPECTIVE BIODOSIMETRY FOLLOWING OCCUPATIONAL AND ACCIDENTAL EXPOSURE

These studies were designed to investigate the feasibility of the FISH translocations assay for retrospective dosimetry in (1) populations with no prior biological and physical dosimetry investigation; (2) populations with known physical dosimetry estimates; and (3) populations with known biological dosimetry estimates using conventional dicentric analysis immediately following exposure. The data from the last group are considered to be the most reliable ones for comparison with translocation frequencies in order to define the stability of translocations.

Four study groups selected were composed of (1) nuclear power plant workers; (2) populations living in the contaminated areas; (3) Chernobyl cleanup workers; and (4) individuals or groups of persons accidentally exposed.

### 9.6.1. Retrospective biological dosimetry in population groups without prior personal dosimetry

In the case of the Chernobyl radiation accident, in view of the size of the exposed population and the inaccessibility of some early data obtained by Soviet scientists, it is difficult to discuss the results of later FISH studies in comparison with contemporary dosimetric information. In order to perform retrospective estimations of radiation doses, the frequency of chromosomal aberrations was determined in 15 individuals known to be severely exposed at Chernobyl, and all were treated for symptoms of the delayed stage of the cutaneous radiation syndrome.

These studies began in 1991 and follow-ups were performed until 1994 [116–118]. In 1991, biological dose equivalent estimates were determined, either by

measuring the frequency of dicentric and ring chromosomes using the Qdr method or by measuring the frequency of stable translocations using FISH with composite whole chromosome specific DNA libraries and a pan-centromeric DNA probe. With both methods, fairly comparable individual dose estimates between 1.1 and 5.8 Gy were obtained for 12 out of 15 individuals, whilst three of them showed no elevated aberration frequencies [117]. For the follow-up studies the frequencies of translocations were examined in the same donors during a period of three years from September 1991 until July 1994, when, in 11 out of 12 cases, they remained fairly constant. This permitted comparable dose estimates from the various sampling times to be made [118].

From these studies a direct conclusion on the stability of translocations cannot be made because there are no reference data immediately following exposure (i.e. biological and physical dosimetry). However, the follow-up studies indicate that translocations can remain constant from five years post-exposure time and at different dose levels.

### **9.6.2. Retrospective biological dosimetry in population or occupational exposure groups with physical dose estimates**

Several studies designed primarily to estimate absorbed doses have been carried out on the frequencies of chromosome aberrations in the lymphocytes, e.g. of the atom bomb victims of Hiroshima and Nagasaki (Japan) or in Chernobyl cleanup workers. The frequencies of translocations recorded in atom bomb survivors seemed close to expected values derived from the individuals' DS86 estimated doses compared with an *in vitro* dose–response curve [81]. These studies, therefore, supported the idea of long term persistence of translocations. However, by contrast, a significant difference was found for four workers irradiated during the Oak Ridge (USA) Y12 accident in 1959, where some years later the translocation frequencies were substantially below expected values [97].

A pilot study carried out in 1994 of about 60 personnel recruited from Estonia for cleanup work in Chernobyl in 1986 or 1987 with registered doses ranging from 0 to 300 mSv was undertaken to determine whether both dicentric and translocation analyses might verify their recorded doses [119]. In another set of investigations, 52 cleanup workers were studied with FISH painting [120]. The dicentric estimates were no longer valid but translocations could be used to verify early dosimetry carried out on only the more highly irradiated persons. For the vast majority of lesser exposed subjects, FISH was found to be impractical as an individual dosimeter. However, it has been suggested as having some value for discriminating groups of subjects exposed to different doses [119], and this is supported by the study of the Estonian cleanup workers [51, 121].

There is another data set on 75 Mayak workers for whom physical dosimetry was available and who had received their main exposure between 1948 and 1963



[122]. Cumulative external doses between 0.02 and 9.91 Sv and plutonium burdens ranging between 0.26 and 18.5 kBq were reported. At 35 to 40 years after protracted exposure using whole chromosome painting probes for chromosomes 1, 4 and 12 in combination with a pan-centromeric probe, the translocation frequencies were determined. The results showed a higher frequency of translocations in the Mayak workers in comparison with a matched control group. However, the range of translocation yields was generally lower than expected from the registered personal doses and calibration curves [119, 122].

FISH painting was carried out on metaphase preparations obtained from 73 radiation exposed residents from settlements along the Techa river. The study group comprised two subgroups living in settlements either 7 to 60 km or 78 to 148 km downstream from the facility. Both were distinguished from controls, and significantly higher mean translocation frequencies were observed [123].

Biological dosimetry studies of radiation workers at the Sellafield nuclear site with accumulated lifetime whole body doses ranging from 173 to 1108 mSv, all but three being >500 mSv, were carried out in the period 1991 to 1994. When the workers were divided into dose range cohorts the groups' mean translocation frequencies showed a significant increase with dose categories. However, by contrast, the cumulative lifetime doses were unrelated to dicentric frequencies [124].

In Hiroshima atom bomb survivors a good correlation was found between electron spin resonance dosimetry and cytogenetic dosimetry using translocation frequencies from lymphocytes of 40 survivors who lived close (approximately 2 km) to the hypocentre, and who were at least 10 years old at the time of bombing [125]. The Hiroshima atom bomb survivors studies indicate the persistence of stable translocations. However, from the other studies mentioned above, it can be concluded that some fraction of translocations seems to decrease with post-exposure time.

### **9.6.3. Retrospective biological dosimetry in persons with known biological dose estimates made shortly after accidents, using conventional dicentric analyses**

#### *Tritium accident*

The accidental overexposure to tritiated water described earlier (Section 8.8.4.7) was also examined retrospectively by FISH [126]. Initially, dicentrics had indicated an average dose of 0.38 Gy, which compared well with 0.47 Gy obtained by measuring tritium in urine. These values are average doses to soft tissues of the body as tritium incorporates into body water, delivering a more or less uniform exposure to all the soft tissues. Subsequent blood samplings showed an expected reduction in dicentric yields consistent with a disappearance half-time of 3.3 years.

Six and eleven years after the accident, FISH dosimetry was attempted using the combined yields of 1- and 2-way translocations. On the first occasion, one laboratory made the analysis by single colour painting chromosomes #1, 2 and 4, and for the second analysis the work was shared with a second laboratory that painted chromosomes #2, 3 and 5. Dose estimates were made by reference to an in vitro calibration made with tritium in one of the laboratories, which yielded a linear dose–response curve for full genome corrected total translocations of  $Y = c + (5.26 \pm 0.49) \times 10^{-2}D$ . The combined data from all the FISH scoring produced a dose estimate of 0.48 Gy.

### *Goiânia accident*

In the Goiânia (Brazil, 1987) radiation accident a large number of persons was exposed when a spent  $^{137}\text{Cs}$  radiotherapy source was broken open [90, 91]. These persons provided a good cohort for a follow-up study. Immediately after detection of the accident, lymphocytes from 129 affected individuals were analysed for the frequencies of dicentrics and rings. 29 persons had an estimated dose in the range of 0.3 to 5.9 [127]. Although most of the individuals received an inhomogeneous exposure, suggested by the presence of localized skin lesions, all cases except six showed a Poisson distribution of aberrations. Some of these victims were followed up over the years by examining the frequencies of dicentrics (analyses started immediately) as well as translocations using FISH (started after five years) for retrospective radiation dosimetry [128].

Data on translocation frequencies (using various cocktails, covering about 80% of the genome) obtained by FISH could be directly compared with the baseline original frequencies of dicentrics from the same persons [128]. The frequencies of translocation observed years after radiation exposure (from 1992 onwards) at higher doses ( $>1$  Gy) were two to three times lower than the initial dicentrics determined in 1987. For exposure levels estimated to be  $<0.9$  Gy small differences were found between the frequencies of translocation and the initial dicentric yields. The accuracy of these dose estimates might be increased by scoring more cells. However, factors such as the persistence of translocation carrying lymphocytes, translocation levels not proportional to chromosome size, and interindividual variation reduce the precision of these estimates. No decline in FISH 1- and 2-way translocations during the follow-up was found [128], which is similar to the Chernobyl studies. Straume et al. [129] also evaluated two victims of the Goiânia accident one year after their exposure using FISH. When the data were compared with dicentrics frequencies obtained immediately after the accident, lower translocation frequencies were observed.

### *German and Estonian accidents*

By contrast, in another study [130] undertaken 11 years after an accident to three radiation workers, FISH, using chromosomes #2, 4 and 8 and pan-centromere

probe, gave stable translocation frequencies that were not significantly different from the mean dicentric frequencies determined by conventional FPG staining shortly after detection of the accident. About 75% of the translocations were identified as 2-way types. Following a radiation accident in Estonia in 1994, chromosomal analyses were carried out after one month and subsequently 2, 6, 10, 12, 17, 22 and 24 months after exposure of five individuals assessed to have received approximately 1 to 3 Gy [131, 132]. In the follow-up studies, 2-way translocations remained relatively stable in all five subjects, and in one person a significant decrease in 1-way translocations was observed. Dicentrics decreased in all subjects to about 50% of the initial frequencies by 12 months post-exposure [131].

These studies indicate that translocations can persist with time. However, the issue of long term persistence of translocations depends on several factors, an important one appearing to be dose. At dose levels of >3 Gy, there is a decline in the translocation frequencies with post-exposure time.

### *Istanbul accident*

In Section 8.8.4.6, a case is described where several persons were irradiated by an unshielded  $^{60}\text{Co}$  source mixed with scrap metal. One month elapsed between the incident and recognition by the authorities that exposures to radiation had occurred. The patients had considerably depressed blood cell counts. For the five most seriously exposed persons, dicentric analysis indicated doses ranging from 2.2 to 3.1 Gy. This includes using the G function adjustment to the dose–response curve to take account of their exposures being protracted over several hours. In Section 8.8.4.5, it is noted that when exposures are sufficiently large to cause deterministic effects, such as lowered blood counts, the dicentric yields may decline appreciably over a period of a few weeks.

FISH dosimetry was also performed with the same blood specimens as were used for the dicentric assay. The analyses were performed in three laboratories [87], and the resultant dose estimates were based on the combined yields of 1- and 2-way translocations pooled from the laboratories. The FISH dose estimates, which also include the G function adjustment, were 20 to 30% higher than the values derived from dicentrics.

FISH is usually considered for deployment as a retrospective dosimeter where blood sampling occurs on a timescale of years after irradiation or where long term exposures have occurred, for example, from environmental contamination with radionuclides. This case has, however, illustrated rather well that FISH also has a role in cases where high doses are received, with moderately delayed sampling, on a timescale where, for lesser doses, the dicentric assay is usually considered to be quite adequate.

## 9.7. PERSISTENCE OF TRANSLOCATIONS WITH POST-EXPOSURE TIME IN ANIMALS

For *in vivo* studies, female Swiss mice were whole body X ray irradiated with doses of 0, 0.5, 1, 2 and 3 Gy. FISH using composite DNA libraries specific for chromosomes 1, 11 and 13 was performed on metaphase chromosomes from isolated splenocytes at 0, 7, 28, 56, 112 and 224 days post-irradiation to detect chromosomal exchanges [133]. Equal induction of dicentrics and translocations was observed immediately after irradiation for all the doses used. Dicentrics and fragment frequencies decreased exponentially with time, reaching the control level by 112 days. Genomic translocation frequencies declined with post-irradiation time, reaching stability during later intervals (112 and 224 days). The decay of reciprocal translocations (2-way) followed a slower rate than incomplete (1-way) ones (up to 20% and 50%, respectively).

In another set of experiments, male and female Swiss mice were exposed to 1 and 3 Gy of X rays. Chromosomal aberrations in bone marrow cells were analysed at 1, 7, 21 and 100 days following irradiation by FISH with mouse chromosome specific DNA libraries for cocktails of #1, 13; #2, 8; #6, 15 and X, Y; in total, 38% of the mouse genome was painted. Pooled data indicated that the frequencies of dicentrics and fragments decreased with time and reached the control level by day 21. The frequencies of translocations, for the 3 Gy group, were significantly decreased (about 40%) at day 7 and thereafter were constant up to day 100 [134].

Following irradiation with doses of 1 and 3 Gy, the frequencies of reciprocal translocations (2-way) found between days 7 and 100 post-exposure were not significantly different from those observed at day 1, whereas for incomplete (1-way) translocations a difference was found (a reduction of 45 to 60%).

Unlike dicentrics and fragments, frequencies of trisomic cells increased with time, indicating that X ray induced aneuploid cells persist with time. These results confirm the observation reported in the Chernobyl and Goiânia study groups about six years after the exposure [119, 128].

These data also indicated that, following irradiation, induced translocations are randomly distributed among the painted mouse chromosomes. However, the persistence of translocation involving individual chromosomes was found to be heterogeneous [134].

In another set of experiments, rhesus monkeys were whole body X ray irradiated with a dose of 5 Gy. Lymphocytes from unexposed monkeys were used to establish an *in vitro* dose response curve (under identical irradiation conditions). DNA libraries for human chromosomes #1 and #4 were used to detect structural aberrations in the metaphase monkey chromosomes. These two human specific chromosome probes completely labelled the monkey chromosomes #1 and #4, without any cross-hybridization to the other chromosomes. The frequency of reciprocal translocations (2-way) was determined in *in vitro* experiments and *in vivo* 2 and 5 years after

exposure. The translocation frequencies up to two years post-exposure were marginally (about 10%) less than those measured from the *in vitro* dose–response curve. However, five years after exposure they dropped to about 50% [116, 135]. In an earlier report also using rhesus monkeys, stability of the translocation frequency following whole body irradiation (at doses in the range of 0.56 up to 2.25 Gy with 2.3 GeV protons) after 28 years was reported [136]. However, in the latter study the *in vitro* calibration curve used for dose estimates was generated by using a different quality of radiation and dose rate from the *in vivo* study.

From these animal experiments performed with acute doses, it can be concluded that radiation induced 2-way translocations can persist with time, certainly after doses up to 2 Gy.

#### 9.8. PERSISTENCE OF STABLE TRANSLOCATIONS IN IN VITRO EXPERIMENTS WITH HUMAN LYMPHOCYTES

Attempts were made to investigate the fate and stability of chromosome aberrations through mitotic divisions following *in vitro* X ray irradiation of human lymphocytes. Induction, distribution and persistence of chromosomal aberrations in  $G_0$  lymphocytes were analysed in the first and up to the fourth cell division (from 48 up to 110 h). Different groups used different cocktails for chromosome painting, such as #1, 2, 4; #2, 8; #2, 3, 5 in the presence and for the first cocktail absence of a pan-centromeric probe. All cells that had been analysed by FISH were relocated to determine, by using differential staining of chromatids, that they had passed through cell divisions 1 to 4 [28]. In these studies lymphocytes were exposed to one or two doses of X rays varying between 1.5 and 3 Gy [114, 137–139].

The results of these studies are not fully in agreement. In general, it was found that translocations decline in culture more slowly than dicentrics (50 to 60% for dicentrics and 0 up to 25% for translocations per cell generation).

Guerrero-Carbajal [137] reported persistence of translocations through three cell cycles in cultures of lymphocytes that had all been irradiated. However, if irradiated and unirradiated samples were mixed (which may be representative of a non-uniform exposure), frequencies of both 1- and 2-way translocations decreased.

In one study [114] after 2 Gy, both 1- and 2-way translocations persisted equally whilst in the other two studies [137, 139] persistence of the 2-way type was more evident. Additionally, 1-way translocations were more frequent at a higher dose (3 Gy) and in first division.

In these studies, it is possible that some of the translocations scored as 1-way were actually 2-way, but the exchanged material either from painted chromosome(s) or DAPI background chromosomes was too small and thus below the limit of resolution. This problem can be solved by using a combination of chromosome paint

probes with telomeric and centromeric probes for the whole genome [35]. Following irradiation of the lymphocytes with a dose of 3 Gy, when all three probes were used, the true estimate of 1-way translocations was reduced to approximately 5% from 21% when the telomere probe was omitted.

In view of the better stability of translocation frequencies, it is clear that FISH based translocation measurements are superior to the use of conventional dicentric analysis for retrospective biological dosimetry. However, it is clear that, unlike the conventional dicentric assay, FISH retrospective biodosimetry is still in its developmental phase. To further define the stability of translocations and to validate the use of FISH based translocations for retrospective dosimetry, more studies are desirable to define (1) interindividual sensitivity and variation in control level, the influence of (2) dose, (3) chronic and acute exposure, (4) whole and partial body exposure, (5) to perform an intercomparison study to determine the reproducibility of data among different laboratories, using an identical scoring criterion (including distinction between 1- and 2-way translocations), (6) to examine the relative persistence of 1- and 2-way translocations and also to investigate whether their persistence characteristics vary with radiation quality [139].

## **10. PREMATURE CHROMOSOME CONDENSATION (PCC) ANALYSIS**

### **10.1. INTRODUCTION**

Biological dosimetry is generally performed by analysing dicentrics and/or translocations at the first mitosis following *in vitro* PHA blastic transformation. These assays have several recognized problems, namely radiation induced mitotic delay and cell death during the two day assay culture that operate specially after high doses, which can cause considerable underestimation of the radiation exposure dose [41].

The induction of PCC by fusing human lymphocytes with Chinese hamster ovary (CHO) mitotic cells in the presence of a fusing agent, polyethylene glycol (PEG), enables one to measure the chromosomal aberrations immediately following irradiation without the perturbing influence of processes associated with cell cycle progression to mitosis [36]. The use of the PCC method, in combination with conventional techniques such as C banding or FISH with chromosome specific DNA libraries with or without a pan-centromeric probe, permits the detection of breaks, dicentrics and rings as well as translocations. This assay has been proposed as a biodosimetric tool by analysing the frequencies of chromosomal aberrations, i.e. excess of breaks, dicentrics and translocations [37, 39, 43, 140–142].

The PCC assay is useful to determine exposure to low doses as well as following life threatening high acute doses of low and high LET radiation. Moreover, it can discriminate accurately between total and partial body exposures [32, 141]. Since with the PCC assay the number of normal cells reflects more accurately the proportion of unirradiated lymphocytes, this method is efficient for detecting even a small spared fraction (as low as 5%). The PCC assay similarly could also be better able to quantify small localized burns from partial body exposures. Recently, specific inhibitors of protein phosphatase (i.e. okadaic acid and calyculin A) were employed to induce PCC [38, 39, 45, 143]. When protein phosphate inhibitors are used for drug induced PCC, it is necessary to stimulate lymphocytes to grow under similar conditions as with metaphase analysis (48 h).

## 10.2. CELL CULTURE AND CELL FUSION CONDITIONS

### 10.2.1. Using CHO mitotic cells

Chinese hamster ovary (CHO) mitotic cells should be prepared before performing the PCC analysis. CHO cell cultures are typically set up from stock lines. These are readily available, very easy to handle and have a short cell cycle of approximately 12 h. CHO cells can be grown in 750 mL tissue culture flasks or roller bottles, in complete medium (composed of F10, 15% newborn calf serum and antibiotics). Mitotic cells can be obtained by adding Colcemid (at a final concentration of 0.1  $\mu\text{g}/\text{mL}$ ) for 4 to 6 h when cultures are half-full, followed by mitotic shake-out. Mitotic cells from one flask or roller bottle can be isolated several times per day; therefore, after each isolation, fresh medium supplemented with Colcemid should be replaced in the flasks containing the remaining cells. The mitotic cells can be prepared in large quantities in advance and kept in a freezer at  $-80$  to  $-110^\circ\text{C}$  before use.

### 10.2.2. Isolating lymphocytes

Generally, Ficoll Hypaque should be used for isolating lymphocytes as described in the earlier Section 8.1.5.2. This has the advantage that when enough lymphocytes are isolated, a part can be used immediately and the rest frozen at  $-120^\circ\text{C}$  for future use, if found necessary.

### 10.2.3. Fusing agent

Generally, polyethylene glycol (PEG) of molecular weight 1450 should be used, and the desired concentration for fusion is 40% v/w (in F10 medium or PBS).

#### **10.2.4. Fusion and chromosome condensation processes**

To induce prematurely condensed chromosomes, lymphocytes should be fused with mitotic CHO cells (ratio 5:1) that possess a mitotic promoting factor in the presence of PEG. The fusion process takes only 4 min (1 min in PEG alone, and then wash medium F10 should be added gradually). This is followed by a one hour incubation in complete medium with Colcemid at 37°C [37, 43].

#### 10.3. FIXATION PROCEDURES

In principle, this method is similar to the method used for metaphases but the optimum timings and concentrations vary slightly. Lymphocytes should be treated with a hypotonic solution of KCl (0.075M) and kept in a prewarmed waterbath (at 37°C) for 3 to 4 min, and, following centrifugation, cells can be fixed in a mixture of acetic acid:methanol (1:4).

Slide preparation is performed by using the standard technique that is similar to other assays described earlier (see Section 8.2).

#### 10.4. STAINING PROCEDURES

The choice of staining technique depends on the biological end point to be analysed, as follows:

##### **10.4.1. Standard chromosome breaks analysis**

For the purpose of analysing chromosomal aberrations as radiation induced chromosome breaks, slides can be stained with conventional Giemsa (Gurr improved R66) or the FPG technique as was already described in Section 8.3 [37, 144].

##### **10.4.2. Dicentric analysis using C banding**

In addition to chromosome breaks, dicentrics can also be visualized. For this, slides should be pretreated with barium hydroxide and salt solution, followed by Giemsa staining, which highlights the centromeric region of all chromosomes so that dicentric chromosomes can be easily distinguished from monocentrics [40, 140].



### 10.4.3. Translocation and dicentric analysis using the chromosome painting assay

The frequency of radiation induced translocations and dicentrics can also be determined with the PCC preparations by employing the FISH technique, using either chromosome painting probes alone or in combination with a pan-centromeric probe. The latter gives more accurate discrimination between translocations and dicentrics, as described in Section 9.3 [43].

## 10.5. ANALYSIS OF SLIDES

Criteria for analysis of slides are similar in part to those described in Section 8.4 (i.e. coding slides, scanning parameters, etc.). PCC spreads can be located manually or by use of automated metaphase finder systems that are in more general use [141]. It is advisable to facilitate scoring by using a recording system that permits marking each chromosome piece on a drawing or image of the PCC spread. A drawing tube microscope attachment can be used to visualize PCC spreads on a much larger scale and record markings on a drawing. Some metaphase finding systems are equipped with specialized applications that allow annotation of digitized images. The microscope stage co-ordinates of PCC spreads on slides should be recorded and the selection method of candidate PCC spreads for scoring should not introduce bias to distort aberration yields.

Analysis involves counting the number of chromosome elements, which appear as single chromatids and can be discriminated easily from the CHO mitotic chromosomes in the human interphase PCC spreads following Giemsa staining. When the FPG technique is used the human chromosomes appear darkly stained while the CHO cells, which were grown for more than two cell cycles in medium supplemented with BrdU, display the harlequin effect and appear very lightly stained (see Fig. 10, in Section 6.4). When the FISH assay is used, cot-I hamster DNA can be used to mask all signals in the CHO chromosomes so that only the appropriate human PCC are highlighted (Fig. 14).

## 10.6. SCORING CRITERIA

The appearance of the PCC can be used to define easily the cell cycle position of the lymphocytes at the time of their treatment. Cells that were in  $G_1$ , S and  $G_2$  appear as single chromatid, pulverized chromosomes and having two chromatids, respectively [125]. For biological dosimetry with Giemsa stained preparations, one scores only the spreads comprising single chromatids, i.e. human lymphocytes that were treated in  $G_0/G_1$ , and each element represents one human chromosome.

Therefore, in unirradiated lymphocytes 46 elements will be scored. The number of chromosome elements in the exposed samples is recorded, and the induced frequency is estimated by simply subtracting the value obtained in untreated samples. In cases of suspect partial body exposures an alternative analysis method, Qpcc, which involves the analysis of the yield of excess PCC fragments in damaged cells (containing excess PCC fragments) has been introduced [141]. This method is identical in concept to the Qdr method introduced by Sasaki and Miyata [75]; see Section 8.8.4.4. Following C banding or using a pan-centromeric probe and chromosome specific DNA libraries employing the FISH technique, slides can be scored for the presence of dicentrics and/or translocations (see Fig. 14), recorded and analysed, as described in Sections 8.4 and 9.4.

## 10.7. APPLICABILITY OF THE PCC ASSAY FOR BIOLOGICAL DOSIMETRY AND ESTIMATION OF WHOLE AND PARTIAL BODY EXPOSURE

The scientific and medical response to an accidental overexposure to ionizing radiation should include an attempt to estimate absorbed doses to irradiated persons. This can assist in the planning for possible treatments of high dose injuries or the counselling and reassurance of persons who received low doses. Using conventional metaphase and MN analysis this can be done only when lymphocytes reach mitosis (i.e. after 48 and 72 h, respectively), and heavily damaged cells, which grow slowly, may not have divided by this time. Moreover, after a radiation accident, there is sometimes an urgent need to determine the absorbed dose as quickly as possible. In this respect, the PCC method opens up the possibility of scoring aberrations within a few hours (3–4) of blood sampling. Therefore, mitotic delay and cell death during in vitro culture following a high dose of radiation have no influence on the yield of PCCs. Additionally, since this assay reflects the initial damage, it improves the level of detection of lower doses [37, 40, 42].

In the case of a severe radiation accident involving high acute doses, it is necessary to determine whether the exposure is uniform or not, in order to help the clinician to make decisions on medical management including transplantation of allogenic bone marrow or treatment with drugs. If there is a likelihood that some of the patients' own bone marrow cells have survived because of inhomogenous exposure, then transplantation is not indicated.

### 10.7.1. Experimental studies

Attempts were made to investigate the efficiency of the three cytogenetic methods (dicentrics, MN and PCC) for biological dosimetry and for assessment of an unirradiated fraction in the case of an acute exposure at 5 and 8 Gy in vitro (using human

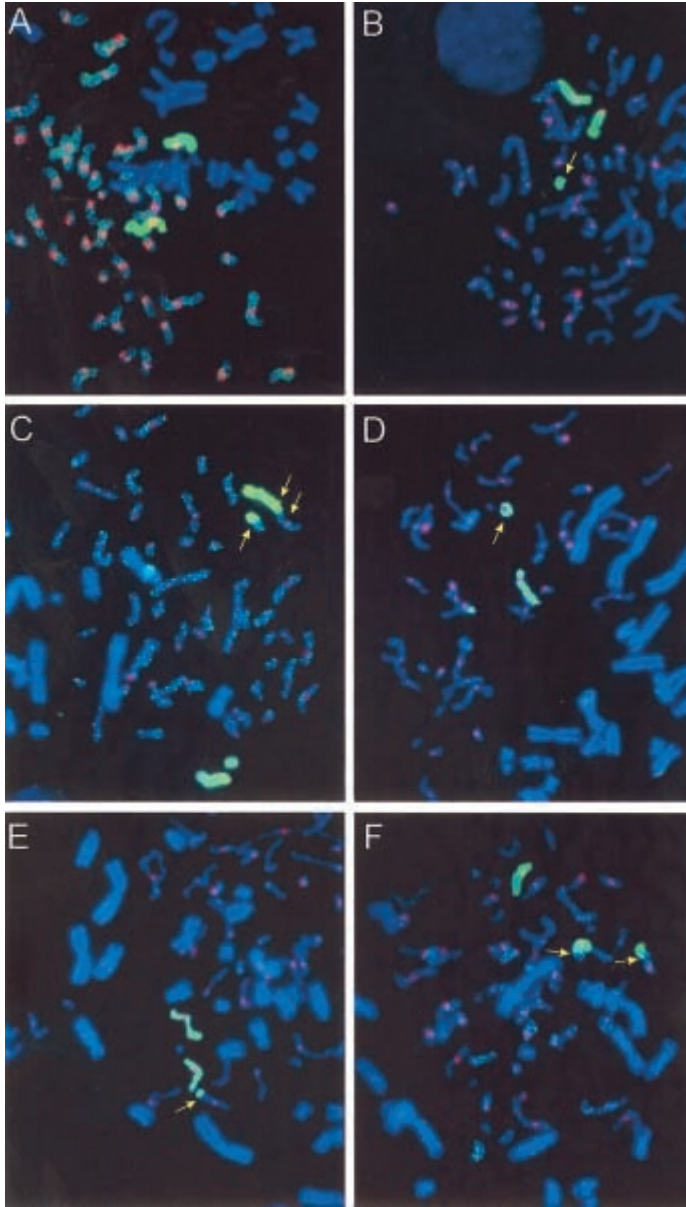


FIG. 14. PCCs with double coloured FISH, the combination of chromosome paint (#8) and a pan-centromeric probe for whole genome. In unirradiated control (A) normal PCC. In irradiated cells, arrows indicate (B) excess of break in PCC,  $ace(b)^*$ ; (C) dicentric,  $dic(BA)^*$ , bicoloured fragment,  $ace(ba)^*$ ; (D) ring,  $r(B)^*$ ; (E), (F) terminal translocation,  $t(BA)$  and  $t(AB)^*$ . (Definition of painting, according to the Protocol for Aberration Identification and Nomenclature Terminology, PAINT [43].)

lymphocytes) and in vivo (using rhesus monkeys). Although all three methods appeared to be able to estimate the dose in the case of 100% (whole body) irradiated samples, only the PCC technique was able to detect inhomogenous exposure when a small fraction, as low as 5% in vitro and 6% in vivo, had been irradiated [41, 144, 145]. By contrast, the dicentric method required larger volumes to be exposed and calculational adjustments to the data using the Qdr or contaminated Poisson procedures (Section 8.8.4.4). The MN method was least effective for interpreting partial exposures.

### 10.7.2. A radiation accident investigation

Soon after the technique, calibrated in vitro with 200 kV X rays, was published [38], the opportunity arose to examine a serious radiation accident by the PCC method where condensation is induced by okadaic acid. Biological dosimetry was performed on three seriously exposed victims of the Tokai-mura criticality accident in Japan in 1999 [146]. It had been proposed that the method could be used with PCC rings being scored in the event of a very high overdose. The perceived advantages of using rings are their easier identification than dicentrics in Giemsa stained PCC preparations and their lower induced frequencies at doses where yields of dicentrics could be so high that reliable scoring becomes difficult.

The frequencies of PCC rings per 100 cells from samples obtained 9 h after the accident were 150, 77 and 24, which, respectively, led to dose estimates of >20, 7.4 (95%C.I. 6.5–8.2) and 2.3 (1.8–2.8) Gy Eq. The unit designated Gy Eq is gray equivalent to 200 kV X rays. One should bear in mind that the exposures were to a mixed field of gamma and neutron radiation, and the equivalent dose unit Sv (Section 2) is inappropriate to use at such high doses because it is based on the judged risks of stochastic effects at low doses. For the most highly irradiated person the dose could only be approximated to >20 Gy Eq because the published in vitro calibration [38], showed a levelling off (saturation) in the linear quadratic dose–response approaching 20 Gy of 200 kV X rays.

Parallel analyses of the blood samples were also made by conventionally scoring dicentrics and rings in metaphases. Because such high exposures had occurred, with a consequential rapid fall in peripheral lymphocyte counts, the cells were cultured by a method to maximize the likelihood of obtaining metaphases [147]. This method concentrates the lymphocytes using a Ficoll Hypaque column and is similar to that described in Section 8.1.5.2. This yielded, for the most heavily irradiated patient, 715 dic (dicentrics) and 188 rc + ra (centric + acentric rings) in 78 cells where every metaphase was damaged. The corresponding yields for the other two persons were 479 dic and 55 rc in 175 cells and 191 dic + rc in 300 cells. These led to dose estimates of 22.6 Gy Eq by dic alone or 24.5 Gy Eq by dic + rc + ra for the most seriously exposed person, 8.3 Gy Eq by both dic alone and dic + rc for the second person and 3.0 Gy Eq by dic + rc for the least serious case. For the first two patients Gy

Eq is relative to published dose–response data for 1.9 MeV X rays [148] and to  $^{60}\text{Co}$  gamma rays for the third. The reason for referring to 1.9 MeV X rays is that these dose response data included measurements at very high doses. It may be concluded that the two methods of biological dosimetry produced comparable dose estimates. They were also in reasonable agreement with physically reconstructed neutron doses made by sodium activation analysis.

## 11. THE MICRONUCLEUS (MN) ASSAY

### 11.1. INTRODUCTION

Ionizing radiation can induce the formation of acentric chromosome fragments and malsegregation of whole chromosomes. Acentric chromosome fragments and whole chromosomes that are unable to interact with the spindle lag behind at anaphase, and as a result they are not included in the main daughter nuclei. A lagging chromosome fragment or whole chromosome forms into a small separate nucleus; hence the term micronucleus.

The peripheral blood lymphocyte MN assay was first described by Countryman and Heddle [46]. However, in this original method no attempt was made to determine whether the cells scored had actually completed nuclear division *in vitro*. A more reliable approach was eventually developed on the basis of the use of the cytokinesis inhibitor, cytochalasin-B. Using cytochalasin-B, Fenech and Morely were able to demonstrate [47, 48] that cells that had completed one nuclear division could be accumulated and recognized as binucleate (BN) cells and that MN could be specifically and efficiently scored in these BN cells while excluding non-dividing mononuclear cells that were unable to express MN *in vitro* (Fig. 11, Section 6). Consequently, the results obtained with the MN assay are not so confounded by interindividual and interexperimental variation in the frequency of dividing cells which has been shown to have a profound effect on the observed MN frequency [48, 149–151]. The resulting cytokinesis blocked MN (CBMN) assay has since become the standard method for measuring MN in cultured lymphocytes.

Lymphocytes collected in a blood sample are themselves the result of cell divisions occurring *in vivo*. One might therefore expect that some may already contain MN. Thus, it has been suggested that scoring MN in mononuclear lymphocytes in conventional blood smears could be particularly useful for evaluating long term exposure to ionizing radiation when a steady state level of such cells may occur [152, 153].

To identify whether the MN formed was due to chromosome breakage or chromosome loss, antibodies to kinetic proteins or centromeric DNA specific probes were applied [154–156].

One of the notable advantages of the MN assay is that it enables the enumeration of nucleoplasmic bridges (NPBs) between the two main nuclei in a BN cell [157]. NPBs are thought to originate from dicentric chromosomes in which the two centromeres are pulled to opposite poles of the cells.

## 11.2. CELL CULTURES

The lymphocyte culture method is similar to that described in Section 8 for obtaining metaphases. The main differences, however, are that cytochalasin B is added to the cultures, bromodeoxyuridine and Colcemid are not used, the culture time is extended to 72 h, and hypotonic treatment, fixation and centrifugation are modified to preserve the cell cytoplasm. The preparations are either conventionally stained with Giemsa or processed to highlight centromeres using FISH and pan-centromeric probe. Detailed protocols are given in Annex IV.

## 11.3. SCORING CRITERIA

### 11.3.1. Criteria for selecting binucleate cells which can be scored for micronucleus frequency

The cytokinesis blocked cells that may be scored for MN frequency should have the following characteristics:

- (a) The cells should be binucleate.
- (b) The two nuclei in a BN cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.
- (c) The two nuclei in a BN cell should be approximately equal in size, staining pattern and staining intensity.
- (d) The two nuclei within a BN cell may be unconnected or may be attached by one or more fine nucleoplasmic bridges which are no wider than 1/4th of the nuclear diameter.
- (e) The two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of either nucleus are distinguishable.
- (f) The cytoplasmic boundary or membrane of a BN cell should be intact and clearly distinguishable from the cytoplasmic boundaries of adjacent cells.

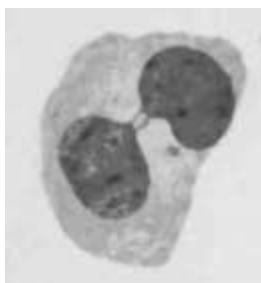
### 11.3.2. Criteria for scoring micronuclei

MN are morphologically identical to but smaller than the main nuclei. They also have the following characteristics:

- (a) The diameter of MN in human lymphocytes usually varies between 1/16th and 1/3rd of the mean diameter of the main nuclei, which corresponds to 1/256th and 1/9th of the area of one of the main nuclei in a BN cell, respectively.
- (b) MN are non-refractile and can therefore be readily distinguished from artefacts such as staining particles.
- (c) MN are not linked or connected to the main nuclei.
- (d) MN may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.
- (e) MN usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

### 11.3.3. Criteria for scoring nucleoplasmic bridges

Nucleoplasmic bridges are observed in binucleated cells following exposure to clastogens such as ionizing radiation. They are a continuous link between the main nuclei in a BN cell and are thought to be due to dicentric chromosomes in which the centromeres were pulled to opposite poles during anaphase. The width of a nucleoplasmic bridge may vary considerably but usually does not exceed 1/4th of the diameter of the nuclei within the cell. The nucleoplasmic bridge should have the same staining characteristics as the main nuclei. On very rare occasions more than one nucleoplasmic bridge may be observed within one BN cell. A BN cell with a nucleoplasmic bridge often contains one or more micronuclei. An example of a BN cell with two nucleoplasmic bridges is illustrated in Fig. 15.



*FIG. 15. A cytokinesis blocked binucleate lymphocyte with two nucleoplasmic bridges and a micronucleus (Giemsa stained).*

## 11.4. DATA HANDLING

### 11.4.1. Dose-response

The procedures for producing *in vitro* dose-response calibration curves are as previously described in Section 8.7. However, ideally they should be made with lymphocytes from male and female donors from different age groups to take account of possible age and gender effects. Linear and linear-quadratic models should be used for fitting the dose-response relationship [48, 158–160]. The coefficient of variation for replicate measurements within the same experiment and for different experiments should be estimated to derive the number of replicate cultures required to obtain a reliable measure of MN frequency. Typical low dose response data for X rays are shown in Table VII. By taking into account inhomogeneity between persons within each dose point, these data fit best, by the iteratively reweighted least squares method, to the linear model  $Y = (0.0093 \pm 0.0009) + (0.0781 \pm 0.0071)D$ .

Published dose-effect curves from different laboratories for acute low LET radiation show considerable variations. Some of these curves are even inconsistent with basic radiobiophysical expectations of the effectiveness of various radiation qualities. This marked interlaboratory variability highlights an important confounding factor that currently limits the accuracy of using MN as a biological dosimeter [149].

### 11.4.2. Background frequency

The background frequency of MN is reported to be quite variable; values ranging from 2 to 36 per 1000 CB cells have been recorded [149]. Age and gender dependences have been noted [48, 161]. A number of possible causes has been suggested, such as dietary factors [162] as well as variable exposure to a wide range of environmental clastogens and aneugens.

The variability clearly poses limitations on using MN as a biological dosimeter, particularly for low doses where pre-existing individual background frequencies are not known. Estimates have been made, suggesting that the cytokinesis blocked (CB) MN assay in its basic form could only detect *in vivo* exposures in excess of 0.3 Gy X rays [163]. Ways in which lower and possibly less variable background frequencies can be obtained have been suggested. These rely on considering just certain subsets of the scoring data. For example, one might apply the pan-centromere FISH probe and considered, just those MN that do not exhibit a positive signal for centromeres [164]. These MN probably derive from chromosome fragments rather than whole chromosomes. Inclusion of NPBs may be an advantage as their spontaneous frequency is very low, 50 to 90% lower than that of the MN [157].



TABLE VII. DOSE–RESPONSE DATA FOR MN IN BN LYMPHOCYTES AFTER EXPOSURE IN G<sub>0</sub> TO LOW DOSE X RAYS DELIVERED AT 0.5 Gy/min [48]

	MN per 1000 BN cells				
	0 Gy	0.05 Gy	0.10 Gy	0.20 Gy	0.40 Gy
Subject A	12.6	12.8	23.4	19.0	48.8
Subject B	4.2	9.8	12.6	31.2	42.0
Subject C	9.6	11.2	17.2	23.0	48.8
Subject D	14.6	18.8	20.6	27.0	40.2
Subject E	10.6	13.2	19.8	20.6	43.8
Subject F	8.2	13.8	19.6	25.4	30.9
Mean (±SE)	9.9 (±1.5)	13.2 (±1.3)	18.9 (±1.5)	24.4 (±1.8)	42.4 (±2.7)

### 11.4.3. Nuclear division index (NDI)

When scoring CB lymphocyte preparations one observes cells with 1, 2, 3, etc., main nuclei. The relative frequencies of the cells may be used to define cell cycle progression of the lymphocytes after mitogenic stimulation and how this has been affected by the exposure.

This is referred to as the NDI [155].

Five hundred viable cells are scored to determine the frequency of cells with 1, 2, 3 or 4 nuclei and the NDI is calculated by using the formula:

$$\text{NDI} = (M1 + 2 \times M2 + 3 \times M3 + 4 \times M4)/N$$

where M1 to M4 represent the number of cells with one to four nuclei and N is the total number of viable cells scored.

## 11.5. APPLICATION OF THE MN ASSAY FOR BIOLOGICAL DOSIMETRY

### 11.5.1. Triage

It has been proposed that the CBMN method has a potential for application in a mass screening role for rapid biological dosimetry to confirm the initial triage (sorting) of large numbers of persons possibly exposed during a major radiation accident [150].

However, it has not yet been reported as having been used for such a purpose. The perceived advantage is the speed with which data can be collected. The CBMN assay has been evaluated for this role in simulated accidents with in vitro irradiated blood samples.

Thierens et al. [160] compared the sensitivity of the CBMN assay to that of conventional dicentric analysis and translocation analysis by FISH of chromosomes #2, 4 and 8. The conclusion was that, with restriction of scoring time to one day for biomonitoring purposes, the CBMN assay with centromere detection had the best dose detection limit (0.1–0.2 Gy), compared to the CBMN assay without centromere detection (0.2–0.5 Gy), conventional analysis of dicentrics (0.5 Gy) and FISH analysis of translocations (1.0 Gy).

### **11.5.2. Studies with patients**

The opportunity has been taken for testing the CBMN assay with cancer patients receiving radiotherapy. In groups of prostate and cervical cancer patients undergoing fractionated teletherapy regimes, doses estimated by MN agreed quite well with averaged whole body doses calculated from the radiation treatment plans plus cumulative dose–volume histograms [165, 166].

### **11.5.3. Investigation of radiation accidents**

#### *11.5.3.1. Chernobyl*

The CBMN assay has also been used successfully for assessing the protracted exposure due to incorporation of long lived radionuclides by residents in the vicinity of the Chernobyl nuclear power plant. Eighty individuals who were located between 100–200 km from Chernobyl at the time of the accident in 1986 were tested for their MN frequency in BN lymphocytes between 1989 and 1991 after migration to the USA [167]. In this study whole body counts for  $^{134}\text{Cs}$  and  $^{137}\text{Cs}$  were performed, so that the MN frequency could be related to body dose. Multiple regression analysis of the data from the 80 subjects showed that (a) the MN frequencies were significantly associated with the radiocaesium activity level ( $P = 0.004$ ) and (b) the estimated internal absorbed dose (which ranged from 0.6 to 9.2 mGy) was significantly and positively correlated with MN frequency ( $R = 0.71$ ).

#### *11.5.3.2. The Istanbul accident*

For accidents involving a few subjects and where speed in obtaining results has not been so vital, most laboratories have chosen to use the dicentric assay. Thus there are few published accounts of MN being used as a biological dosimeter soon after an

accident. One example, however, is the accident in Istanbul [87, 88] previously described in Sections 8.8.4.6 and 9.6.3.4, where ten scrap metal workers were irradiated by an unshielded former radiotherapy  $^{60}\text{Co}$  source. Lymphocytes sampled ~1 month after the exposures were assayed for MN as well as for dicentric and FISH translocations. Using data pooled from two laboratories, MN derived dose estimates were made for eight of the subjects and gave values in the range 0.7–2.7 Gy, in excellent agreement with doses obtained from dicentrics. It was noted in Section 9.6.3.4 that the dose estimates from FISH were about 20 to 30% higher than those based on the dicentric yields and this was probably due to the subjects' severely depressed blood cell counts. The same tendency to underestimate doses in such a situation would also apply with the MN assay because this too is a class of damage that has a limited in vivo persistence.

## **12. AUTOMATIC ANALYSIS OF CHROMOSOMAL ALTERATIONS**

### **12.1. INTRODUCTION**

Automated analysis for cytogenetic assays is not yet routinely used by many laboratories for biodosimetry, although it is likely to increase as systems improve. In the following section a brief description is given of the development of automation and its current status. The primary incentive for automating analysis is labour saving where, particularly for assessing exposure to low radiation doses, large numbers of cells need to be scored. Attempts have been made to automate scoring for all four assays described in this manual.

### **12.2. DICENTRIC ASSAY**

Giemsa solid stained metaphase spreads were analysed by using a metaphase finder [168–170]. This was approached as a two step procedure in which metaphase spreads were first fully automatically located and quality ranked to discriminate well spread, probably complete metaphases from incomplete clusters of chromosomes and artefacts. The images were then presented automatically on demand in focus to the operator for routine scoring by eye. Use of the metaphase finder at least halved the technician time required for routine dicentric scoring and was considered a worthwhile investment in itself.

Attempts were then made to use an image analyser for automatically detecting dicentric chromosomes [171]. Overall the system found almost 40% of dicentrics known to be on the slides by previous manual scoring. In another study the metaphase finder combined with the 'dicentric hunter' was used [172]. This was again with Giemsa solid stained chromosomes, and the software looked at the outline of objects, trying to detect 2 'waists' centromeres, measured the distance between the waists and also the overall length of the object. Again a similar value of ~40% efficiency for recognizing dicentrics on slides was obtained

One of the main difficulties encountered in this approach was composite objects comprising chromosomes touching at their telomeres and so posing a problem for correct discrimination of dicentrics. 'Automatic scissors' software, developed for interactive karyotyping, is available for separating touching and overlapping objects but this slows down the analysis time and does require some operator intervention. More recent developments show promise for improving the resolution of dicentrics identified automatically by using a human pan-centromeric DNA probe and colour pigment immunostaining [173].

### 12.3. MICRONUCLEUS ASSAY

Fully automated analysis of MN in CB binucleate lymphocytes has been approached by image analysis methods concentrating on pattern recognition procedures [174]. A PC based system was developed with an image processing board and a board for microscope control. In this system the main nuclei plus MN and cytoplasm are analysed separately and sequentially by capturing images from gallo-cyanin stained nuclei plus MN and naphthol yellow-S stained cytoplasm from one microscope field by using different filters. The current capacity to detect 63% of binucleated cells and 57% of the MN within them is quite acceptable. However, it is essential to avoid false positives; therefore artefact rejection procedures need to be improved [174–176]. Attempts have also been made to analyse MN automatically with a non-microscope based approach by using flow cytometry [177].

### 12.4. PREMATURE CHROMOSOME CONDENSATION ASSAY

The approach here is essentially similar to that for dicentric analysis by using automated metaphase finding on Giemsa stained preparations [178]. The images are then passed to the operator for scoring by eye. The speed of analysis is approximately three times faster than fully manual analyses.

## 12.5. FISH BASED TRANSLOCATION ASSAY

Some considerable success has been obtained by using FISH staining of 3 to 4 pairs of chromosomes [178]. A system consisting of a PC and a cooled CCD camera was developed. It was based on a two step approach: the finding of metaphases with counterstain fluorescence, followed by the detection of translocations involving chromosomes labelled with whole chromosome paint. From the candidate list of translocations, similar false positive and false negative rates have been measured on fluorescence stained lymphocyte preparations (about 10%), as were reported with for candidate dicentrics on Giemsa stained slides [180]. A longer screening time was needed for fluorescence; 1 h per slide, of which 25 min are necessary for autofocussing, compared with a few minutes per slide with bright field microscopy. Therefore, a larger chip size is being used with the CCD camera to increase the speed of scoring. For detection of chromosome paints, a relatively simple threshold based on the grey value histogram combined with some morphological operations seems to be sufficient to detect the chromosomes or chromosome parts labelled with the whole painting probe [179]. The suitability of the system for scoring translocations was tested in a study to detect X ray induced translocations involving chromosome #4. A comparison was made between automatic and manual scoring, and the efficiency of the automatic assay was found to be approximately 90% of that obtained manually.

By increasing the number of hybridized chromosomes in one colour, the sensitivity of the method can be improved. However, when more chromosomes are painted, procedures to separate eventual touching and/or overlapping chromosomes are essential [181]. Piper et al. [182] reported on the construction of a fluorescence metaphase finder with commercially available hardware and a standard Unix workstation. A cocktail of the three chromosomes #1, #2 and #4 was used and a comparison was made with manual scoring. The results showed that the amount of time required for analysis was reduced by a factor of three. Furthermore, the metaphase finder found more scorable spreads than did visual scanning. Machine assisted scoring had additional benefits notably that digitized images of metaphases sometimes assisted the analysis of chromosome rearrangements because cells could be revisited easily for re-examination and further analysis. This system is further modified by using a binary decision tree for classification of observed metaphases and for improving scanning accuracy [183]. Another advantage found with digitized coloured images held in a computer is that they can be enhanced electronically, and this can sometimes permit better discrimination than can be achieved by eye, of very small translocated pieces of chromosomes.

An obvious extension that is being addressed is to analyse multicolour FISH preparations by combining chromosome and centromere specific DNA libraries for automated analysis of translocations and dicentrics simultaneously.

### 13. SAFETY OF LABORATORY STAFF

Staff should be conforming with their national and institutional legislation or regulations regarding safe working generally in laboratories. There are some particular features concerning safety in cytogenetics laboratories that are worth highlighting.

#### *Infection*

Handling human blood poses some risk of blood borne parasites and infections being acquired by laboratory staff. All specimens should be regarded as being potentially infective even if they are known to be derived from healthy persons. Specimens should be unpacked and manipulated in a class 2 microbiological safety cabinet. Setting up cultures in such a cabinet has the added benefit of minimizing culture failure due to microbial contamination. Use of sharps, e.g. hypodermic needles, should be kept to a minimum to reduce risk of needle stick injuries. Suitable disinfectants should be available to deal with spills. All biological waste and used disposable plasticware should be sterilized, for example by autoclaving, before disposal.

Staff should be offered vaccination against hepatitis B. Currently, there is unfortunately no vaccine for human immunodeficiency virus (HIV). The legal and ethical position regarding HIV testing of blood samples upon receipt differs between countries, and researchers should ascertain their national requirements. It should be noted that when blood samples are accepted from abroad, depending on the country of origin airlines may require the sender to provide a certificate confirming that the samples have tested HIV negative.

#### *Optical*

Ultraviolet lamps may be used in

- (a) sterilizing the interior of microbiological safety cabinets;
- (b) exposing slides during the FPG staining procedure;
- (c) fluorescence microscopes.

For (a) and (b), shielding and working procedures should be in place to avoid direct irradiation of the skin or eyes of laboratory staff. For (c), microscopes are engineered to be inherently safe during normal use.

#### *Chemical*

Certain fine chemicals and pharmaceuticals are used routinely in the procedures covered in this Manual. When present in cultures or used in staining procedures, they

are mostly used in small volumes and in dilutions that generally present no health hazard. They are, however, made up and stored in concentrated stock solutions. The main reagents of concern and their internationally agreed risk phrases (R numbers) are listed below:

Benzylpenicillin	R 42; 43
Bromodeoxyuridine	R 20; 21; 22; 46; 61
Calyculin A	R 23; 24; 25; 38
Colcemid	R 25; 63
Cytochalasin B	R 26; 27; 28; 63
Formamide	R 37; 38; 41; 61
Giemsa stain	R 20; 21; 22; 40; 41
Heparin	R 36; 37; 38
Hoechst stain	R 23; 24; 25; 36; 37; 38
Hypaque	R 42; 43
Okadaic acid	R 23; 24; 25; 38
Pepsin	R 36; 37; 38; 42
Phytohaemagglutinin	R 20; 21; 22; 43
Ribonuclease A	R 20; 21; 22; 38
Streptomycin sulphate	R 20; 21; 22; 61

Key:

R20	Harmful by inhalation
R21	Harmful in contact with skin
R22	Harmful if swallowed
R23	Toxic by inhalation
R24	Toxic in contact with skin
R25	Toxic if swallowed
R26	Very toxic by inhalation
R27	Very toxic in contact with skin
R28	Very toxic if swallowed
R36	Irritating to eyes
R37	Irritating to respiratory system
R38	Irritating to skin
R40	Possible risk of irreversible effects
R41	Risk of serious damage to eyes
R42	May cause sensitization by inhalation
R43	May cause sensitization by skin contact
R46	May cause heritable genetic damage

- R61 May cause harm to the unborn child
- R63 Possible risk of harm to the unborn child



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# ANNEXES –WORKING PROTOCOLS

## Annex I

### DICENTRIC ASSAY

It is not the intention of this Manual to attempt to impose a strictly detailed protocol for making and processing lymphocyte cultures. The variety of materials and methods which are commonly used by laboratories around the world have been described and discussed, and probably no two laboratories adopt precisely the same technique. Nevertheless, it is felt that some workers would find a detailed, step by step description of a reliable method to be helpful.

#### I-1. LYMPHOCYTE CULTURE

##### I-1.1. Materials

- (1) Heparinized whole blood.
- (2) Phytohaemagglutinin (PHA), commercially available. If supplied freeze dried, it should be reconstituted with sterile analytical grade water.
- (3) Eagle's minimum essential medium (MEM) culture medium, commercially available: ready to use,  $\times 10$  concentration or powdered. Working concentrations should be made up with sterile analytical grade water. L glutamine may need to be added according to the manufacturer's instructions. The pH will need to be adjusted with sterile sodium bicarbonate.
- (4) Antibiotics may need to be added to the medium made from concentrate. Add 1 mL of a stock solution of antibiotics in saline to 100 mL of medium. The stock solution should contain 10 000 IU/mL of benzylpenicillin and 10 mg/mL streptomycin sulphate and can be stored frozen.
- (5) Bromodeoxyuridine (BrdU). Add 1 mL of a stock solution to 100 mL of medium. The stock solution is 6.4 mg of BrdU dissolved in 10 mL of medium and membrane filtered. This will give a final concentration in the culture of 15  $\mu\text{M}$ . The stock can be stored for one month in the dark at 4°C or for several months at -20°C.
- (6) Heat inactivated (56°C for 0.5 h) foetal calf serum, commercially available and stored frozen.
- (7) Colcemid: stock solution of 25  $\mu\text{g}/\text{mL}$  in sterile physiological saline. It can be stored at 4°C for 6 months.

- (8) Sterile culture vessels. There are various options, e.g. glass bacteriology bottles or disposable plastic containers. The volume should be 15 to 20 mL.
- (9) Cultures should be set up in a clean workplace, such as a microbiological safety cabinet, under subdued lighting. Liquids can be transferred between vessels using sterile disposable syringes or graduated pipettes. If blood needs to be passed through a hypodermic needle, this should be done slowly by using a wide bore (19 gauge) needle to minimize shearing forces on the cells.

### **I-1.2. Method**

- (1) Place 0.3 mL of heparinized blood into a culture vessel;
- (2) Add 4.0 mL of culture medium to which antibiotics and BrdU have already been added;
- (3) Add 0.1 mL of reconstituted PHA;
- (4) Add 0.5 mL of foetal calf serum;
- (5) Seal the lid securely;
- (6) Mix the contents of the vessel by gentle shaking;
- (7) Incubate at  $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  in the dark for 45 h;
- (8) Add 0.1 mL of Colcemid stock solution to the culture and shake gently;
- (9) Return to the incubator for three more hours.

## **I-2. FIXATION AND SLIDE PREPARATION**

### **I-2.1. Method**

- (1) Tip the contents of the culture vessel into a centrifuge tube.
- (2) Spin at 200g for 10 min (to convert g into rev/min, use  $g = r\omega^2/981$ , where  $r$  = radius in cm and  $\omega = (2\pi \times \text{rev/min})/60$ ).
- (3) Remove the supernatant by suction and resuspend the cell button in 5 to 10 mL of 0.075M potassium chloride solution.
- (4) Leave to stand at room temperature for 15 to 20 min.
- (5) Spin again at 200g for 10 min.
- (6) Remove supernatant and resuspend the cells in 5 to 10 mL of freshly prepared 3:1 methanol/acetic acid fixative. The fixative must be added slowly, but at constant rate with vigorous agitation, ideally using a vortex mixer, to prevent the cell button from becoming a solid clump.
- (7) Spin again.
- (8) Remove supernatant and resuspend in 5 to 10 mL of fixative.
- (9) Spin again.
- (10) Remove supernatant and resuspend in 5 to 10 mL of fixative.

- (11) Spin again.
- (12) Remove all but 0.25 mL of the supernatant and resuspend the cell button in the remaining fluid.
- (13) Draw up the cell suspension into a Pasteur pipette.
- (14) Take a clean, grease free slide that has previously been stored in a freezer.
- (15) Melt the frost on the slide with your breath.
- (16) Allow one or two drops of the cell suspension to drip onto the slide from a height of at least 10 cm.
- (17) Prepare at least two such slides from each culture.
- (18) Place the slides to dry in gentle heat over a hotplate.

### I-3. STAINING

#### I-3.1. Materials

- (1) Hoechst 33258 stain. A 1000 × concentrated stock solution of 50 µg/mL in pH 6.8 phosphate buffer can be stored at 4°C in the dark.
- (2) Giemsa stain.
- (3) Phosphate buffer (pH 6.8) made up from commercially available tablets.
- (4) 2 × SSC (sodium chloride and trisodium citrate): 17.53 g sodium chloride, 8.82 g sodium citrate, distilled water to make 1.0 L.
- (5) Xylene and DPX mountant.
- (6) An ultraviolet lamp (>310 nm) or a fluorescent strip lamp.

#### I-3.2. Methods

A few (up to five) days should elapse between preparation of the slides and commencement of FPG staining, while the conventional Giemsa stain can be used as soon as the slides are dry.

##### *Fluorescence plus Giemsa (FPG)*

- (1) Place approximately 10 drops of Hoechst stain (diluted from the stock solution to 0.5 µg/mL) onto the slide and cover with a coverslip;
- (2) Place the slide on a sheet of aluminium foil and beneath an ultraviolet lamp for 0.5 h;
- (3) Carefully remove the coverslip;
- (4) Wash well with pH 6.8 buffer;
- (5) Place in 2 × SSC at 60°C for 20 to 30 min;
- (6) Wash in distilled water;

- (7) Place slides in Giemsa stain — a 5 to 10% solution in pH 6.8 buffer for 3 min;
- (8) Rinse briefly in buffer;
- (9) Rinse briefly in distilled water;
- (10) Air dry;
- (11) Clear and mount under a coverslip.

*Conventional Giemsa*

- (1) Place slide in 2% Giemsa stain in pH 6.8 buffer for 5 min;
- (2) Wash in buffer;
- (3) Rinse briefly in distilled water;
- (4) Air dry;
- (5) Clear and mount under a coverslip.



## Annex II

### FISH BASED TRANSLOCATION ASSAY

Two protocols are presented here, using either blue scribed probes made in the laboratory or commercially available probes.

#### PROTOCOL 1. USING BLUE SCRIBED PLASMID

##### II-1.1. Single colour FISH (Fig. 8)

###### II-1.1.1. Probe labelling (using Promega kit)

Mix 1  $\mu\text{L}$  Biot-16-dUTP (1 mM) with 897  $\mu\text{L}$  super Q water.

Prepare a labelling mixture in an Eppendorf tube as follows:

- 26.9  $\mu\text{L}$  of above mixture,
- 3.3  $\mu\text{L}$  deoxynucleotides triphosphate solution (dATP, dCTP, dGTP),
- 2.2  $\mu\text{L}$  dTTP, 5  $\mu\text{L}$  nick translation buffer (10  $\times$ ),
- 5.0  $\mu\text{L}$  enzyme mix,
- 12.6  $\mu\text{L}$  super Q water,
- plasmid DNA.

Place the labelling mixture for 2 h at exactly 15°C in a waterbath. To stop the reaction add 50  $\mu\text{L}$  (0.1 M) TE buffer. This buffer comprises 10 mM Tris/HCl pH 8.0, plus 0.1 mM EDTA. Then add the following:

- 25  $\mu\text{L}$  total human DNA (2.0  $\mu\text{g}/\mu\text{L}$ )
  - 5  $\mu\text{L}$  salmon sperm DNA (10  $\mu\text{g}/\mu\text{L}$ ) (Sigma)
  - 13  $\mu\text{L}$  sodium acetate (pH = 5.6)
  - 325  $\mu\text{L}$  100% ice-cold ethanol (kept at -20°C).
- Mix thoroughly and store the tube overnight at -20°C.

Next day, spin down the pellet at 13 000 rev/min for 30 min at 4°C. Remove all the supernatant, dissolve the pellet in 40  $\mu\text{L}$  hybridization buffer, for at least for 30 min at 42°C. Keep the biotinylated probe at -20°C (25 ng/ $\mu\text{L}$ ).

### *II-1.1.2. In situ hybridization*

#### (a) RNase treatment

Wash the slides for 5 min with PBS- (without  $Mg^{++}$  and  $Ca^{++}$ ) at room temperature.

Dehydrate the slides in an ethanol series (70, 90 and 100%), with 5 min intervals.

Incubate the slides with RNase A (100  $\mu\text{g}/\text{mL}$ ) in  $2 \times \text{SSC}$  (saline sodium citrate), add 100  $\mu\text{L}$  on a 24 mm  $\times$  50 mm coverslip for 1 h in a moist chamber at 37°C. Wash three times for 5 min each, with  $2 \times \text{SSC}$  remove the coverslip during the first wash.

Finally, wash once for 5 min with PBS-.

#### (b) Pepsin treatment

Incubate slides with a pepsin solution 0.005% in 10 mM HCl, for 10 min in a waterbath (at 37°C).

Wash with PBS at room temperature for 5 min.

Wash with PBS/ $MgCl_2$  at room temperature for 5 min.

Post-fixation should be done for 10 min with 1% formaldehyde (3 mL in 100 mL) in PBS/ $MgCl_2$  (50 mM), keep slides at room temperature. Wash for 5 min with PBS.

Dehydrate the slides in an ethanol series (70, 90 and 100%) with 5 min intervals.

Keep the slides at room temperature in the dark in a dust free container.

#### (c) Probe competition

Take the tube with biotinylated probe from -20°C. Put the amount of each probe that is required into a sterile tube and incubate this tube for 5 min at 70°C in a waterbath (without shaking).

Immediately put the tube in ice water for 5 min.

Incubate the tube for 2 h in a waterbath at 37°C.

#### (d) Prehybridization of slides

Prehybridization can be started about 30 min before the end of probe competition.

Incubate slides with 100  $\mu\text{L}$  of 70% formamide/2  $\times$  SSC under a 24 mm  $\times$  50 mm coverslip for 4 min at 80°C. SSC can be prepared as follows: for 10  $\times$  SSC dissolve sodium chloride 17.5 g and sodium citrate 2 hydrate 8.8 g in 100 mL distilled water.

Immediately remove the coverslip and put the slides in 70% ethanol (kept at  $-20^\circ\text{C}$ ) for 5 min.

Dehydrate the slides in an ethanol series (90 and 100%), with 5 min interval at room temperature.

(e) Hybridization

Incubate the slides with 4  $\mu\text{L}$  probe (the concentration of probe is 25 ng/ $\mu\text{L}$ ), then make up to 20  $\mu\text{L}$  with hybridization buffer under a 24 mm  $\times$  50 mm coverslip. Seal slides with rubber glue or nail varnish, store them overnight in a moist chamber with 60% formamide in 2  $\times$  SSC, pH = 7.0.

Next day remove the glue, wash four times for 5 min each, with 50% formamide in 2  $\times$  SSC, pH = 7.0, in a waterbath at 42°C. The coverslip can be removed during the first wash.

Wash three times for 5 min each with 0.1  $\times$  SSC in a waterbath at 60°C.

Wash for 5 min with 4  $\times$  SSC in 0.05% Tween 20 (Sigma, polyoxyethylene sorbitan monolaurate) at room temperature.

*II-1.1.3. Immunofluorescence detection*

Preincubate slides with immunological buffer (100  $\mu\text{L}$ ), 5% non-fat dry milk in 4  $\times$  SSC under a 24 mm  $\times$  60 mm coverslip, for 15 min at room temperature in a moist chamber.

Wash twice, 5 min each with 4  $\times$  SSC in 0.05% Tween 20 (after the first wash remove the coverslip).

Incubate with 100  $\mu\text{L}$  of Avidine-FITC (Vector Laboratories) (diluted 1:1000 in the immunological buffer) under a 24 mm  $\times$  60 mm coverslip for 30 min at room temperature in the dark.

Wash three times, 5 min each with  $4 \times$  SSC in 0.05% Tween 20 at room temperature.

Incubate with 100  $\mu$ L biotinylated goat anti-avidine (Vector Laboratories) (diluted 1:100 in the immunological buffer) under a 24 mm  $\times$  60 mm coverslip for 30 min at room temperature in the dark.

Wash three times, 5 min each with  $4 \times$  SSC in 0.05% Tween 20 at room temperature.

Incubate with 100  $\mu$ L Avidine-FITC (Vector Laboratories) (diluted 1:1000 in the immunological buffer) under a 24 mm  $\times$  60 mm coverslip, for 30 min at room temperature in the dark.

Wash three times, 5 min each with  $4 \times$  SSC in 0.05% Tween 20 at room temperature.

Repeat all the steps from first labelling with Avidin-FITC onwards once more.

Dehydrate in an ethanol series (70, 90 and 100%) for 5 min each, at room temperature.

Embed the air dried slides in propidium iodide (PI)-DABCO (Sigma) [with 20 mM tris-HCl (pH = 8.0), 90% glycerol containing 2% of 1,4-diazabicyclo[2,2,2]-octane, 35  $\mu$ L/slide)], in Vectashield™ antifade mountant under a 24 mm  $\times$  50 mm coverslip.

## II-1.2. Double and triple colour FISH

The double and triple FISH procedures are facilitated considerably by the introduction of digoxigenin and fluorescein. Biotin, digoxigenin and fluorescein dUTP can be incorporated by the same labelling method, and the hybrids can be detected in a one step procedure. This technique has advantages of combining chromosome paint probes with a pancentromeric probe, and discriminating different chromosome paint probes from each other (Fig. 9; see Section 6.3.2).

*Digoxigenin* (Boehringer, Mannheim) is a good alternative for biotin. It can be used as label alone or in combination with biotin. Unlike biotin, digoxigenin is not present in biological material except for the plant from which it is extracted. Like biotin, digoxigenin modified dUTP and UTP as well as a photoreactive digoxigenin derivative are available for probe labelling. The labelling procedures of probes with digoxigenin are the same as described for biotin. Detection of digoxigenin can be achieved with specific high affinity monoclonal antibodies and conjugates of polyclonal sheep antibodies (sheep- $\alpha$ -digoxigenin alkaline phosphatase) (Boehringer, Mannheim).

*Fluorescein* (Boehringer Mannheim) can be incorporated efficiently in DNA probes by nick translation or random priming. The fluorescein labelled probes can be examined direct after hybridization in a fluorescent microscope.

## PROTOCOL 2. USING DIRECTLY LABELLED (COMMERCIALY AVAILABLE) PROBES

### II-2.1. Pretreatment

Wash slides with PBS for 5 min at room temperature. Dehydrate slides in an ethanol series (70, 90 and 100%) 2 to 5 min each step, at room temperature, and air dry.

### II-2.2. RNase and pepsin treatment

Mix 445  $\mu\text{L}$  water, 50  $\mu\text{L}$   $20 \times \text{SSC}$  and 5  $\mu\text{L}$  RNase A (10  $\mu\text{g}/\mu\text{L}$ ) (the mixture can be prepared in advance and should be kept at  $-20^\circ\text{C}$ ). Pipette 100  $\mu\text{L}$  of RNase A per slide, overlay with a coverslip. Incubate in a moist chamber for 60 min at  $37^\circ\text{C}$ .

Wash three times with  $2 \times \text{SSC}$  (5 min each at room temperature). During the first wash remove coverslip. Afterwards, wash with PBS for 5 min at room temperature. For pepsin treatment (0.005% in 10 mM HCl), prepare in advance a mixture consisting of 50  $\mu\text{L}$  pepsin (10%), 99 mL water and 1 ml 1N HCl. This mixture can be kept at  $-20^\circ\text{C}$  before use. Prewarm the mixture in a waterbath at  $37^\circ\text{C}$  and put 100  $\mu\text{L}$  onto each slide for 8 to 10 min. Wash with PBS for 5 min at room temperature. Wash with 50 mM  $\text{MgCl}_2$  (5mL  $\text{MgCl}_2$  and 95 mL PBS) for 5 min at room temperature. Wash with 1% formaldehyde in  $\text{MgCl}_2$ -PBS for 10 min at room temperature. Rinse in  $2 \times \text{SSC}$ , twice and for 5 min each at room temperature. Air dry in an ethanol series (70, 90 and 100%) 2 to 5 min each at room temperature.

### II-2.3. FISH protocol for chromosome paint probes in combination with a pan-centromeric probe

Warm chromosome paint probes to  $42^\circ\text{C}$  and shake well before use. A sufficient amount of every chromosome paint should be prepared in a separate Eppendorf tube with hybridization buffer; shake well and spin down.

#### (a) *Denaturation*

Chromosome paints can be denatured by incubation at  $65^\circ\text{C}$  for 10 min in a waterbath. Then put on ice for 2 to 3 min, and transfer to a waterbath ( $37^\circ\text{C}$ ) and incubate for 60 to 90 min.

For using the chromosome paints in combination with a pan-centromeric probe (CP), start warming the CP and hybridization buffer at  $37^\circ\text{C}$  30 min before probe

competition. Denature the CP by incubating at 85°C for 10 min in a waterbath, then immediately put on ice for 2 to 3 min.

For triple colour FISH with a pan-centromeric probe, the final volume of 18 to 20  $\mu\text{L}$  of hybridization mixture per slide should be used (i.e. for 3  $\mu\text{L}$  of each of the three concentrated paint probes add 1.6  $\mu\text{L}$  of its appropriate buffer and add 2 to 3  $\mu\text{L}$  of concentrated CP).

For example, when three chromosomes #1, 4 and 8 are being used:

Chromosome #1 (biotin), #4 biotin/FITC, #8 FITC and CP FITC, they will generate red, yellow, green and green colour signal, respectively.

### (b) *Prehybridization*

Prehybridization of slides should be started approximately 30 min before the end of probe competition. Put 100  $\mu\text{L}$  of 70% formamide in 2  $\times$  SSC and 50 mM PBS per slide and overlay with a coverslip [350  $\mu\text{L}$  deionized 100% formamide (store at  $-20^\circ\text{C}$ ), 50  $\mu\text{L}$  0.5 M PBS (store at  $-20^\circ\text{C}$ ) and 50  $\mu\text{L}$  of 20  $\times$  SSC. The formamide should be deionized shortly before its use].

Denature slides at 80°C for 3 min on the hot plate. Air dry the slides in an ethanol series (stored at  $-20^\circ\text{C}$ ) of 70% for 5 min, 90 and 100% for 2 to 5 min each at room temperature. Allow the slides to be air dried.

### (c) *Hybridization*

Mix well all chromosome paints and CP in one Eppendorf tube. Spin down for a few seconds, and put 20  $\mu\text{L}$  of the mixture onto each slide, overlay with a coverslip, seal with rubber glue and air dry. Slides should then be incubated overnight in a moist chamber at 42°C. This can be extended to two days.

Detection:

Prepare a wash solution (WS) as follows:

Add 0.5 mL of detergent to 1 mL of 4  $\times$  SSC and mix with 0.05% Tween 20 in 4  $\times$  SSC (200 mL 20  $\times$  SSC and 5 mL 10% Tween 20 in 800 mL water).

- (1) Dilute blocking protein (BP) to 10–15% (v/v) in WS.
- (2) Use WS for diluting antibodies as follows:  
First layer B3 (1:500), Texas red Avidin,  
Second layer B4 (1:250) biotinylated goat anti-avidin  
F1 (1:200) rabbit anti-FITC  
Third layer B3 (1:500) F2-FITC, goat anti-rabbit IgG  
F2 (1:100)

- (3) Incubate in the dark for 10 min at room temperature, microcentrifuge at 11 000g for 10 min, and use the supernatant.
- (4) Prewarm the supernatant to 42–45°C in 2 × SSC, followed by one washing step using 50% formamide in 2 × SSC, pH 7.0, followed by three washes in 0.1 × SSC.
- (5) Carefully remove coverslips in a jar of 2 × SSC.
- (6) Additional washing steps are required:  
in 50% formamide in 2 × SSC, 2 to 3 times, 5 min each at 42°C; 0.1 × SSC, three times, 5 min each at 42°C, and 0.05% Tween/4 × SSC for 5 min at 42°C.
- (7) Put 100 μL of solution A onto each slide and overlay with a coverslip, incubate in a moist chamber for 15 to 20 min at 37°C.
- (8) Wash slides with 0.05% Tween 20 in 4 × SSC for 2 to 5 min at 42°C.
- (9) Put 100 μL of the first layer of antibody onto each slide and overlay with a coverslip. Incubate in a moist chamber for 20 to 30 min at 37°C.
- (10) Wash slides in 0.05% Tween 20 in 4 × SSC, three times, 5 min each at 42°C.
- (11) Put 100 μL of the second layer of antibodies onto each slide and overlay with a coverslip. Incubate in a moist chamber for 20 to 30 min at 37°C.
- (12) Wash slides with 0.05% Tween 20 in 4 × SSC, three times, 5 min each at 42°C.
- (13) Put 100 μL of the third layer of antibodies onto each slide and overlay with a coverslip, incubate in a moist chamber for 20 to 30 min at 37°C.
- (14) Wash slides with 0.05% Tween 20 in 4 × SSC, three times, 5 min each at 42°C.
- (15) Repeat steps 12 to 15 once.
- (16) Dehydrate slides in an ethanol series of 70, 90 and 100%, 2 to 5 min each at room temperature.
- (17) Allow the slides to air dry.
- (18) Counterstain with DAPI (0.15 μg/mL in Vectashield mountaint), 25 μL per slide under a coverslip.

## Annex III

### PREMATURE CHROMOSOME CONDENSATION ASSAY

Human peripheral blood mononuclear cells are fused with mitotic Chinese hamster ovary (CHO) cells in the presence of polyethylene glycol (PEG). As a result of the cell fusion in only one hour, the mononuclear blood cells undergo chromatin condensation which is rapidly followed by dissolution of their nuclear membrane and further condensation of chromatin into 46 ( $2n = 46$ ) single chromatid chromosomes.

#### III-1. ISOLATION OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES

For the separation of mononuclear cells from anticoagulated whole blood, a LeucoPREP™ brand, cell separation tube (Becton Dickinson Labware) can be used.

##### A. **LeucoPREP**

###### A.a. *Test principles*

The LeucoPREP product is a tube system containing a separation medium that, like Ficoll-Hypaque, takes advantage of the lower density of mononuclear cells and platelets to separate these from the remaining components of anticoagulated whole blood. The separation occurs when blood is placed in the tube over the gel layer and the tube is subjected to a specified centrifuge force for a given duration. Subsequent washings and centrifugations reduce the quantity of platelets present. The resulting preparations of viable mononuclear cells can be used for PCC.

###### A.b. *Specimen collection and processing*

- (1) Store LeucoPREP tubes (10 mL) upright at room temperature (18–25°C).
- (2) Collect blood by venipuncture using a heparinized tube. An EDTA vacutainer may also be used.
- (3) Heparin anticoagulated blood should be separated within two hours of blood drawing. EDTA anticoagulated blood should be separated within six hours of blood drawing but no sooner than half an hour.
- (4) Add undiluted blood (8 to 10 mL) to each LeucoPREP tube, then centrifuge for 15 min at 2000 to 2500 rev/min at room temperature.
- (5) After centrifugation, mononuclear cells and platelets will be in a fluffy, white layer just under the plasma layer. Aspirate plasma as much as possible without



- aspirating cells. Collect cell layer with a Pasteur pipette and transfer to a 10 mL size conical centrifuge tube with cap.
- (6) Resuspend cell pellet by gently vortexing. Add F10 medium (10 mL), mix cells by inverting tubes 3 to 4 times, then centrifuge for 10 min at 1000 rev/min.
  - (7) Repeat step 5 once again.

## B. Ficoll-Hypaque gradient system

### B.a. Test principles

Ficoll-Paque is an aqueous solution of density  $1.077 \pm 0.001$  g/mL containing 5.7 g Ficoll 400 and 9 g sodium diatrizoate calcium disodium ethylenediamine-tetracetic acid (EDTA) in every 100 mL.

#### PREMATURE CHROMOSOME CONDENSATION TECHNIQUE

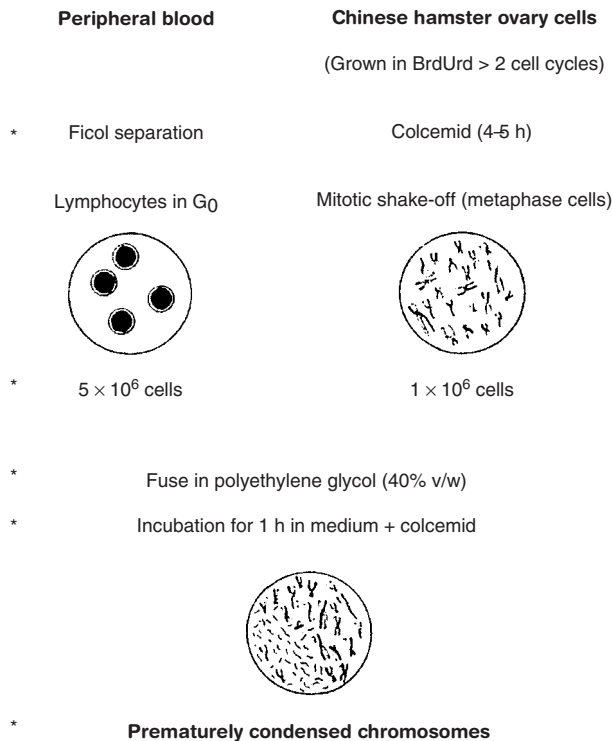


FIG. 16. A schematic diagram of the PCC technique.

- (1) Collect blood specimens by venipuncture and place into heparinized tubes (about 8 mL).
- (2) Dilute blood samples with an equal volume of balanced salt solution.
- (3) Put about 5 mL of diluted blood (drop by drop) on top of Ficoll-Hypaque (3 mL) without intermixing.
- (4) Centrifuge the tubes for 30 min at 2500 rev/min at 8–10°C.
- (5) Collect lymphocytes (middle layer) and wash three times (centrifuge at 1000 rev/min for 10 min) with 5 mL F10 culture medium plus 5% foetal calf serum.

The isolated lymphocytes may be used immediately for performing PCC experiments or frozen for future use.

### III-2. FREEZING THE ISOLATED LYMPHOCYTES

After the second wash with F10 and centrifugation, resuspend the cell pellet by gently vortexing and make a cell suspension in 1:1, F10 + 40% foetal calf serum (FCS):F10 + 40% FCS + 20% DMSO. Make cell suspensions in a manner so that each ampoule (1.5 mL) contains about  $8 \times 10^6$  isolated lymphocytes. For freezing the best method is to use a machine that can gradually decrease the temperature. Finally store frozen ampoules at  $-110^\circ\text{C}$  or in liquid nitrogen.

### III-3. THAWING THE ISOLATED LYMPHOCYTES

Take lymphocyte ampoules out of the freezer and put them directly into a waterbath ( $37^\circ\text{C}$ ). When they are slightly melted, transfer the whole suspension into a centrifuge tube (10 mL). Add 10 mL cold ( $4^\circ\text{C}$ ) RPMI + 40% FCS onto lymphocyte suspensions, drop by drop (in about 30 min), then centrifuge for 10 min at 1000 rev/min. Resuspend the cell pellet in 5 mL RPMI + 5% FCS. These mononuclear lymphocytes can be used for PCC experiments.

### III-4. COLLECTION AND PREPARATION OF MITOTIC CHINESE HAMSTER OVARY CELLS

Chinese hamster ovary (CHO) cells are grown in roller bottles or flasks (750 mL) in complete medium (F10 + 15% new-born calf serum and antibiotics (penicillin 100 U/mL and streptomycin 0.1 mg/mL)). Colcemid (0.1  $\mu\text{g}/\text{mL}$ ) is added to the exponentially growing cells, and mitotic cells are harvested by a standard selective detachment (shake-off) procedure 4 to 5 h later. CHO cells can also be grown for

more than two cell cycles (~32 h) in complete medium supplemented with 5-bromodeoxyuridine (5-BrdU, final concentration of 5  $\mu$ M). Mitotic CHO cells obtained will all be differentially stained and look pale in colour following FPG staining. Therefore, lymphocyte PCCs will be better differentiated among CHO mitotic cells (Fig. 10; see Section 6.4).

(a) Freezing mitotic CHO cells

Mitotic CHO cells can either be prepared and used immediately for fusion or taken from stock frozen in complete medium supplemented with 8% DMSO. Put them in small aliquots ( $2.5 \times 10^6$ /ampoule in 1.5 mL) and store them at  $-110^\circ\text{C}$ .

(b) Thawing mitotic CHO cells

Take ampoules of CHO mitotic cells out of the freezer and put them into a waterbath at  $37^\circ\text{C}$ , then transfer the cell suspension into a centrifuge tube and add 10 mL medium. Centrifuge for 10 min at 800 rev/min. Discard the supernatant, add medium (5 mL) and keep them on ice until use.

### III-5. PREPARATION OF POLYETHYLENE GLYCOL (PEG) SOLUTION

Put 400 mg of PEG (M.W. 1450, Sigma, 40% w/v) into a small (10 mL) centrifuge tube (round bottom) and add 600  $\mu$ L Hank's balanced salt solution (HBSS) or phosphate buffered saline (PBS) or F10 medium, and leave the tubes in the waterbath at  $37^\circ\text{C}$  for 15 min. PEG can also be melted first in an oven and then mixed with HBSS or PBS or F10 medium.

### III-6. CELL FUSION

- (1) Interphase lymphocytes and mitotic CHO cells are washed once with HBSS or F10 (5 mL) separately. Centrifuge for 5 min at 800 rev/min, then discard supernatant. In a round bottom culture tube, mix interphase cells with mitotic CHO cells (5:1) in 10 mL F10 medium and centrifuge for 5 min at 800 rev/min (higher speeds may cause the pellet to compact too much).
- (2) Pour off the supernatant and keep the tube inverted. Blot the residual drops of medium by placing the tubes upside down in a test tube rack on a paper towel.
- (3) If air bubbles are formed on top of the pellet in the tube, they should be removed with a Pasteur pipette.

- (4) Using a micropipette (200  $\mu$ L), take 0.150 mL PEG and put directly into the cell pellet, place in the test tube rack for 1.5 min. Shake the tube very gently, only three times (30 s interval). At this point, the cell pellet should appear detached from the bottom of the tube, forming big clumps in the PEG solution.
- (5) Subsequently, add 1.5–2 mL F10 or PBS very slowly over 3 min (0.5 mL per min). Mix the cell suspension gently by tapping the tube.
- (6) Centrifuge the tube for 5 min at 800 rev/min.
- (7) Pour off supernatant completely and add 0.5 mL of culture medium (F10 plus 15% foetal calf serum). Finally add 50  $\mu$ L of Colcemid (final concentration 1  $\mu$ g/mL), gently tapping the tube to form small clumps. Incubate test tube at 37°C for 1 h. By this time cell fusion and induction of PCC are completed.

### III-7. FIXATION PROTOCOLS

- (1) Add 7–8 mL prewarmed hypotonic (KCl, 5.6 g/L) to each tube and incubate for 10 min at 37°C.
- (2) Centrifuge the tube for 5 min at 800 rev/min.
- (3) Discard supernatant until 0.5 mL above pellet. Cells are fixed in 5 mL methanol:acetic acid (4:1).
- (4) Centrifuge the tube for 5 min at 800 rev/min.
- (5) Repeat steps 3 and 4 two more times.
- (6) Following the last centrifugation, discard supernatant and leave about 0.3 mL fixative solution on top of the pellet. Then break the pellet gently and add about 0.5–1 mL fixative by lightly tapping the tube.

### III-8. SLIDE PREPARATIONS

Drop cells with a drawn-out Pasteur pipette onto precleaned slides. By observing Newton rings gently blow under an infrared lamp.

### IX. STAINING PROTOCOLS

- (a) When mitotic CHO cells are not prelabelled with 5-BrdU, slides can be stained with an 3% aqueous Giemsa solution (Gurr Improved R66) for 5 min.
- (b) When mitotic CHO cells are prelabelled with 5-BrdU, slides can be stained according to the FPG technique. Finally, rinse slides in distilled water, allow them to dry and then mount with Depex and a 24  $\times$  60 mm coverslip.

- (c) For C banding of PCCs (for dicentric analysis), freshly prepared slides should be treated with 1N HCl for 5 min, followed by washing in 0.2N HCl for 5 min. Slides are then dried with a paper towel and treated with Ba(OH)<sub>2</sub> solution (5%) for 3 min at room temperature. They are then washed in 0.2N HCl for 5 min. Afterwards incubate slides in 2 × saline sodium citrate (SSC) at 60°C for 30 min. Wash with Gurr's buffer (pH = 6.8) and stain with 6% Giemsa for 30 min. Finally, rinse in tap water, allow them to dry and mount with Depex and a coverslip.
- (d) For the detection of translocations, chromosome specific DNA libraries and/or a pan-centromeric probe can be used (Fig. 14). Therefore, it is possible to detect dicentrics and translocations simultaneously (see Annex II).

## Annex IV

### MICRONUCLEUS ASSAY

A simple standard protocol that works well is given below. There are other methods involving more procedural steps and employing cultures of isolated lymphocytes, but for routine biodosimetry purposes whole blood cultures are adequate.

- (1) The blood sample is collected using lithium heparin anticoagulant.
- (2) Typically 0.4–0.5 mL of whole blood is added to 4.5 mL of culture medium (F10 or RPMI 1640) supplemented with 10 to 15% fetal calf serum (heat inactivated at 56°C for half an hour), L glutamine, antibiotics and phytohaemagglutinin (PHA). The concentration of PHA has to be optimized (it is 25 µg/mL for Wellcome HA-15).
- (3) The blood is cultured in tissue culture vessels at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere.
- (4) Cytocalasin-B (Cyt B) is added at 44 h post PHA stimulation. The optimum concentration for accumulating BN cells in whole blood cultures is 6 µg/mL. Cyt B is difficult to in aqueous solution. Cyt B stock solution × 100 (600 µg/mL) should be prepared in dimethylsulphoxide (DMSO) and aliquoted in small glass tubes (not plastic!), and stored until required at –20°C.
- (5) The culture is terminated at 72 h post PHA stimulation.
- (6) The cells are centrifuged gently (800 rev/min) for 5 min and the supernatant culture medium is removed.
- (7) The cells are hypotonically treated with 7 mL of cold (4°C) 0.075M KCl to lyse red blood cells, and centrifuged immediately at 800 rev/min for 8 min.
- (8) The supernatant is removed and replaced with 5 mL fixative consisting of methanol:acetic acid (3:1) with 1% formaldehyde. The fixative should be added whilst agitating the cells to prevent clumps forming. The cells are then centrifuged again at 600 rev/min for 8 min.
- (9) The cells are washed with two further changes of fixative, this time without formaldehyde.
- (10) The cells are resuspended gently, and the suspension is dropped onto clean glass slides and allowed to dry.
- (11) Staining of cells can be done using either 8 to 10% Giemsa in potassium phosphate buffer (pH 7.3) for light microscopy or acridine orange (10 µg/mL in phosphate buffered saline pH 6.9) for fluorescence microscopy.

## LIST OF ABBREVIATIONS

BrdU	bromodeoxyuridine
BSS	International Basic Safety Standards (see Ref. [3])
CBMN	cytokinesis blocked MN
CHO	Chinese hamster ovary
CRP	Co-ordinated Research Programme
Cyt B	cytocalasin-B
DMSO	dimethylsulphoxide
DNA	desoxyribonucleid acid
DSB	double strand break
EDTA	ethylenediamine-tetraacetic acid
FISH	fluorescence in situ hybridization
FPG	fluorescence plus Giemsa
ICRP	International Commission on Radiological Protection
ICRU	International Commission on Radiation Units and Measurements
HBSS	Hank's balanced salt solution
HIV	human immunodeficiency virus
LET	linear energy transfer
MEM	minimum essential medium
MN	micronucleus, micronuclei
NDI	nuclear division index
NPB	nucleoplasmic bridge
PBS	phosphate buffered saline
PCC	premature chromosomal condensation
PEG	polyethylene glycol
PHA	phytohaemagglutinin
RBE	relative biological effectiveness
Rnase	ribonuclease
SSB	single strand break
SSC	saline sodium citrate
TLD	thermoluminescence dosimeter

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