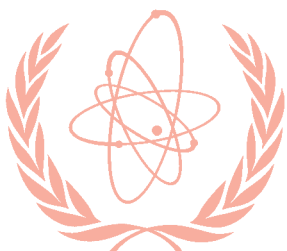


# Cytogenetic Dosimetry: Applications in Preparedness for and Response to Radiation Emergencies



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APPLICATIONS IN PREPAREDNESS FOR AND  
RESPONSE TO RADIATION EMERGENCIES**

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**Emergency Preparedness  
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**CYTOGENETIC DOSIMETRY:  
APPLICATIONS IN PREPAREDNESS FOR AND  
RESPONSE TO RADIATION EMERGENCIES**



INTERNATIONAL ATOMIC ENERGY AGENCY  
VIENNA, 2011

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## FOREWORD

Cytogenetic dosimetry 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 association has been through a sequence of coordinated research programmes (CRPs), the running of regional and national training courses, the sponsorship of individual training fellowships, and the provision of equipment to laboratories in Member States, establishing capabilities in biological dosimetry.

From this has arisen the provision to Member States of advice regarding the best focus for research and suggestions for the most suitable techniques for future practice in biological dosimetry.

One CRP resulted in the publication in 1986 of a manual, entitled *Biological Dosimetry: Chromosomal Aberration Analysis for Dose Assessment* (Technical Reports Series No. 260). This was superseded in 2001 by a revised second edition, Technical Reports Series No. 405.

This present publication constitutes a third edition, with extensive updating to reflect the considerable advances that have been made in cytogenetic biological dosimetry during the past decade.

The IAEA wishes to express its thanks to all authors and reviewers of this publication. The major contributions of Dr. D. Lloyd are especially acknowledged.

This publication has been co-sponsored by the Pan American Health Organization and the World Health Organization.

The IAEA officer responsible for this publication was E. Buglova of the Department of Nuclear Safety and Security.

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# 1. INTRODUCTION

## 1.1. BACKGROUND

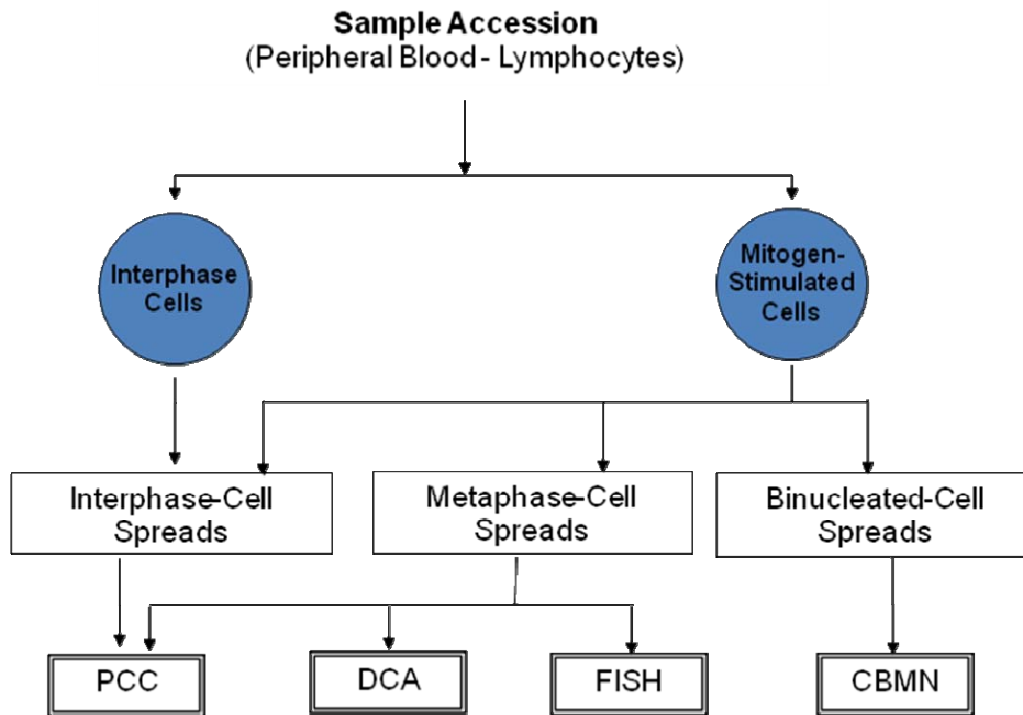
Biological dosimetry, based on the analysis of solid stained dicentric chromosomes, has been used since the mid-1960s. The intervening years have seen great improvements bringing the technique to a point where dicentric analysis has become a routine component of the radiation protection programmes of 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. Biological dosimetry using chromosome damage biomarkers is particularly important because, unlike physical measurement of dose, it takes into account interindividual variation in susceptibility.

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 nuclear or radiological emergency is investigated<sup>1</sup> [2]. Diagnostic sources of information may come from other biologically-based radiation biomarkers as well as clinical signs and symptoms that persons might display, and also from physical measurements such as those made on personal monitoring badges and thermoluminescence, optically stimulated luminescence or electron spin resonance on solid matrix components from (i.e. teeth dental enamel, fingernails, extracted bone, etc.) or associated (i.e. watch or eye glasses, etc.) with the irradiated persons. Information from questioning of patients and witnesses on basic facts such as time in the locality and distance from the radiation source may also assist with dose calculations. All these sources of information may be combined with biological dosimetry to obtain a clearer evaluation of the case.

For many years the dicentric assay using blood lymphocytes was the only method of biological dosimetry available, and still today it is the technique most frequently used. The dicentric and other aberrations may also be observed in other cells such as skin fibroblasts and buccal epithelial cells but this is beyond the scope of this publication which is confined to assays with blood lymphocytes. However there are now a number of other biological endpoints: micronuclei, translocations and aberrations in prematurely condensed chromosomes (Fig. 1 and Table 1) that can also be assayed in lymphocytes and these are described.

---

<sup>1</sup> In this context, a 'radiation emergency' means the same as a 'nuclear or radiological emergency'.



*FIG. 1. Schematic for sample accession of peripheral blood lymphocytes for various cytogenetic-chromosome aberration assays i.e., Premature chromosome condensation (PCC) assay, metaphase spread dicentric (and ring) chromosome aberration assay (DCA), metaphase spread fluorescence in situ hybridization (FISH) translocation assay, and cytokinesis-block micronuclei (CBMN) assay used for dose assessment.*

TABLE 1. COMPARISON OF CYTOGENETIC ABERRATION ASSAYS USED FOR DOSE ASSESSMENT<sup>a</sup>

	Cytogenetic Aberration Assays			
	Premature chromosome condensation (PCC)	Dicentric (and ring) (DCA)	Fluorescent <i>in situ</i> hybridization (FISH)	Cytokinesis-block micronucleus (CBMN)
Typical aberrations scored for biological dosimetry applications	excess chromosome fragments; dicentric <sup>b</sup> and rings  translocations <sup>b</sup>	dicentric <sup>b</sup> (and rings)	dicentric <sup>b</sup> (and rings)  translocations <sup>b</sup>	micronuclei  nucleoplasmic bridges
Typical radiation scenario applications	acute recent exposure	acute protracted recent exposure	acute protracted old exposure	acute protracted recent exposure
Photon equivalent, acute dose range (Gy) for whole-body dose assessment	0.2 to 20	0.1 to 5	0.25 to 4	0.3 to 4
Useful for partial-body exposure applications	Yes	Yes	NA <sup>c</sup>	NA
Useful for triage dose assessment	Yes	Yes	NA	Yes
Status of assay standardization	NA	ISO standards [3, 4]	NA	ISO standard — pending, and [5]

<sup>a</sup> Table modified from TMT Handbook [6].

<sup>b</sup> Specific chromosome aberrations typically detected by use of centromeric and whole-chromosome specific DNA hybridization probes.

<sup>c</sup> NA: not applicable/not available.

Other assays, beyond the scope of this publication, use molecular endpoints that measure DNA breakage, changes in the regulation of some sentinel genes or the presence of protein biomarkers that may be detected within cells or in blood plasma/serum. This is an area of rapidly emerging technologies with a number of assays at differing stages in development and verification. The range of biological dosimetry options now available have led to proposals for a multiparametric approach to investigating an overexposed person [7] and having a variety of assays available may be particularly useful if a laboratory has to deal with an event involving many casualties.

In the investigation of radiation emergencies 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 (tissue injury) 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 stochastic consequences — i.e. cancer. For persons whose exposure is very low, 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 a radiation emergency or terrorist attack where many persons may have been exposed. At this time, triage of casualties using biological and clinical endpoints that initially and rapidly can identify individuals suspected of exposure to life-threatening doses as well as to provide a triage dose is needed.

## 1.2. OBJECTIVE

The primary objective of this publication is to provide the user 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 publication describes the four possible cytogenetic methods (Fig. 1 and Table 1) currently available for biological dosimetry. It is appropriate to have all these techniques readily available in main geographical regions, but, given a degree of international cooperation and networking, it is not necessary to have all of them available in each national biological dosimetry laboratory.

## 1.3. SCOPE AND HISTORY OF DEVELOPMENT

The first manual in this series [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 teaching radiobiology or for persons such as health physicists, lawyers or policy makers who may require some professional understanding of biological dosimetry.

In a revised edition of this manual [8], published 15 years later, FISH chromosome painting, premature chromosome condensation (PCC) and micronuclei (CBMN) assays were included.

Now, a further 10 years later, this document has been produced. Much of the original text on the metaphase spread dicentric (and ring) chromosome aberration assay (DCA) from the earlier editions is still valid and has been retained although where appropriate, updated. The FISH, PCC and CBMN assays have been considerably revised in the light of recent research and experience in using them. It was inevitable that, over the 25 years spanned by these three editions, the topic of cytogenetic dosimetry would expand considerably and become more technically complex. However the present revision is arranged so that a minimally equipped laboratory newly entering the field should not find the publication too daunting. It is possible still to extract material from just those sections that relate to the two most important core assays that should be established, namely the dicentric and micronucleus assays. Clear advice is given on how to apply them in practice, by constructing basic dose response curves and interpreting the data from overexposure case investigations.

A major new development in recent years, reflected in this revision, has been the arrangements to perform triage in radiation mass casualty events. Considerations are provided of how a biological dosimetry laboratory can respond to a sudden surge in cases by using assays in a triage mode, speeding up analyses with computer assisted microscopy and by networking with other laboratories. Coincidentally with this increased provision for



collaborative working in emergency response have come international guidelines on quality assurance, quality control and participation in interlaboratory comparison exercises. These topics are now covered in this revision.

Multiple cytogenetic assays are useful for biological dosimetry since no one single assay is sufficiently robust for all potential radiation scenarios including early-phase acute-exposures, partial-body exposures, retrospective or prior exposure (e.g. biosampling years after exposure) as well as applications involving triage cytogenetics for radiation mass casualty events.

#### 1.4. STRUCTURE

This publication is arranged as follows following this 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 are discussed. In Section 7 the requirements of blood sampling are described and Section 8 considers physical, biological and statistical requirements for constructing dose response curves. Sections 9, 10, 11 and 12 then describe the techniques for performing biological dosimetry with, respectively, the four cytogenetic endpoints of dicentrics, fluorescence in situ hybridization based translocations, prematurely condensed chromosomes and micronuclei. In Section 13 the considerable advances that have been made in recent years in developing automatic analysis of the chromosomal assays are described and in Section 14 another recent development, how the chromosomal dosimetry community can respond most effectively to mass casualty events, is considered. In Section 15 the guidance and procedures for quality assurance are described and the final Section 16 discusses safety of laboratory staff carrying out cytogenetic analysis. The comprehensive up to date reference list is followed by seven Annexes, the first four describing reproducible working protocols for DCA, FISH based translocation, PCC and CBMN assays. Annex V describes the criteria for measuring the mitotic index and Annex VI contains a guide to a number of statistical tests that are commonly employed in biological dosimetry data analysis and its underlying research. The final Annex, VII, presents a worked example of an interlaboratory quality assurance exercise on dicentric scoring and dose estimation. The publication concludes with a list of abbreviations used, a glossary of important technical terms, and finally a list of contributors to the drafting and peer review of this much revised third edition of the publication.



## 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. 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 and neutrons 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. For photons, these are derived from the ratio of mass energy absorption coefficients, and the values to be used may be obtained from standard tables [9]. 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 [10].

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 [11]. 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 dicentrics, 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 publication, it is sometimes possible to refine the whole body dose estimate for situations where non-uniform or partial body irradiations from external sources have occurred.

Most internally incorporated radionuclides also result in non-uniform irradiations but here the dose of concern is not that to the lymphocytes but rather that to the specific organs and tissues where the radioactivity deposits. The usefulness of chromosomal analysis is often somewhat limited because, for example, following an intake of radioiodine, aberrations will be induced in lymphocytes but these cannot be interpreted in terms of dose to the thyroid. Exceptions to this are those nuclides that have a wide distribution in the body such as tritiated water or radiocaesium where experience has shown that lymphocyte aberration analysis provides meaningful dose estimates.

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 intended for use in radiological protection. 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 (Gy) specifying, where appropriate, sufficient details to characterize the radiation type and quality [12–15].

### 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. Refs [16, 17] 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). 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 relative biological effectiveness (RBE) variations for chromosomal damage [18]. 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 the same and 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 LET 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 9.7.4.3 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,

$$RBM = \frac{\text{dose of 200-250 kVp X rays producing effect Z}}{\text{dose of radiation producing effect Z}} \quad (1)$$

It should be noted that X-rays are 2–3 times more effective than gamma rays and therefore the reference radiation should always be defined [19].

Fig. 2 shows the typically shaped linear and linear quadratic dicentric dose response curves obtained with, respectively, high and low LET radiations.

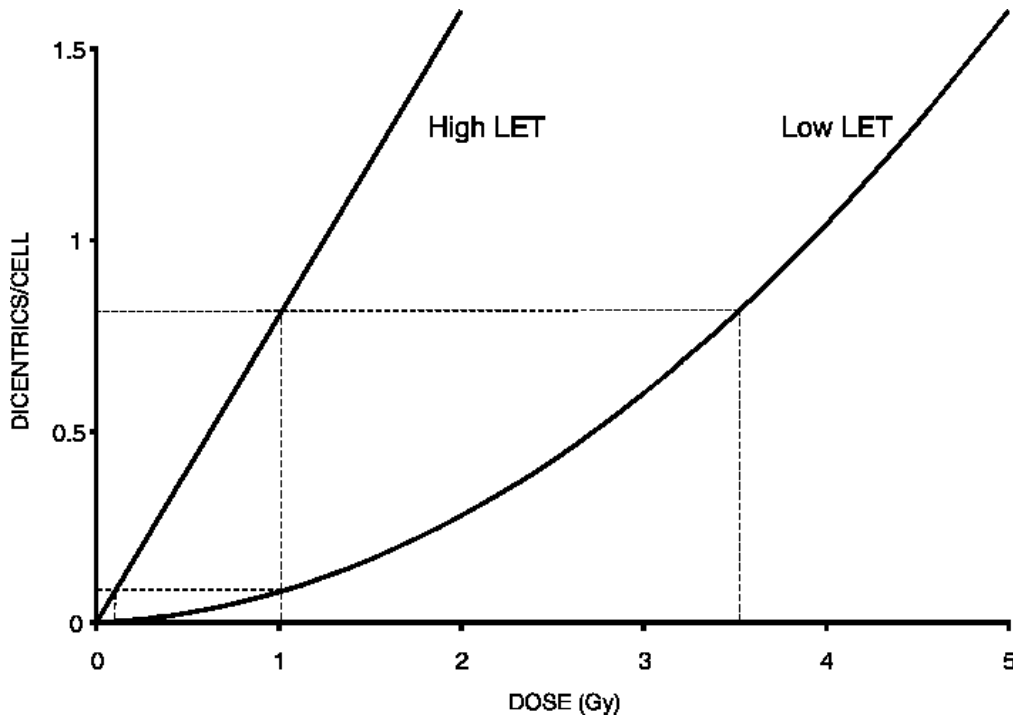


FIG. 2. Typical linear and linear quadratic dose response curves, showing how RBE changes with yield [8].

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, which describes the situation at low doses, usually designated as  $RBE_m$ , would be the ratio of the linear coefficients of the two curves' yield equations.

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. 3).

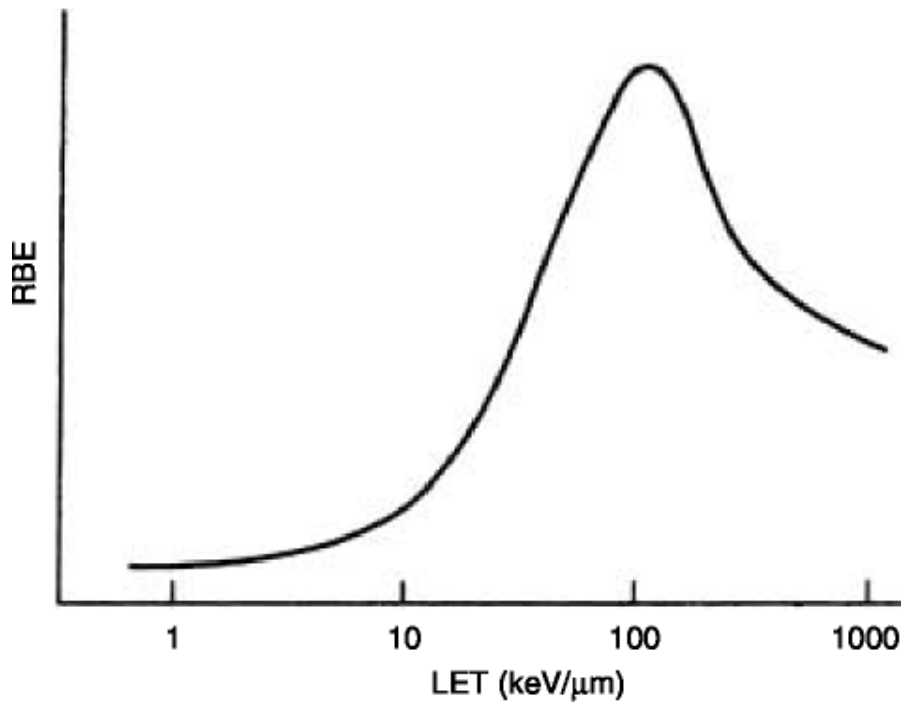


FIG. 3. Generalized relationship between RBE and LET [8].

This curve shows that the RBE increases up to an optimum value of about 100 keV/μm 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 μm 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. Diccenrics 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. 2) 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 Eq. (2)

$$Y = C + \alpha D + \beta D^2 \quad (2)$$

where:

$Y$  is the yield of dicentric,

$D$  is the dose,

$C$  is the control (background frequency),

$\alpha$  is the linear coefficient, and

$\beta$  is the dose squared coefficient.

The ratio of  $\alpha/\beta$  can be referred to as the cross-over dose. It 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 up to a maximum, 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/ $\mu\text{m}$  will be linear (Fig. 2). 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 formation of 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. 3.

Summarizing this discussion, the dose–response curve (Fig. 2) 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 around 100 keV/ $\mu\text{m}$  and decreases at higher LET values (Fig. 3). How does the dose rate 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 dicentric 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 dicentric 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 long enough to allow repair between dose fractions, 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 can be 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 concurrently 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 estimating 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 involved in the emergency. This is an important requirement because there are demonstrable differences in RBE for induced chromosome damage by various low LET radiations even though for radiological protection purposes, they are weighted identically ( $W_R = 1$ ) [19]. 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 [20] was the first to show that peripheral ‘human leukocytes’ can be stimulated to undergo *in vitro* mitoses by phytohaemagglutinin (PHA), a protein derived from the bean plant *Phaseolus vulgaris*. While Carstairs [21] showed that ‘small lymphocytes’ are the target cells for mitogenic initiation by PHA.

Peripheral small lymphocytes when observed in a blood smear have large dense nuclei surrounded by relatively little cytoplasm (Figs 4 and 5). They have a diameter of around  $6\ \mu\text{m}$ , and the volume is estimated to be around  $110\ \mu\text{m}^3$ .

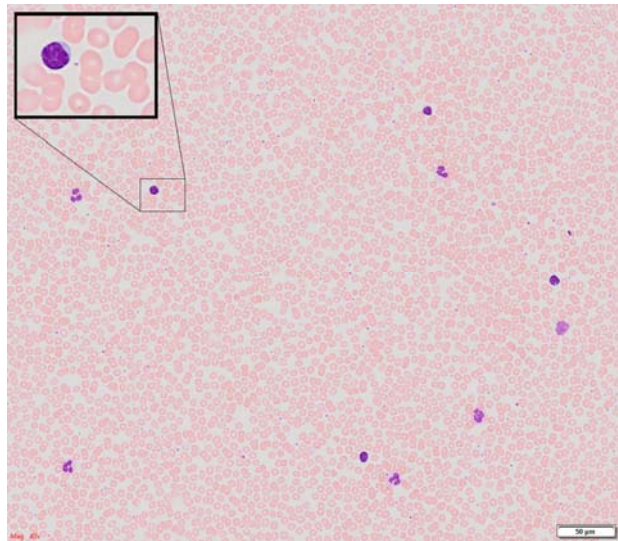


FIG. 4. A typical blood smear with a small lymphocyte and some red cells shown enlarged.

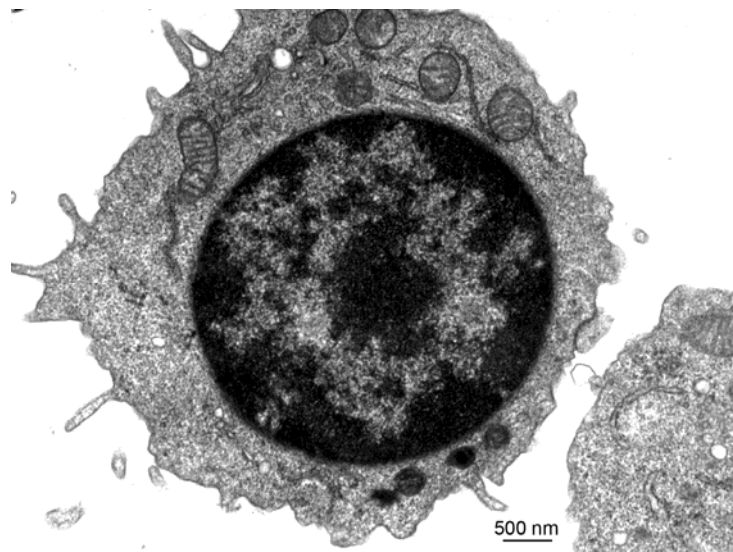


FIG. 5. A small lymphocyte as seen in an electron microscope.

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, undergoing somatic mutations and 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 [22]. It is the T cells, mostly of the CD4<sup>+</sup> and CD8<sup>+</sup> subtypes, which are stimulated *in vitro* by phytohaemagglutinin and are used for biological dosimetry.

Lymphocyte concentrations in the peripheral blood vary as a function of age, ethnicity, presence of pathogens and environmental factors (i.e. smoking, obesity, alcohol use, etc.). For example, certain ethnical populations (i.e. in East Africa) exhibit lower baseline values of lymphocyte counts compared with the overall population reference levels. A trend towards a decrease of the lymphocyte count is observed with age. This is particularly visible during childhood when a continuous drop is observed to reach around  $2 \times 10^9$ /L at 15 years old. The decrease tendency is also observed for adults but the drop is slower and at 75 years old and more, the lymphocyte number is below  $2 \times 10^9$ /L [23].

In general for a healthy adult the normal range of lymphocytes in peripheral blood is 1.5-4.0  $\times 10^9$ /L [24]. 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 count. These factors should be borne in mind for early blood sampling of radiation casualties for biological dosimetry [25].

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<sup>+</sup> and CD8<sup>+</sup> 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 [26]. 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.

For interpreting *in vivo* induced chromosome aberrations in humans, it is of great importance that the bulk of the peripheral lymphocytes belongs to the 'redistributional 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 redistributional 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 redistributional pool and that the overall recirculation time is about 12 hours. 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 [27].

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 phytohaemagglutinin (PHA). 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 hours 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.

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 hours after culture initiation and the first mitoses are found after about another 10 hours. There are two peaks of DNA synthesis measured by tritiated thymidine treatment, one at 34 hours and a second at 40 hours, and two peaks of mitotic activity, one at around 44 hours and a second at around 49 hours. This may represent two subpopulations of cells which show different stimulation patterns in a culture set up with Ham's F-10 and PHA [28]. 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 [29, 30] 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 artefact. A model of the organization of a metaphase chromosome is shown in Fig. 6.

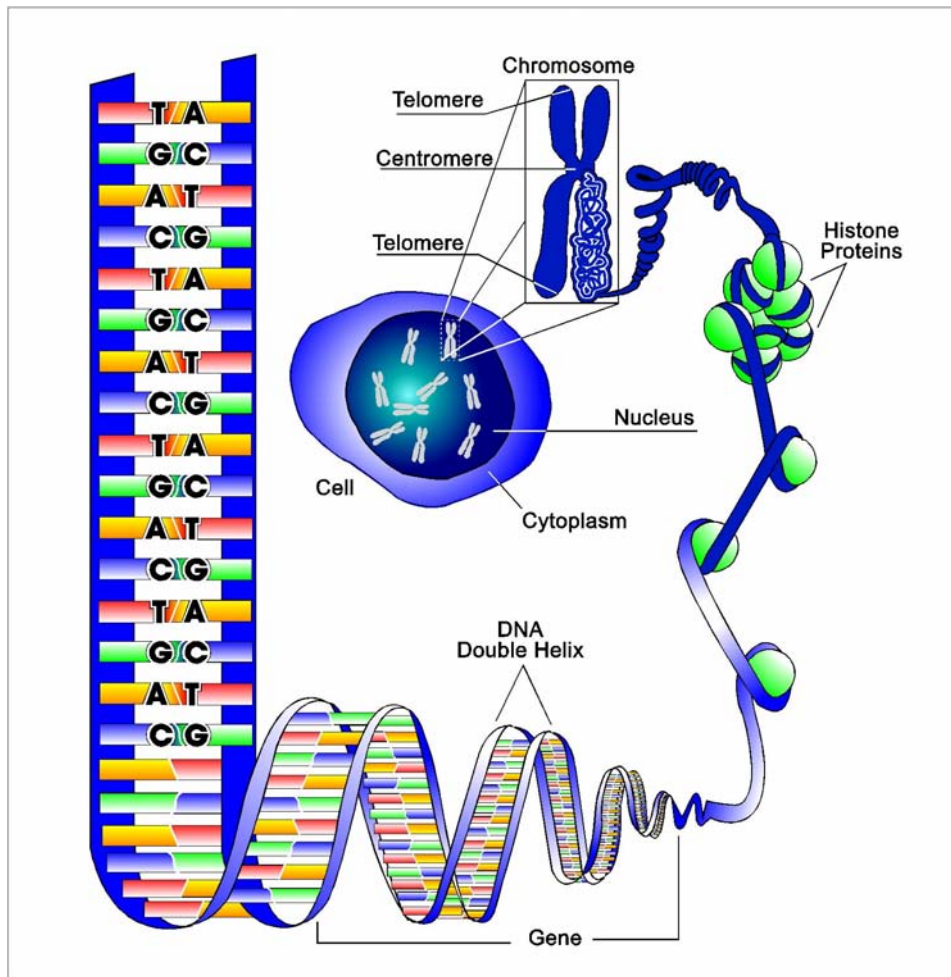
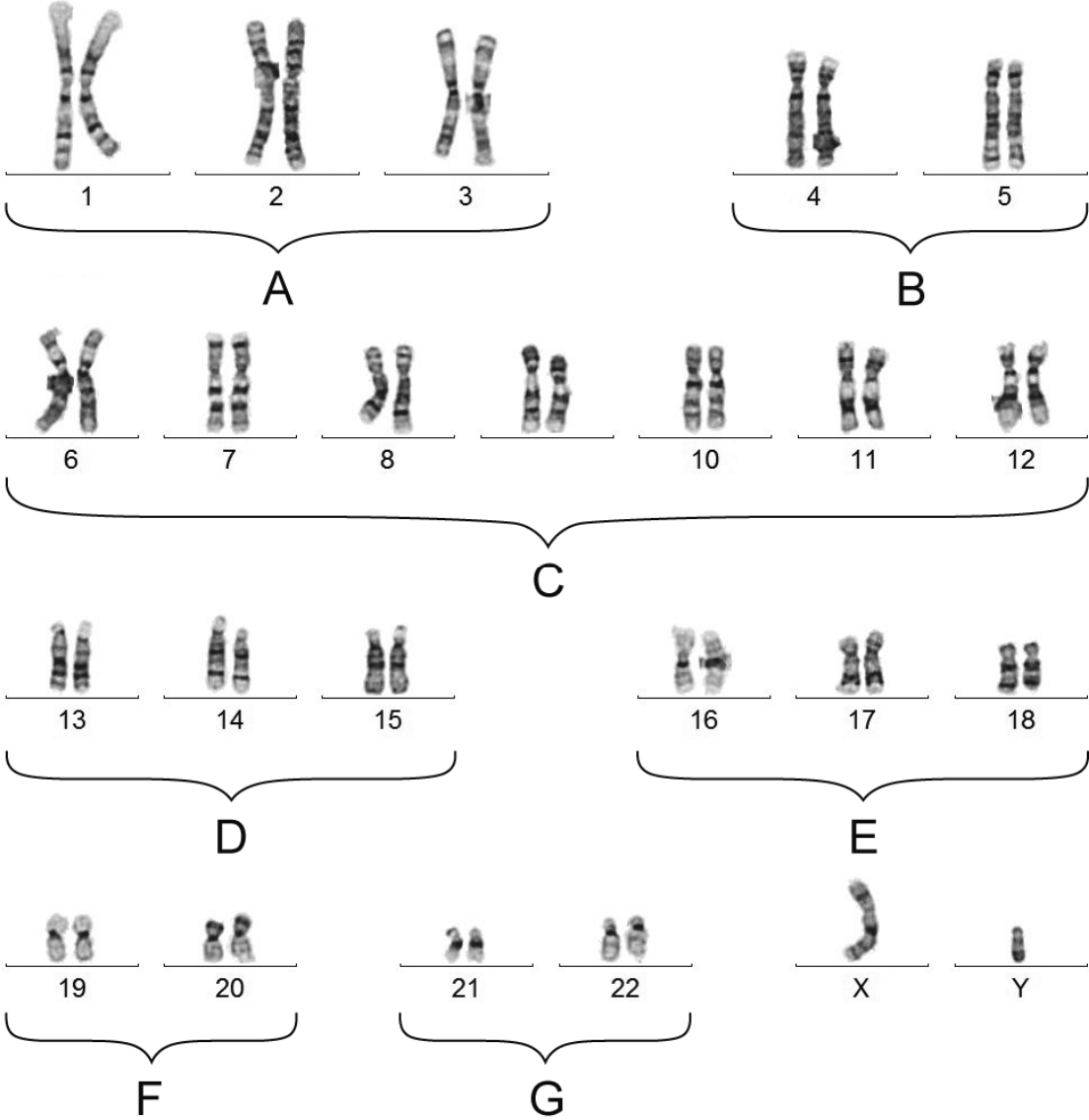


FIG. 6. Schematic illustration of the many different orders of chromatin packing that give rise to the highly condensed metaphase chromosome (courtesy REAC/TS, USA).

### 5.2. HUMAN KARYOTYPE AND DNA CONTENT OF CHROMOSOMES

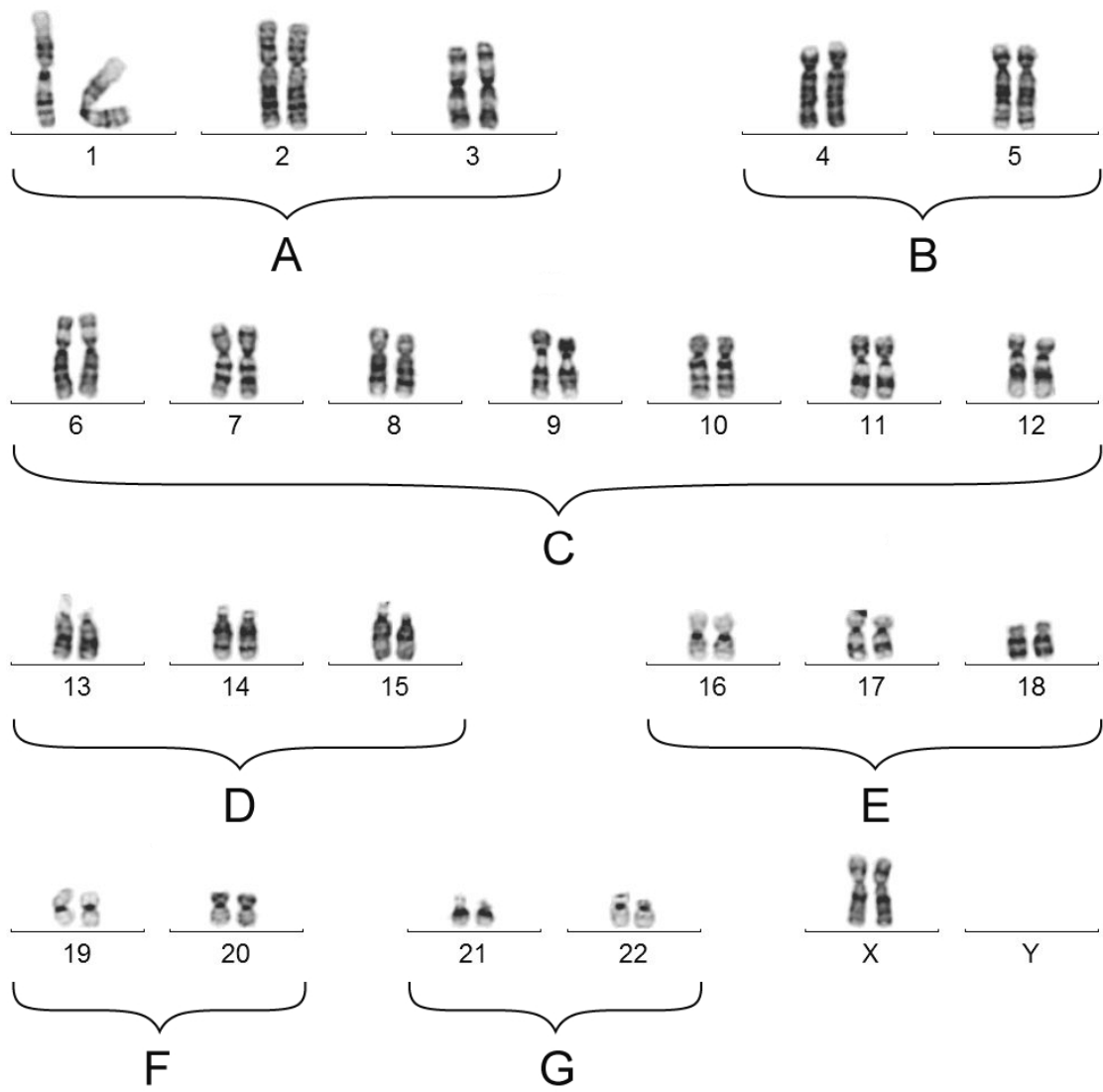
The human karyotype (Fig. 7) is the characteristic chromosome complement for humans, and consists of 23 pairs of large linear chromosomes of different sizes, giving a total of 46 chromosomes in every diploid cell. Human chromosomes are normally combined into seven

groups from A to G plus a pair of sex chromosomes X and Y [31]. The chromosomal groups are: A:1-3, B: 4 and 5, C: 6 -12, D: 13-15, E: 16-18, F: 19 and 20 and G: 21 and 22.



*Male*





*Female*

*FIG. 7. A banded chromosome/karyotype preparation from a normal male, 46, XY and a normal female 46, XX (courtesy Mayo Clinic, USA).*

The relative DNA contents of the human chromosomes for either gender are shown in Tables 2 and 3. These data have been calculated from Morton, 1991 [32].

TABLE 2. 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 3. 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

Chromosome No.	p arm	q arm	Both arms	Chromosome No.	p arm	q arm	Both arms
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	5.08
12	1.21	3.22	4.43			Total	100

### 5.3. 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 at mitosis. The cell cycle has a number of stages which can be distinguished by their appearance and function (Fig. 8).

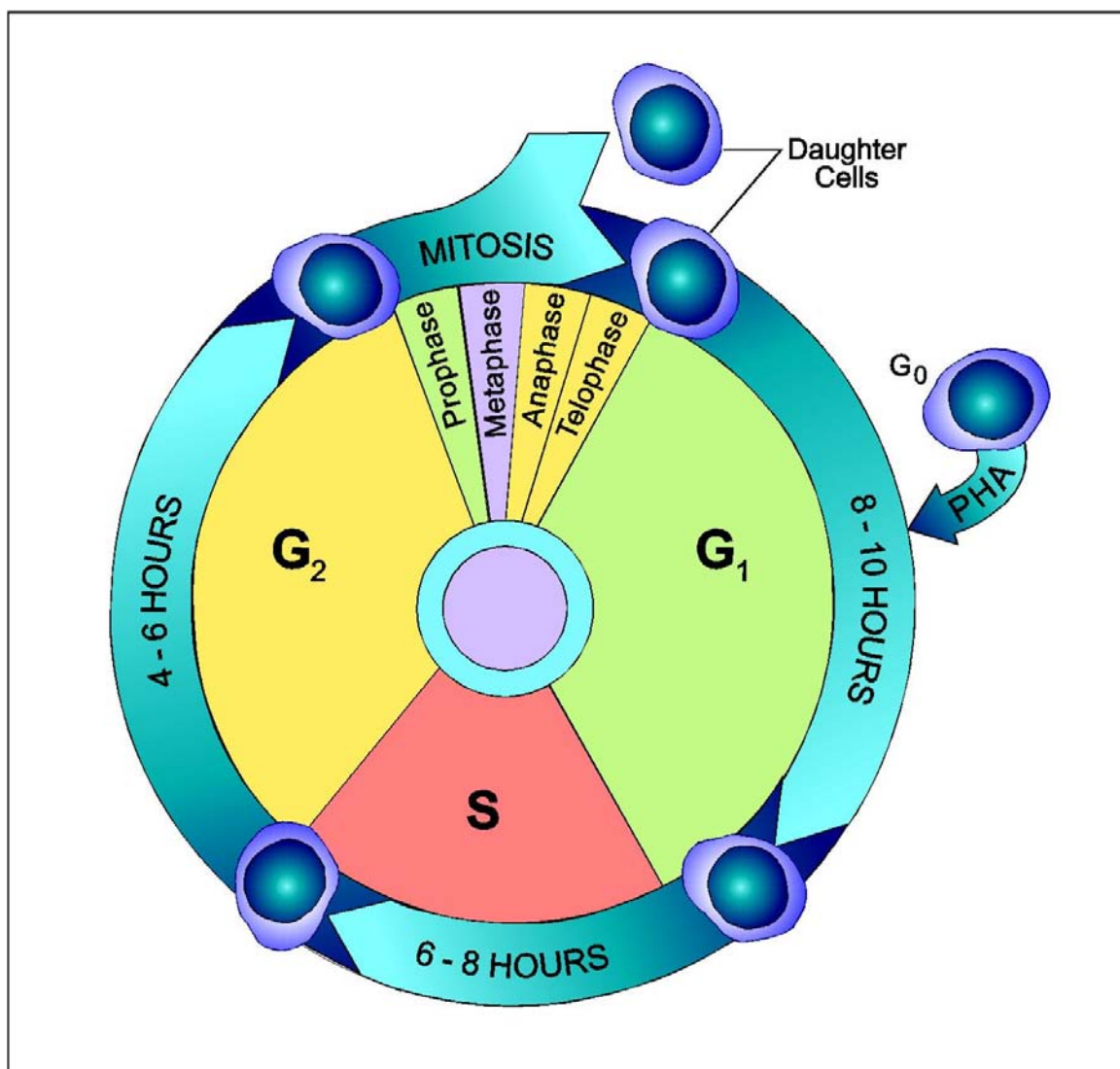


FIG. 8. The cell cycle (courtesy REACTS, USA).

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  $G_1$  (a presynthetic gap) and is followed by  $G_2$  (a postsynthetic gap) within the interphase. In cells which are not cycling, for example peripheral lymphocytes, the cell remains in  $G_0$ .

For cycling cells, interphase is metabolically the most active part of the cell cycle, and most of the energy requiring reactions in the nucleus takes place at this stage. The duration of each stage in the cycle varies with the type of cell and the conditions of growth. One can determine the lengths 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 [33]. 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.

The progression through the cell cycle is controlled at different checkpoints to ensure maximum fidelity in the DNA integrity and proper chromosome segregation to daughter cells. The main checkpoints act at the end of  $G_1$ , prior to replication, at the end of  $G_2$ , prior to mitosis and at the metaphase/anaphase transition, prior to chromosome segregation and cell division. At these checkpoints, the cell cycle progression can be blocked if DNA damage, incomplete replication or abnormal spindle structure are detected.

## 6. RADIATION INDUCED CHROMOSOMAL ALTERATIONS

The first reported evidence that X rays could induce chromosomal aberrations came from the genetic studies by Müller [34] of *Drosophila*. This was confirmed by the cytological studies of Painter and Müller [35]. Sax [36] later developed his 'breakage first' hypothesis on the origin of X ray induced chromosome aberrations, followed by Revell [37] who proposed the alternative exchange hypothesis. In essence, Sax [36] 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 [37] 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 [38], of a lesion/non-lesion interaction whereby a damaged site, in the Revell sense, may interact with an undamaged chromosome to form an exchange.

### 6.1. RADIATION INDUCED DNA LESIONS

Ionizing radiation is characterized by the production of discrete energy deposition events (i.e. spurs, blobs, and tracks) in time and space that damage DNA directly and indirectly by the generation of reactive species mainly produced by the radiolysis of water [39]. Biophysical studies of track structure show that low-LET radiation can produce localized clusters of ionizations within a single electron track. High-LET radiation produces a somewhat larger number of ionizations that are close in spatial extent [17] (Fig. 9A). Ionizing radiation induces a wide range of damage in DNA including base damage (BD), single strand breaks (SSB), abasic sites (AS), DNA-protein cross-links (DPC), and double strand breaks (DSB) (Fig. 9B).

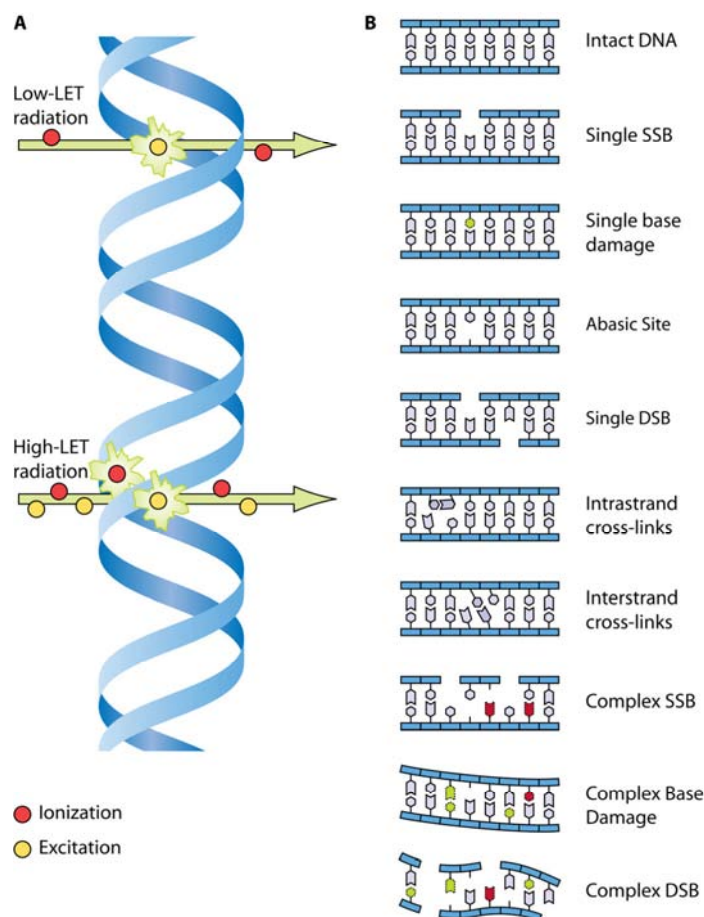


FIG. 9. Ionization pattern for low- and high-LET radiation (A) and radiation induced DNA lesions (B) [40, 41].

The energy to form an ion pair ( $\text{H}_2\text{O}^+ + \text{e}^-$ ) from the radiolysis of water is  $\sim 20$  eV, and ionizing radiation deposits energy in events that range in energies up to hundreds of eV, with the average amount being 60 eV. Because this energy is sufficient to produce approximately three ion pairs, the radicals formed will react in the vicinity of a concrete region. The resultant DNA lesions for all types of radiation can be single DNA lesions involving SSB, AS, or BD as well as Multiple Damage Sites (MDS) or clustered DNA lesions [42]. MDS lesions produced in DNA might involve one or more DSB, several SSB as well as BD. Complex clustered DNA lesions may be more difficult to repair or indeed fail to repair and hence potentially lead to the generation of lethal chromosome aberrations [43].

The cell has complex signal transduction, cell-cycle checkpoint and repair pathways to respond to the DNA damage. BD, AS, and SSB are repaired by different processes like base excision repair (BER), nucleotide excision repair (NER), and single strand break repair (SSBR) [44, 45]. DPC are repaired by NER and homologous recombination repair (HRR) [46]. DSB are critical lesions and their misrepair or non-repair are involved in the formation of chromosome aberrations like dicentrics or translocations [47]. The HRR and DNA non-homologous end-joining (NHEJ) are the two major DSB repair mechanisms [48, 49]. These two mechanisms act at different phases of the cell cycle. Whereas NHEJ contributes substantially to DSB repair in all cell cycle phases, HRR contributes modestly in  $G_1$  and progressively more as cells move through the cycle into  $G_2$  [50]. A comprehensive review of the biophysical and molecular processes leading to the formation of chromosomal aberrations by radiation has been published by Sasaki [51].

## 6.2. CHROMOSOME-TYPE ABERRATIONS

Schemes for the classification of chromosomal aberrations have been presented [52, 53].

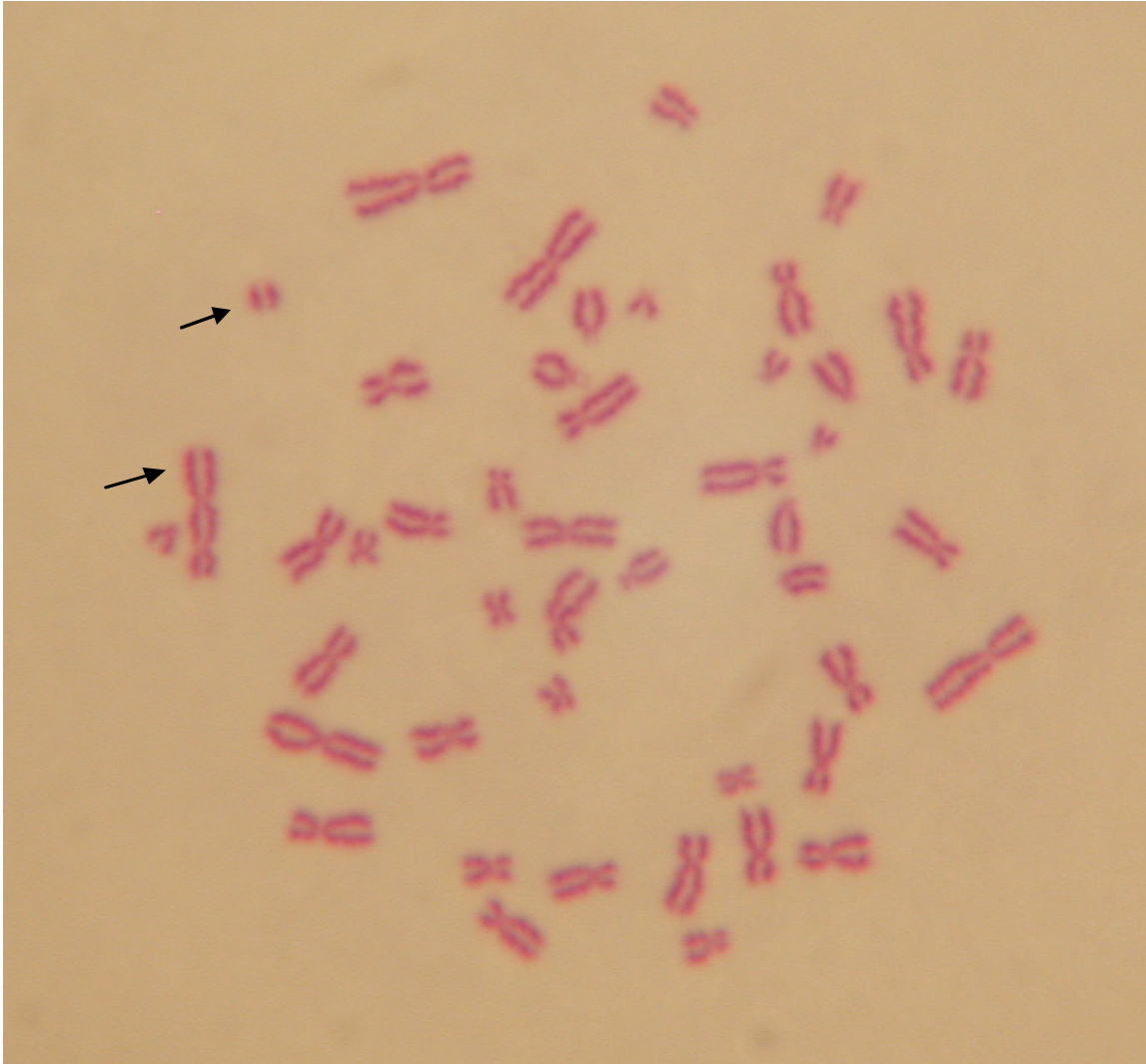
The peripheral lymphocyte population that is mitogenically stimulated is normally non-cycling and resides in the  $G_0$  stage of the cell cycle. The chromosome aberrations induced by radiation will consequently be of the chromosome-type, i.e. they involve both chromatids of a chromosome. It is well 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 mostly 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.

Chromatid-type aberrations therefore have little place in biological dosimetry because they are not induced by irradiation of  $G_0$  lymphocytes. Nevertheless they do occur as part of the overall background frequency of chromosomal damage and may be present in excess if the person being investigated for suspected irradiation also has history of exposure to chemical clastogens. It is therefore important for the microscope scorers to be fully cognisant of chromatid-types and not to confuse them with the chromosome-types. In addition, with the increasing research interest in delayed chromosomal instability and bystander phenomena there is renewed interest in the chromatid aberrations. Chromosome-type aberrations are therefore covered in this Section and chromatid-type aberrations are addressed in Section 6.4.

### 6.2.1. Unstable aberrations

#### *Dicentrics*

The dicentric (Fig. 10) is the main aberration used for biological dosimetry.



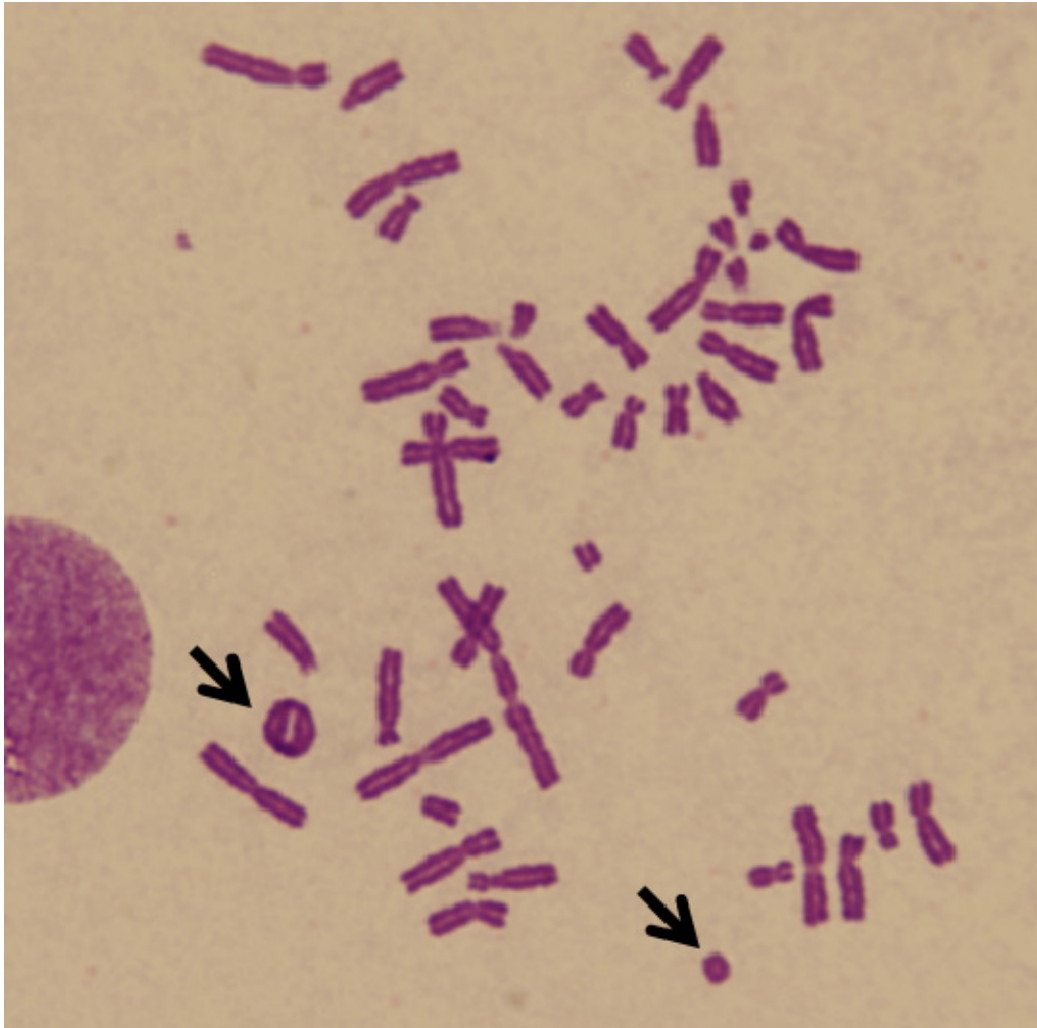
*FIG. 10. A dicentric chromosome with its accompanying acentric fragment, (Giemsa staining).*

It is an exchange between the centromeric pieces of two broken chromosomes which in its complete form is accompanied by an acentric 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 9.

#### *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. 11).





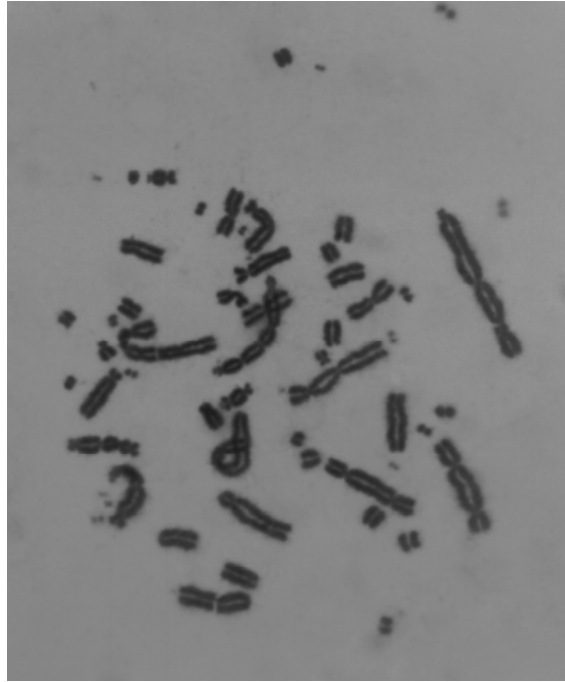
*FIG. 11. A metaphase spread with two rings (arrowed), a dicentric and acentric fragments (Giemsa staining).*

#### *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. Acentric rings where clear spaces may be seen within the dots are normally considered to be interstitial deletions whereas minutes which appear as double dots are mostly terminal deletions [54, 55].

#### *Rogue cells*

Rogue cells are defined as metaphase cells prepared from cultured blood lymphocytes which exhibit extremely high levels of chromosome damage in the absence of an overt cause. An example is shown in Fig. 12 where chromosome breakage and rearrangements are so extensive that it is difficult to identify more than one or two normally appearing monocentric chromosomes.



*FIG. 12. A rogue cell observed among 500 otherwise normal metaphases taken from a healthy, non-smoker, control person with no occupational or medical history of radiation exposure and living in a low radon area. The metaphase displays the characteristic features of many polycentric chromosomes and acentric fragments including a large number of double minutes.*

In contrast, the cell has numerous polycentric chromosomes, acentric fragments and double minutes. Double minutes are tiny bodies of chromatin containing a few megabases of DNA and can be defined as cytogenetic equivalents of amplified DNA sequences [56].

These unique cells have been observed in all races and ethnic groups throughout the world. For example, rogue cells were first observed in 1968 in blood samples collected from the Yanomami Indian tribes that inhabit the rainforests of Venezuela [57]. Subsequently they have been reported in the inhabitants of many countries including England, Japan, Ukraine, Lithuania and the Russian Federation. The term 'rogue' cells was coined by Awa and Neel [58] who described these cells in the offspring of both irradiated and non-irradiated control subjects from the bombing of Hiroshima. Similar to the atomic bomb survivor studies, cytogenetic evaluations performed on exposed and unexposed populations living near Chernobyl showed that rogue cells did not associate with radiation exposure since they were also found in the non-exposed control groups [59, 60].

Although the worldwide occurrence of rogue cells in the human population has been firmly established, their clinical significance, if any, is unknown. In studies where serial sampling was performed rogue cells have been found to be transitory appearing intermittently in brief bursts simultaneously in certain individuals of discrete populations. It is noteworthy that rogue cells have not been reported in the clinical cytogenetics literature which is likely due to the longer three-day culture allowing 2 or 3 cell divisions by which time rogue cells would probably be lost. In addition, clinical cytogeneticists analyse relatively few metaphase cells (i.e.15–20) to reach a diagnosis on the normal or abnormal state of an individual's karyotype. In contrast, radiation cytogeneticists routinely analyse hundreds even a thousand or more first-division metaphase cells from an individual which greatly increases the probability of detecting rogue cells which are known to occur at low frequency. In conclusion, the

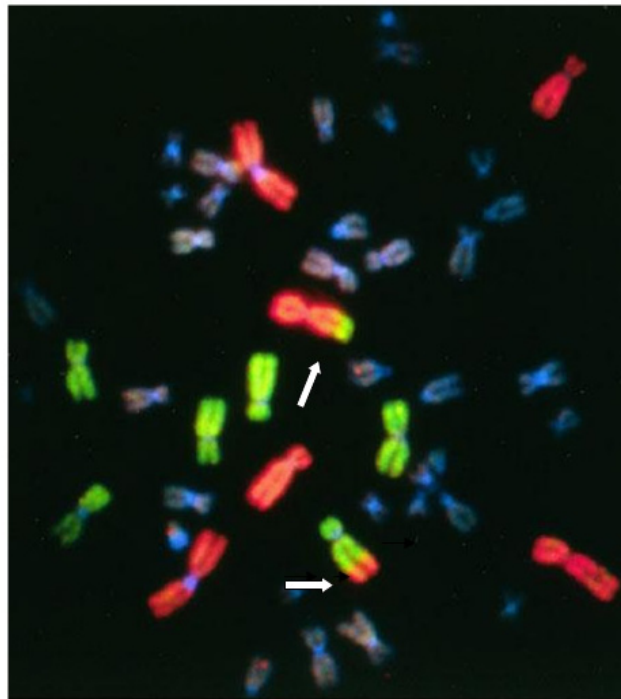
etiology and medical significance of rogue cells in human lymphocytes remains an enigma although some evidence suggests that viruses such as the JC human polyoma virus may play a role in their expression [61].

In view of the occurrence of rogue cells, the recommendation is therefore that, for most biological dosimetry purposes, isolated metaphases having the appearance of rogue cells should be excluded from the dose evaluation. An exception to this could be where there is additional evidence of exposure to high LET radiations and then, ideally, several multiply damaged cells present with a continuous spectrum of damage.

### 6.2.2. Stable aberrations

#### *Reciprocal translocations*

The reciprocal 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 procedure is too laborious for routine biological dosimetry. With solid Giemsa staining, translocations are not observed so reliably. Their application to dosimetry is now possible with the FISH method (see Section 10). By the FISH method these are visualized as bicoloured monocentric chromosomes (Fig. 13).



*FIG. 13. A metaphase illustrating FISH-based chromosome 'painting' to detect translocations. Chromosome pairs 1, 2 and 4 are 'painted' red and chromosome pairs 3, 5 and 6 are 'painted' green. A reciprocal translocation is illustrated by the two bicolored chromosomes (2 and 5) which have exchanged segments at the ends of their long arms (courtesy Ramsey and Tucker, LLNL, USA).*

#### *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 whole chromosome, centromere and telomere probes, a number of translocations designated as terminal or

incomplete were 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% [62].

#### *Interstitial translocations (insertions)*

This is a bicoloured chromosome where an acentric piece of one chromosome has been inserted within an arm of another chromosome. An example is shown in Fig. 14.

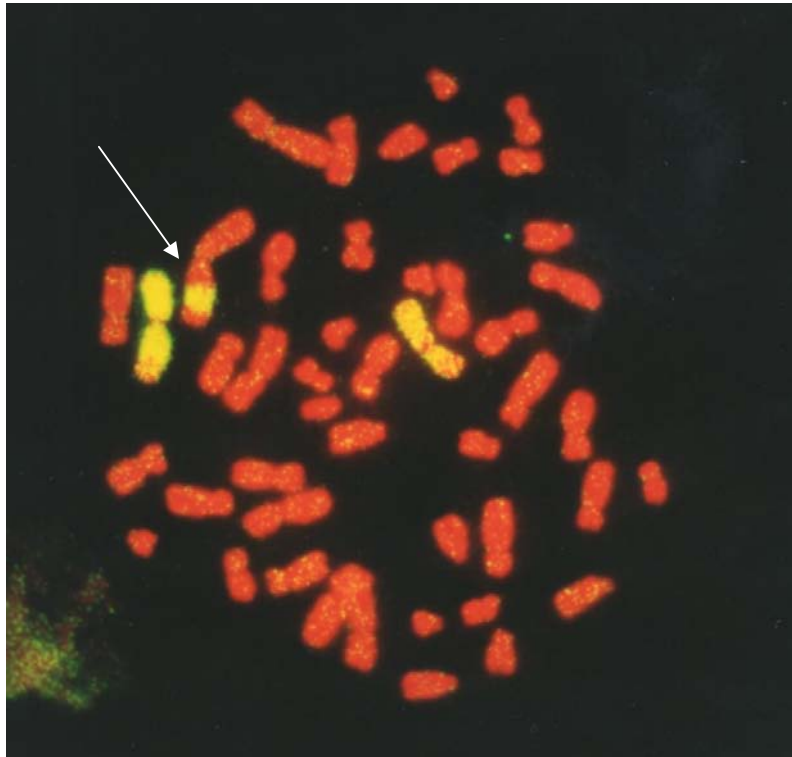


FIG. 14. A human metaphase spread with an insertion. Chromosome pair 1 is painted yellow and all other chromosomes are counterstained with propidium iodide.

#### *Stable and unstable cells*

Retrospective FISH biological dosimetry is possible because stable aberrations such as a reciprocal translocation will pass successfully through mitosis and into the daughter cells. However for this to succeed the complete genome needs to be stable. A translocation can still fail to negotiate division if there is an unrelated and unstable structure such as a dicentric or an excess acentric also present in the same cell. This has led to the need to consider stability not only of individual types of aberrations but of the cell as a whole. This is a concept recognized many years ago by Buckton et al. 1967 [63] who introduced the designations Cs and Cu for stable and unstable cells respectively. The concept has again come to prominence with the development of retrospective biological dosimetry by FISH where it has been demonstrated that reciprocal (two-way) translocations seem to be more stable than incomplete (one-way) ones [64, 65].

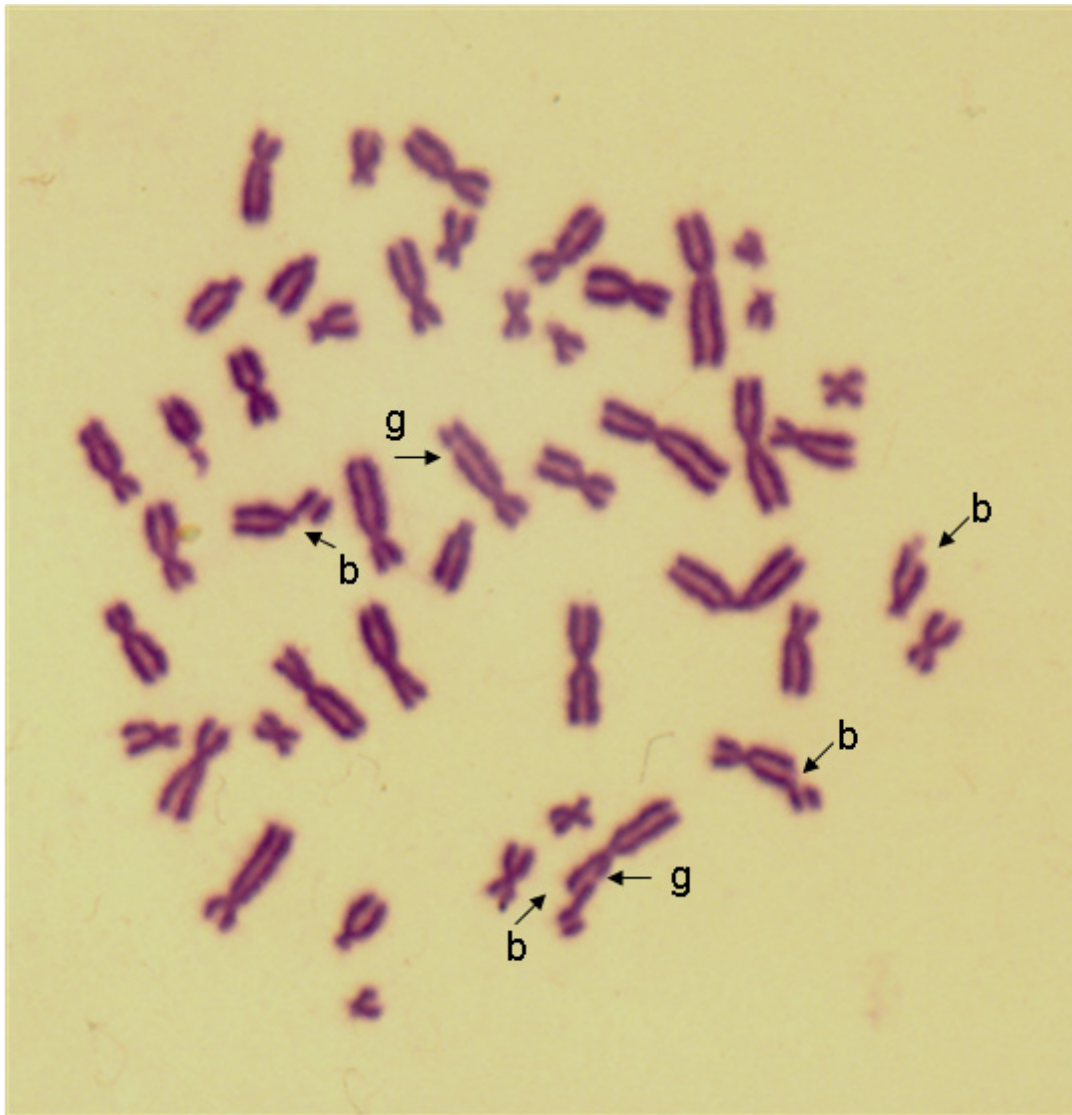
### 6.3. CHROMATID-TYPE ABERRATIONS

Chromatid-type aberrations are generally classified in the same way as chromosome-type aberrations; the apparent unit of involvement in a chromatid-type aberration is, in most cases,

the single chromatid and not the whole chromosome, as is the case for chromosome-type aberrations.

*Terminal and interstitial deletions*

A terminal deletion is a distinct displacement of the chromatid fragment distal to the lesion (Fig. 15).



*FIG. 15. A metaphase spread with chromatid breaks (b) and gaps (g).*

If there is no displacement, the non-staining region between the centric and acentric regions must be of a width greater than the width of a chromatid to be considered a terminal deletion. This latter definition is used to distinguish between terminal deletions (chromatid breaks) and achromatic lesions (gaps).

Chromatid-type interstitial deletions are not as readily observable as their chromosome-type counterparts, in part due to the fact that the small deleted fragment is often separated from the deleted chromosome and is not observed.

### *Achromatic lesions*

Achromatic lesions (or gaps), Fig. 15, are non-staining or very lightly stained regions of chromosomes present in one chromatid (single) or in both sister chromatids at apparently identical loci (double). If the non-staining region is of a width less than that of a chromatid, the event is recorded as an achromatic lesion. This is clearly only a working definition. It is generally suggested that achromatic lesions be recorded, but always separately from chromatid deletions. Their frequency should not be included in the totals for aberrations per cell since their significance and relationship to other 'true' aberration types is at present unclear.

### *Isochromatid deletions*

Isochromatid deletions appear as exceptions to the class of chromatid-type aberrations, since they involve both chromatids, apparently with 'breaks' at the same position on both. However, in suitable material they can be shown to be induced by radiation in the S and G<sub>2</sub> phases of the cell cycle, as is the case for other chromatid-type aberrations.

There are several possible types depending on the nature of the sister unions that occur. If a sister union occurs, it is possible to distinguish isochromatid aberrations from chromosome-type terminal deletions. In mammalian cells, however, a sister union is a rare event and most of the isochromatid deletions are of the non-union proximal and distal types. The acentric fragment is most often not associated with the deleted centric part of the chromosome. The convention for analysis stipulates that since the radiation-induced aberrations in G<sub>0</sub> lymphocytes are of the chromosome-type, all paired acentric fragments are to be classified as chromosome-type terminal deletions. Since the frequency of isochromatid deletions will in any case be low in lymphocytes, this convention is not unreasonable.

### *Asymmetrical interchanges*

Asymmetrical interchanges (interarm interchanges and asymmetrical chromatid exchanges) are the chromatid-type equivalents of chromosome-type dicentrics.

### *Symmetrical interchanges*

Symmetrical interchanges (symmetrical chromatid exchanges), Fig. 16, are the chromatid-type equivalents of chromosome-type reciprocal translocations.



FIG. 16. A metaphase spread with a chromatid symmetrical exchange, Giemsa stained.

In the case of chromatid-type symmetrical exchanges, somatic pairing maintains an association between the chromosomes involved in the exchange and thus they can be readily observed in the absence of any chromosome-banding procedures.

#### *Asymmetrical and symmetrical interchanges*

There are two forms of symmetrical and asymmetrical interarm interchanges, but when analysing metaphase cells only one of each is distinguishable. Somatic pairing allows the symmetrical interchange to be observed.

#### *Triradials*

A triradial (three-armed configuration) can be described as the interaction between one chromosome having an isochromatid deletion and a second having a chromatid deletion.

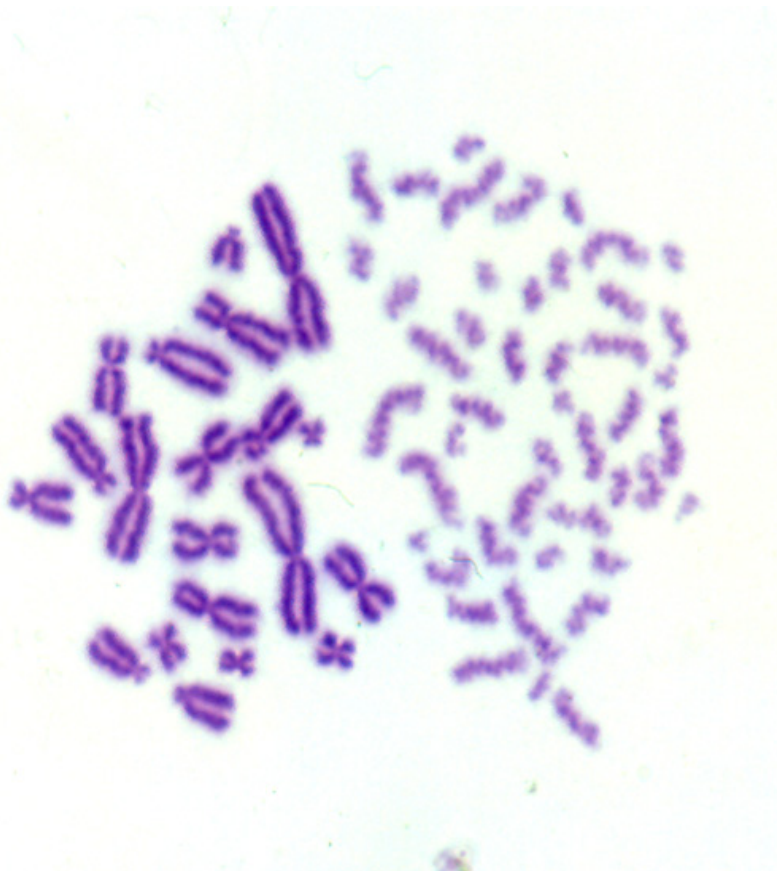
This classification scheme is clearly not exhaustive, since there are many types of complex aberrations that can be produced. The ones described are by far the most commonly observed. A more complete classification is given by Savage [52].

### 6.4. PREMATURE CHROMOSOME CONDENSATION

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 to mitotic Chinese hamster ovary (CHO) or HeLa cells using Sendai virus or polyethylene glycol (PEG) as the fusing agent [66]. However, fusion by means of Sendai virus requires cells with membranes especially receptive to the virus particles and it has been reported that G<sub>0</sub> lymphocytes cannot be satisfactorily fused using Sendai virus. This difficulty was overcome for the purpose of biological dosimetry with the use of PEG for PCC induction [67].

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 [68, 69].

The PCC technique, which is described in detail in Section 11, is 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. dicentrics and translocations) [70–72]. These studies demonstrate that the dicentrics, 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 dicentrics 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).



*FIG. 17. Premature chromosome condensation induced by PEG-mediated fusion in an unirradiated human lymphocyte fused with a mitotic CHO cell. Forty-six distinct single chromatid PCCs can be seen.*

#### **6.4.1. PCC techniques**

The different PCC techniques can be divided as follow:

*Fusion-PCC assay* is the first that has been described in the literature in 1974 [73, 74]. In this assay, lymphocytes are fused with mitotic cells, often CHO cells are used, in order to induce premature condensation of the human chromosomes [67]. By this approach, it is possible to score the number of human chromosomal pieces and therefore, the number of radiation induced chromosomal fragments in excess of background frequency. It has also been used to



estimate non-uniform exposure [75]. The major advantage of this method is that damage can be observed shortly after blood sampling.

*Rapid Interphase Chromosome Assay (RICA)* allows the visualisation of radiation-induced damage using FISH probes. DNA of chromosomes is artificially condensed in order to identify the chromosome domains and to detect exchanges between two different domains [76–78].

*Dic-PCC assay* allows the observation of dicentrics in other phases of the cell cycle (mainly in the G<sub>2</sub> phase) than the classical M phase and therefore to visualize cells that would not have been seen with the conventional dicentric assay [79]. This is particularly interesting when the lymphocyte count has dropped following exposure and when it is difficult to obtain classical mitoses. Using the fusion-PCC method the time between sampling and dose estimation can be reduced [79], however using the chemical techniques for inducing PCC most laboratories culture for 48 hours and hence there is no reduction in time.

*Ring-PCC assay* corresponds to the visualisation of radiation-induced rings in cells in different phases of the cell cycle (Fig. 18).

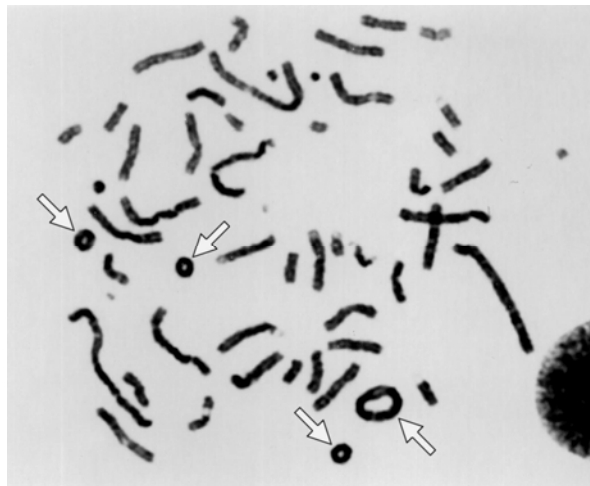
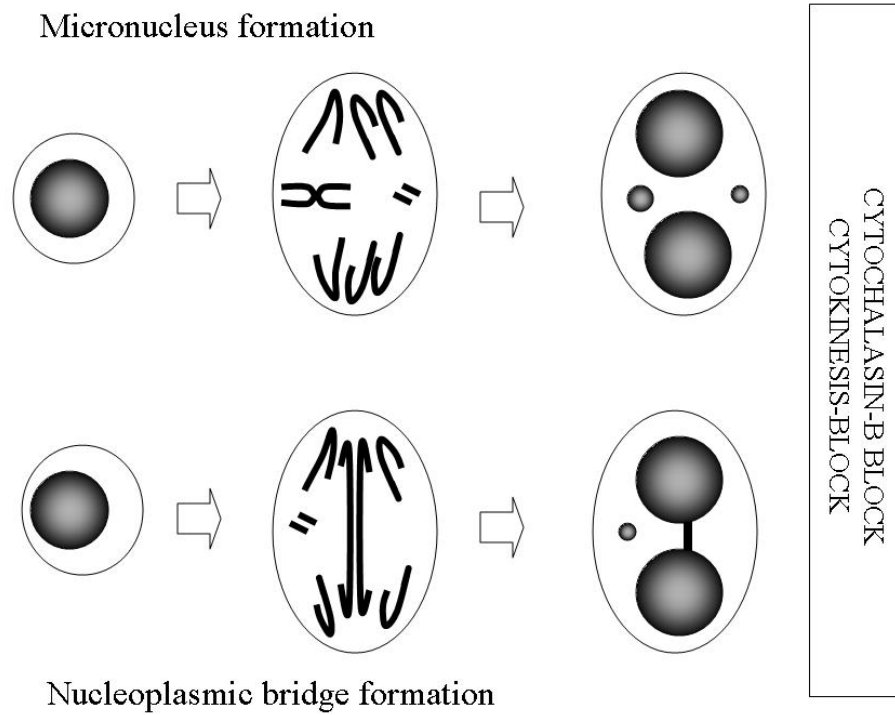


FIG. 18. PCC rings (arrows) in a lymphocyte taken from patient A of the Tokai-mura accident (see Section 11.4).

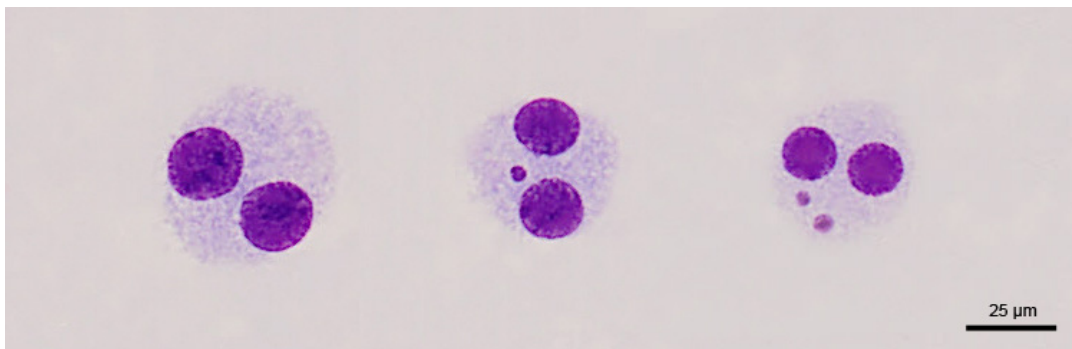
The major advantage of this approach is the measurement of much higher doses than with the classical dicentric assay as the saturation of ring number appears only at doses above 20 Gy for low LET radiation [70, 80, 81].

## 6.5. MICRONUCLEI

Micronuclei (MN) are formed from lagging chromosomal fragments or whole chromosomes at anaphase which are not included in the nuclei of daughter cells (Fig. 19A, B). They are therefore seen as distinctly separate small spherical objects that have the same morphology and staining properties of nuclei, within the cytoplasm of the daughter cells [82].



A



B



C

**FIG. 19.** (A) Schematic diagram showing the mechanism of origin of micronuclei and nucleoplasmic bridges in the cytokinesis-block micronucleus assay. (B) Examples of binucleated cells without and with 1 and 2 micronuclei. (C) Examples of binucleated cells with 1 and with 2 nucleoplasmic bridges; in each case the nucleoplasmic bridge is accompanied by a micronucleus.

In the mid-1980s a major technical innovation was introduced. This was the method for blocking cytokinesis in cultured lymphocytes by adding cytochalasin B (Cyt-B) to the medium without inhibiting nuclear division. The cytokinesis block [83, 84] produces binucleate (BN) cells rather than permitting the two daughter cells to separate. With this protocol it is therefore possible to distinguish between proliferating (following the first mitosis) and non-proliferating cells and to specifically score MN in those cells capable of expressing MN i.e. BN cells. The modified assay allows identification and quantification of MN in binucleate cells with preserved cytoplasm (Fig. 19B).

Measurement of micronuclei within BN cells can be further refined with the use of centromere probes which allows micronuclei originating from acentric chromosome fragments to be distinguished from those originating from whole chromosomes [85, 86].

Current developments in the automation of MN scoring, give new perspectives for the use of the assay in mass radiation casualties and routine biomonitoring [87] (Section 13.3.3).

The cytokinesis-block micronucleus assay has now also evolved into a cytome assay where a spectrum of chromosomal damage can be assessed including breakage, asymmetric chromosome rearrangement, chromosome loss and non-disjunction as well as necrosis, apoptosis and cytostasis [86]. This method is also specifically used to measure nucleoplasmic bridges (NPB), (Fig. 19A, C) a surrogate biomarker of dicentric chromosomes which result from either telomere end-fusions or misrepair of DNA double strand breaks [86]. NPB measured in the cytokinesis-block cytome assay are thus also applicable for biological dosimetry of ionizing radiation exposure [88]. A strong correlation and similar dose-response curves were observed between NPB, and dicentric chromosomes and centric rings [89].

Detailed information on MN analysis and the cytome assay is given in Section 12.



## 7. BLOOD SAMPLING

### 7.1. TIMING

A venipuncture blood sample, preferably 10 mL, could be taken within a few hours of a whole body radiation exposure. However, 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 hours [90]. This could result in an unrepresentative proportion of irradiated cells in the specimen and therefore delaying sampling until at least the next day is advisable. An effort should be made to ensure that the sample is obtained promptly because even if the patient's haematological parameters are within normal limits after about four weeks have elapsed, aberration yields begin to fall, causing greater uncertainty in any estimates of the radiation dose [91].

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. If medical treatment includes whole-blood or blood fraction transfusions, 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 is ethically acceptable as such sampling would be undertaken 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 [92]. 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 (Leibovitz's L-15 medium is essential for long term transportation because it is buffered by 10 times more amino acids than other common culture media and the pH is stable for a long time).
- (2) Take a blood sample into a conventional heparinized tube.
- (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) The cells are then washed in conventional medium and processed following the same steps described later in Section 9.1 for setting up conventional cultures.

If the Leibovitz medium procedure is used, it will need to be validated with a dose response curve produced under the same conditions.

### 7.2. ANTICOAGULANT

Preservative free lithium heparin is the most commonly used anticoagulant for lymphocyte cultures, although it is possible to use sodium or ammonium heparin. Other commonly available anticoagulants, e.g. ethylenediaminetetraacetic acid (EDTA), often result

in poor cell growth and should not be used. If a sample is received in the wrong anticoagulant it is preferable to request a fresh specimen. However this may not always be possible and in such a situation it is possible to 'rescue' the sample by washing. The procedure is to take 4 mL of the blood, add 6 mL of a balanced salt solution (Hank's or Earle's) or culture medium and spin at 600g for 3 to 5 min. Remove the supernatant and add a fresh 10 mL of the washing liquid to the cell pellet and spin again. After final removal of the supernatant the washed cells can be restored to the original volume of blood by adding culture medium containing 10% foetal calf serum. Cultures can then be set up as described later in Section 9.1, treating the washed specimen as if it were a normal blood sample.

### 7.3. CONTAINERS

Disposable glass or plastic specimen tubes containing the correct amounts of lithium heparin are available from several manufacturers. Both the older style screw-top tubes and vacuum tubes can be used. They must be sterile and many manufacturers routinely supply them sterile but this should be confirmed. 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 correctly addressed proper packaging for the return of the sample.

### 7.4. TRANSPORT

Blood specimens should be maintained ideally between 18 and 24°C during transportation. If temperatures well outside this range are likely to be experienced, the provision of coolant or room temperature packs and temperature loggers is advisable. In any case, freezing during transportation should be avoided.

Transport of specimens should comply with applicable national and/or international regulations for the transport of infectious substances, as outlined in the current WHO guidance on regulations for the transport of infectious substances [93]. This document also explains to shippers how to classify, document, mark, label and package infectious or potentially infectious substances such as diagnostic blood samples.

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 include 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 outer packaging, e.g. a sturdy cardboard box, with suitable labelling. Shipping of blood samples, not known to contain pathogens, for diagnostic purposes is characterized as 'UN 3373. BIOLOGICAL SUBSTANCE, CATEGORY B'. The labelling should therefore include this phrase together with a white diamond label with black letters 'UN 3373'. In addition the package should be marked with the sender's name, address and telephone number; the receiver's name, address and telephone number; and the telephone number of a responsible person, knowledgeable about the shipment [93].

If it is felt that cooling or room temperature 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 kits that conform to the regulations are commercially available.

For international shipments, shippers need to obtain any necessary export or import permits and the receiving laboratory should be notified before shipment of the specimens, in order to arrange for an import license if required. 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.

Transit times of two or three days can be tolerated; however, blood samples need special delivery services to avoid long delays, such as around national holidays.

During air transport the blood should not be X rayed in security checks. If this is likely, a piece of X ray film or a standard Thermoluminescent Dosimeter (TLD) or Optically Stimulated Luminescence (OSL) monitoring badge could be included in the package. A 'DO NOT NOT X-RAY' label should be placed on the package. This condition should be also written on accompanying paperwork.





## 8. PRODUCTION OF AN *IN VITRO* DOSE–RESPONSE CURVE

### 8.1. GENERAL CONSIDERATIONS

Despite improvements in techniques and the adoption by different workers of more comparable statistical programs for data analysis, differences between laboratories' calibration curves still remain. The interpretation of dose using a calibration curve produced elsewhere may introduce extra uncertainty, and therefore it must be recommended that any laboratory intending to carry out biological dosimetry should establish its own dose–response data [94].

Most accidental overexposures involve gamma radiation sources but there are also an appreciable number of events involving X rays. It is well established that the calibration for these two low LET radiations are different particularly at low doses. Therefore, 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. Events involving exposure to neutrons are thankfully rare but the possibility should be considered that a laboratory may be requested to respond to a criticality accident. If so, a calibration curve for fission spectrum neutrons will be required.

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 [95]. Freshly taken blood specimens in lithium heparin tubes should be used and irradiated as whole blood at 37°C. After irradiation they should be held for a further 2 hours at 37°C and then cultured by the standard method identical to that used for assaying dicentric, translocations or micronuclei on specimens from suspected overexposure patients.

### 8.2. PHYSICAL CONSIDERATIONS

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 complications of scattered radiation. The materials should have atomic compositions similar to blood because the dose to blood close to the specimen container 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 exposure set up should be calibrated by physical measurements and most commonly an ionization chamber is used but other methods are possible. Fig. 20 for example illustrates measurements using alanine.

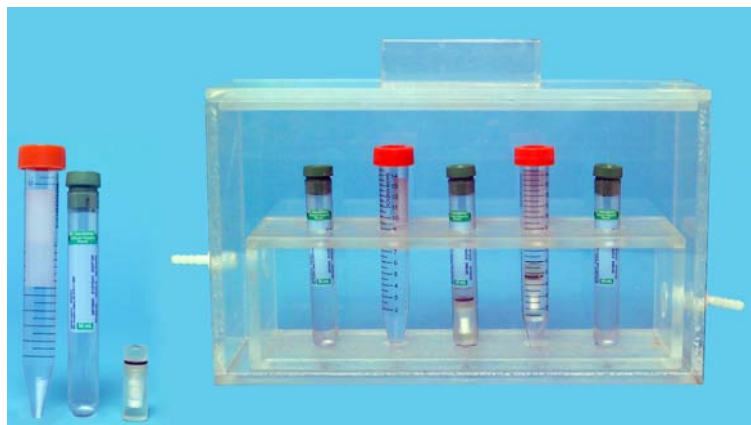


FIG. 20. Exposure array used to hold and position 15 mL test tubes and 10 mL blood vacutainers for exposures to irradiation with  $\gamma$ -ray sources. The box container of the exposure array is constructed of plexiglass with 6 mm wall thickness as part of its design to assure charged particle equilibrium. The box container is also equipped with access ports at both ends to permit water flow with a circulating water bath (not shown) to maintain the contents at 37°C during irradiation. Test tubes and vacutainers filled either with water or vials of alanine used for dosimetry measurements are also illustrated [97] (courtesy AFRRI, USA).

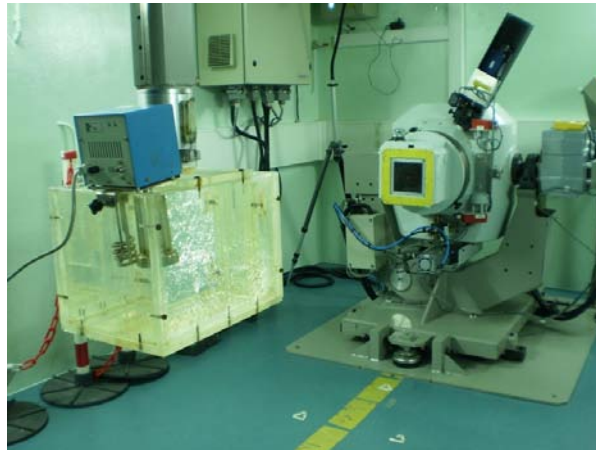
The detector of a physical dosimeter should be surrounded by material equivalent to that which surrounds the blood. If possible it should have similar dimensions to the blood sample so that it can replace the sample for dosimetry purposes. The physical dosimeter would normally be calibrated in air kerma with the unit of Gy and be traceable to a national standard. 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%. Additionally, some researchers choose to produce non-acute calibrations in order to have a better understanding of how the  $\beta$  coefficient should be modified for interpreting aberration yields from accidents involving protracted irradiation. It is even more essential that such calibrations should be carried out at 37°C. If done at room temperature there will be little or no repair during the exposures so that the resulting dose response curve will be the same as that from acute irradiations. An important point to remember is that exposure time, not dose rate, is the critical factor with protraction calibration. Therefore each data point should be from blood irradiated for the same time. This is achieved by varying the distance from the

source which of course requires a lot more physics calibration measurements. The easier alternative, using a constant dose rate and therefore a single irradiation position, requires different delivery times for each dose and the resultant data will not fit so well to the linear quadratic dose response equation [96].

Some laboratories prefer to place the blood sample in a phantom for calibration purposes on the grounds that scatter in a human body is to some extent taken into account (Figs 20 and 21).



*FIG. 21. A water bath heated to 37°C placed in front of a cobalt-60 gamma ray source. In order to achieve electronic equilibrium the blood sample is located inside a plexiglass holder.*

However rather more consideration needs to be given to the dosimetric correction factors above and whether an ionization chamber can be placed beside the blood sample. Water is generally used as the phantom and, it should be maintained at 37°C. If the ionization chamber is placed in the same geometry as the blood sample this will take account of the dose due to the scattered radiation. Using warm water will require significant temperature and pressure corrections to be applied and of course the chamber must not get wet. Neutron calibrations performed in a phantom are particularly problematic. Rather than water, a tissue equivalent material for the phantom is preferable. This produces a radiation spectrum akin to that in the body with possibly a considerable enhancement of the low LET component of dose to the lymphocytes. Specifying the spectrum of the components to the total absorbed dose can be very difficult.

### 8.3. STATISTICAL CONSIDERATIONS

As discussed in Section 3, there is very strong evidence that the yields of chromosome aberrations or micronuclei ( $Y$ ) are related to dose ( $D$ ) by the linear quadratic equation

$$Y = C + \alpha D + \beta D^2 \quad (3)$$

or, for high LET radiation, the  $\alpha$ -term becomes large and eventually the  $\beta$ -term becomes biologically less relevant and also statistically 'masked' and the dose response is approximated by the linear equation

$$Y = C + \alpha D \quad (4)$$

The objective of curve fitting is to determine those values of the coefficients  $C$ ,  $\alpha$  and  $\beta$  which best fit the data points. For dicentrics, irradiation with X or gamma rays produces a distribution of damage which is very well represented by the Poisson distribution [98]. In contrast, neutrons and other types of high LET radiation produce distributions which display overdispersion, where the variance ( $\sigma^2$ ) exceeds the mean ( $y$ ). Whether the ratio of variance to mean ( $\sigma^2/y$ ) is a function of dose is at present an open question. For micronuclei the data tend to overdispersion at all doses even with photon irradiation.

Because curve fitting methods are based on Poisson statistics, the dicentric cell distribution should be tested for compliance with the Poisson distribution for each dose used to construct the calibration curve. Nowadays, the most widely used test is the  $u$  test [99, 100]. The  $u$  test statistic is a normalized unit of the dispersion index ( $\sigma^2/y$ ), which for a Poisson distribution should be unity.  $u$  values higher than 1.96 indicate overdispersion (with a two-sided significance level,  $\alpha = 0.025$ ).

$$u = (\sigma^2/y - 1) \sqrt{\frac{N-1}{2(1 - 1/X)}} \quad (5)$$

where:

$N$  indicates the number of cells analysed, and  
 $X$  the number of dicentrics (or dicentrics plus rings) detected.

$u$  values of  $< -1.96$  indicate underdispersion. Biologically, underdispersion is very unlikely to occur so values of  $u$  lower than  $-1.96$  may be indicative of a problem in data sampling.

Adequate curve fitting requires a sufficient number of degrees of freedom to minimize the error on the curve. Ideally, 10 or more 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 approximately 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 [101]. For high LET radiation a maximum of 2.0 Gy is suggested.

As most radiation accidents involve doses of less than 1.0 Gy, the lower end of the curve is of particular importance in estimating doses. A significant effort should therefore be made to reduce the statistical uncertainty associated with the  $\alpha$  coefficient of yield. It is suggested that several of the calibration doses, certainly a minimum of 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 to detect 100 dicentrics at each dose. However at lower doses this is difficult to achieve and instead several thousand cells per point should be scored; a number between 3000 and 5000 is suggested. In all cases, the actual number of cells scored should be dependent on the number of dose points in the low dose region, with the focus on

minimizing the error on the fitted curve. Table 4 gives example data used to construct dose-effect curves for low LET  $\gamma$ -radiation and high LET  $\alpha$  radiation.

TABLE 4. CYTOGENETIC RESULTS OBTAINED FROM BLOOD SAMPLES IRRADIATED WITH  $\gamma$ -RAYS AND HELIUM-4 PARTICLES [102, 103]

$\gamma$ -rays (Cobalt-60)												
dose (Gy)	N	X	cell distribution of dicentrics						$\sigma^2/y$	u		
			0	1	2	3	4	5			6	
0.000	5000	8	4992	8							1.00	-0.07
0.100	5002	14	4988	14							1.00	-0.13
0.250	2008	22	1987	20	1						1.08	2.61
0.500	2002	55	1947	55							0.97	-0.86
0.750	1832	100	1736	92	4						1.03	0.79
1.000	1168	109	1064	99	5						1.00	-0.02
1.500	562	100	474	76	12						1.06	1.08
2.000	332	103	251	63	17	2					1.14	1.82
3.000	193	108	104	72	15	2					0.83	-1.64
4.000	103	103	35	41	21	4	2				0.88	-0.84
5.000	59	107	11	19	11	9	6	3			1.15	0.81
Average											1.0	

20 MeV $^4\text{He}$ particles												
dose (Gy)	N	X	cell distribution of dicentrics							$\sigma^2/y$	u	
			0	1	2	3	4	5	6			7
0.000	2000	3	1997	3							1.00	-0.04
0.051	900	19	881	19							0.98	-0.44
0.104	1029	27	1004	23	2						1.12	2.84
0.511	1136	199	960	154	21	1					1.07	1.60
1.010	304	108	217	69	15	3					1.09	1.15
1.536	142	96	75	40	25	2					0.98	-0.20
2.050	137	120	63	44	16	12	2				1.20	1.65
2.526	144	148	66	34	25	14	3	2			1.40	3.40
3.029	98	108	47	16	17	17	0			1	1.56	3.93
Average											1.19	

For each dose analysed, total number of cells scored (N), total number of dicentrics observed (X), cell distribution of dicentrics and dispersion index ( $\sigma^2/y$ ) and u-test (u) are presented. u values greater than 1.96 indicate overdispersion.

The technique suggested for determining the best fit coefficients is that of maximum likelihood [104, 105]. Using this method, the best fit value for each coefficient is achieved by assuming a Poisson distribution and maximizing the likelihood of the observations by the method of iteratively reweighted least squares. For overdispersed (non-Poisson) distributions, as obtained after high LET radiation, the weights must take into account the overdispersion. If the data show a statistically significant trend of  $\sigma^2/y$  with dose, then that trend should be used. Otherwise, the Poisson weight on each data point should be divided by the average value of  $\sigma^2/y$ .

The goodness of fit of the curve and significance of fitted  $\alpha$  and  $\beta$  coefficients should then be tested, for instance using the Chi-squared ( $\chi^2$ ) test and an appropriate form of the F-test (e.g. F-test, z-test or t-test) respectively. These tests are detailed in Annex VI. If there is evidence of a lack of fit (i.e. the  $\chi^2$  is greater than the degrees of freedom (df)), then the standard error should be increased by  $(\chi^2/df)^{1/2}$ . Many computer programs calculate SE values

that are based on the sum of squares, instead of the Poisson estimate of the variance, which may lead to a false underestimation of the Poisson error. For this reason, when SE are calculated using this method, and the df is greater than the  $\chi^2$ , it is a good practice to increase the SE by  $(df/\chi^2)^{1/2}$ .

Table 5 indicates the fitted coefficients when the data from Table 4 are used and the SEs calculated using Poisson assumptions.

TABLE 5. THE RESULTS OF FITTING THE DICENTRIC DATA FROM TABLE 4

$\gamma$ -rays (Cobalt-60)				
C $\pm$ SE	$\alpha$ (Gy <sup>-1</sup> ) $\pm$ SE	$\beta$ (Gy <sup>-2</sup> ) $\pm$ SE	$\chi^2$	df
0.00128 $\pm$ 0.00047	0.02103 $\pm$ 0.00516 <i>F</i> = 4.08, <i>p</i> < 0.03	0.06307 $\pm$ 0.00401 <i>F</i> = 15.73, <i>p</i> < 0.01	6.61 <i>p</i> = 0.58	8
20 MeV <sup>4</sup> He particles				
C $\pm$ SE	$\alpha$ (Gy <sup>-1</sup> ) $\pm$ SE	$\beta$ (Gy <sup>-2</sup> ) $\pm$ SE	$\chi^2$	df
0.00143 $\pm$ 0.00093	0.32790 $\pm$ 0.02875 <i>F</i> = 11.41, <i>p</i> < 0.01	0.02932 $\pm$ 0.01636 <i>F</i> = 1.79, <i>p</i> = 0.25	7.40 <i>p</i> = 0.39	6
0,00193 $\pm$ 0,00097	0.37290 $\pm$ 0.01787 <i>F</i> = 20.87, <i>p</i> <sup>1</sup> < 0.01		10,91 <i>p</i> = 0.14	7

<sup>1</sup> *F*<sub>.05 [7, 7]</sub> = 3.77

The *p* values of the  $\chi^2$ -test shown in Table 5 indicate that the fitted data points were not statistically different from the observed ones confirming a good fit. Moreover the significance of the linear and quadratic coefficients was also confirmed by the *F*-test, the ratio between each coefficient and its SE; for each coefficient the *F* value was higher than 3.44 (the cut off value for *F*<sub>.05 [8, 8]</sub>) and the *z* value was higher than 1.96 (the cut off value for the normal distribution; both values can be found in the standard tables). The *F* test, which is described in Annex VI, is a ratio of two Chi-squared distributions and *F*<sub>.05 [8, 8]</sub> means the cut off value for alpha = 0.05, for 8 degrees of freedom for the numerator and 8 degrees of freedom for the denominator. For <sup>4</sup>He particles, weights were decreased by the average value of  $\sigma^2/y$ , 1.19. The  $\beta$  coefficient of the linear-quadratic was not significant (cut off value for *F*<sub>.05 [6, 6]</sub> = 4.28); *z*-test *p* = 0.12) and for this reason a linear fit is also presented.

Opinions vary on how to treat the background level of aberrations in fitting dose response data. In general there are three approaches: a dose point at zero Gy is included in the curve fitting procedure, the zero dose point is ignored, or else the zero dose point is represented in every fitting procedure by a standard background value. If the measured yield at zero dose is used as one of the data points for the curve fitting (as used in the curve fitting presented above), the background becomes a variable parameter. However, since the yield in unirradiated cells is usually low, often none are observed so the measured yield at zero dose is zero. As discussed, at low doses, the statistical resolution of the data points is generally low. Thus, including the zero dose point in the curve fitting procedure can sometimes lead to negative estimates of the background value (*C*) and negative linear coefficients ( $\alpha$ ), 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 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 dicentric is ~0.5–1.0 per 1000 cells [106] whilst for translocations [107] and micronuclei [108] the control values are higher.

There are several programs that can be used for curve fitting such as a Poisson Iteratively Reweighted Least Squares (PIRLS) computer program for additive, multiplicative, power, and non-linear models developed by Peterson [109], or the generalized linear interactive modelling (GLIM, [www.nag.co.uk/stats/GDGE-soft.asp](http://www.nag.co.uk/stats/GDGE-soft.asp)), or using R-based tools<sup>2</sup>. This should be combined with a routine specifically written for curve fitting that is available in this publication in Annex VI-3. Additionally, a number of specialized curve fitting computer programs have been recently developed from within the radiation cytogenetics community [110]. CABAS [111] uses maximum likelihood methods to fit calibration data to the linear quadratic equation. Dose Estimate [112] is a similar tool and this allows both linear quadratic and linear fitting. Apart from curve fitting both CABAS and Dose Estimate have additional tools that assist with processing data from radiation accident cases in order to derive dose estimates when the circumstances depart from recent acute and whole body exposure. These cover the range of calculations described later in Section 9.7.

Whether these or other software are employed, the program should give sufficient information regarding the methods used and provide details of the variances and covariances on the fitted coefficients, as these are required for calculation of the uncertainties on dose estimates (Section 9.7.3).

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<sup>2</sup> R is a free software environment for statistical computing and is available for download from <http://www.r-project.org/>





## 9. DICENTRIC ANALYSIS

The following text concerning cell culture, fixation and slide staining is written in the context of the dicentric assay but much of the material is also relevant to the other assays covered in this publication. By fully discussing the procedures here, the sections covering the other assays need simply to detail how the processes depart from that for dicentrics.

In brief the important differences are:

- (a) for the dicentric assay two day cultures are used whilst they are extended to 3 days for the micronucleus and/or nucleoplasmic bridge assays (cytokinesis-block micronucleus cytome assay (CBMN Cyt)).
- (b) premature chromosome condensation by mitotic fusion requires no cell culturing whilst chemically induced methods generally do.
- (c) the dicentric and other assays on metaphases require mitotic arrest with Colcemid while the CBMN Cyt assay does not.
- (d) instead the CBMN Cyt assay requires cytokinesis blocking with cytochalasin B.

### 9.1. CULTURING

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

#### 9.1.1. 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 [113]. 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 hours.

Medium should be supplemented with L-glutamine, heparin and antibiotics. Penicillin and streptomycin are commonly used (details found in Annex I). Depending on the manufacturer, many media already contain these antibiotics. However, antibiotics may need to be added when diluting the medium to working strength, 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 sterile blood specimen tubes, are essential.

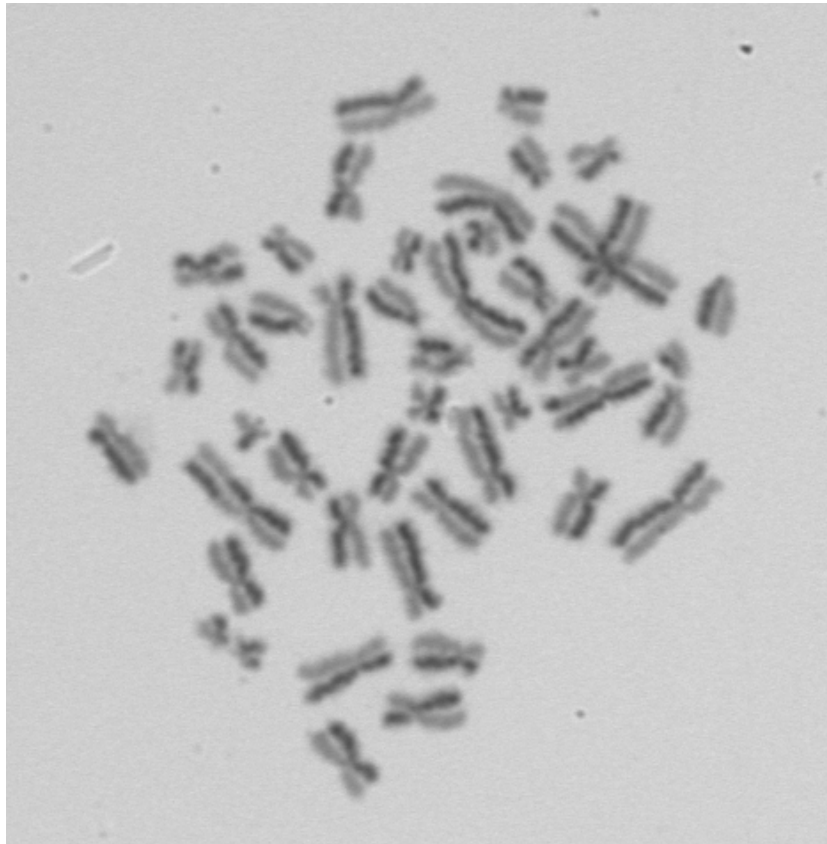
#### 9.1.2. Choice of serum

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  $56 \pm 1^\circ\text{C}$  for 0.5–1 hours 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.

#### 9.1.3. Bromodeoxyuridine

Bromodeoxyuridine (BrdU) should be included in the cultures in order to permit fluorescence plus Giemsa (FPG) staining [114]. 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. (Fig. 22).



*FIG. 22. A second division metaphase stained by the FPG method, exhibiting differential staining of the sister chromatids; the 'harlequin' effect.*

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 [115]. With fresh (<24 hours) 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 hours old, the BrdU concentration may have to be increased to, say, 40  $\mu\text{M}$  in order to achieve reliable FPG staining [116]. 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.

When using the BrdU method, an optimized fixation time should be chosen for which a high proportion of analysable cells are at the first division stage. Unfortunately, it is not always possible to predict the optimal fixation time. Differences may be encountered not only because of individual variations but also because of radiation effects on cell cycle times. Highly damaged cells may have a significantly stalled response to mitogenic stimulation. In practice, laboratories culture for a single time, usually 48 hours. The alternative would be to set up a large number of cultures and fix replicates at a range of different incubation times and then select that which contains the highest frequency of cells in first mitosis. This is however time consuming, costly and impractical especially in situations where many patients

may need to be evaluated rapidly. Therefore a slight modification to the culture has been proposed. Cytochalasin B (Cyt-B), which is normally used for micronucleus preparations (Section 12) may be added to the metaphase cultures and this enables cells after different cell divisions to be distinguished and, for analysis purposes, select only those cells in first mitosis. The Cyt-B is added at 24 hours into the culture period at a final concentration of 2 µg/mL. This technique was first used by Hayata et. al. [117] to identify cells in the first cell cycle, where differentiation was based on the number of chromosomes rather than the harlequin staining of sister chromatids with BrdU. The Cyt-B technique is not widely used for the dicentric assay but has been employed successfully in some dose estimation interlaboratory comparison exercises [118,119].

#### **9.1.4. 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, sometimes called types M and P. The more expensive and highly purified material (P) is not necessary for routine whole-blood cultures; some laboratories, however, consider it 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.

#### **9.1.5. The cultures**

Autoclavable glass or sterile, disposable plastic containers may be used. It is common practice to culture in 10 mL round-bottomed disposable tubes. These should be held at about a 45° angle with loosened caps in an incubator at 37°C with 5% CO<sub>2</sub>. It is also possible to culture the cells without a CO<sub>2</sub> incubator but then the caps should be closed.

Cells should be incubated at 37.0 ± 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 hours. 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 hours [120]. In a busy laboratory where a communally used incubator may be opened and closed frequently there is a danger that even with fan assistance the temperature of the cultures may fall below the optimum for an appreciable time. An alternative is to incubate in a thermostatically controlled water bath. This provides a more rapid heat transfer to the culture than via air and greater thermal stability throughout the 48 hours. If this method is used, the head space above a 5 mL culture should be at least 10 mL, and gassed with filtered 5% CO<sub>2</sub> in air. The vessels' lids should then be sealed tight. The water bath should have a lid so that the cultures containing BrdU are in darkness.

The culturing methods are based, with modifications, on the techniques originally published by Moorhead et al. [121] and Hungerford [122]. 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. The criteria for determining mitotic index is found in Annex V.

#### 9.1.5.1. 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.

#### 9.1.5.2. 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 3 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 wide bore 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 placed in culture. The concentration of viable cells can be established by dye exclusion of a small aliquot counted in a haemocytometer chamber so that the cell concentration in the cultures can be adjusted to an optimum value. This value is likely to vary between laboratories and so should be independently established but it is likely to be in the range  $0.5\text{--}2.0 \times 10^6$  /mL. A detailed protocol for this has been given by Hayata et al. and McFee et al. [123, 124], who point out that the method is particularly suitable for producing clean preparations with a lot of metaphases. Some laboratories find it better to use separated lymphocyte cultures for FISH analysis and also when preparing slides for scanning with an automated metaphase finder (Sections, 10 and 13.3.1). It is probably unnecessarily complicated for scoring conventional Giemsa staining with a normal light microscope, where method (a) above, or whole-blood cultures, are sufficient.

#### 9.1.6. Mitotic arrest

Colchicine or its synthetic analogue, demecolcine (Colcemid) can be used, with the latter being the arresting agent preferred by most researchers. A suitable stock solution will contain 10 µg/mL of Colcemid in physiological saline and, if prepared aseptically and stored at 4°C, will keep for six months. Adding 25–50 µL of this solution to each culture of 5.0 mL (final concentration: 0.05–0.1 µg/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 hours before terminating the cultures. A few researchers [125] prefer to add the Colcemid midway through the culture period, i.e. after about 24 hours or in some cases at the start of cultures [123]. 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 unless the final concentration in the culture is substantially lowered to about

0.05 µg/mL [123]. Early addition of Colcemid could allow cultures to be prolonged beyond 48 hours to allow for longer cell cycle times in some individuals, e.g. the elderly.

## 9.2. FIXATION PROCEDURE

Lymphocyte cultures are conventionally incubated for 48 hours, although the exact time may vary between laboratories from 46 to 52 hours. 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, except where specified, further processing may be carried out at room temperature. However, it is important to maintain safe-handling practices as the blood samples may contain human pathogens. 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 approximately 15 min at 37°C but when isolated lymphocytes are used, 3–5 min are enough. It is also possible to add about 1 mL of fixative to the hypotonic solution for 5 to 10 min to minimize cell lyses upon centrifugation. 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. Experience has shown that spreading of the chromosomes can be strongly influenced by the ambient temperature and relative humidity in the laboratory. Variable quality due to these factors can be overcome by dispensing the cells in a controlled environment cabinet. Cabinets designed specifically for cytogenetics laboratories are commercially available.

The cells should be thoroughly suspended in the remaining fixative by bubbling with a pipette and dispensing two or three drops 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).

### 9.3. STAINING

Fluorescence plus Giemsa (FPG) staining is recommended as this permits the analysis to be confined to the first *in vitro* division metaphases (M1) [126]. However, this method has certain drawbacks which can be overcome by using conventional Giemsa staining, as well as FPG. Many workers have noted considerable variation in the quality of FPG staining between replicate slides and also between different patches on 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°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 hours) 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 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 provided that the level of M2 cells is less than 5%, as assessed by FPG. If the level is higher, this may require an 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 people behave differently in their lymphocytes' stimulation and proliferation capacity [127].

Adaptation to alternative culture techniques such as culturing with Cyt-B with Colcemid or with early Colcemid may provide an easier and faster alternative to FPG staining [117–119, 125]. These techniques may be of particular use in triage scenarios where rapid dose estimates are required.

#### 9.3.1. Pretreatment

A pre-treatment of slides with RNase A, prior to staining, can remove residual stainable cytoplasmic material [128]. 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 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 [128].

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

This method is derived from that published by Perry and Wolff [114] 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 [129], 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 hour or, alternatively, a 30 W fluorescent strip lamp for about 1.5 hours. 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 x SSC (0.3M sodium chloride and 0.03M trisodium citrate) at 60°C for about 20–30 min.

Experience has shown that this SSC stage can be omitted if it results in an undesirable swelling of the chromatids which makes microscope analysis more difficult. The use of 2 x 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.

### **9.3.3. 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 a mounting medium. Figs 10 and 11 show Giemsa-stained metaphases.

It is possible to modify the staining specifically to highlight centromeres although for experienced scorers this is not normally necessary [130]. Such highlighting can be achieved by FISH, using a pancentromeric probe (Fig. 27) or with Giemsa stain using the C-banding method (Fig. 23).



*FIG. 23. A metaphase stained with Giemsa by the C-banding method which highlights centromeres.*

The C-banding protocol is:

- (1) Place the slides in 0.2 N hydrochloric acid at room temperature for 30 min.
- (2) Wash three times in distilled water.
- (3) Place slides in 5% barium hydroxide at 60°C for 1 min.
- (4) Wash in 0.2 N HCl for 2 min.
- (5) Wash in distilled water for 2 min.
- (6) Place in 2x SSC at 60°C for 45 min.
- (7) Wash in distilled water.
- (8) Allow to air dry and stain in 2% Giemsa in pH 6.8 phosphate buffer for 10 min.
- (9) If the staining intensity is insufficient, the slides can be reimmersed in the Giemsa stain for a further 5–10 min.

#### 9.4. ANALYSIS OF SLIDES

##### **9.4.1. Conventional microscopy**

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 x 100 to x 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 x 1000 to x 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. It is recommended that only complete metaphases be recorded, i.e. those with 46 centromeres. 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.

#### **9.4.2. Computer assisted microscopy**

Metaphase finding by automated pattern recognition systems has been introduced into many labs and several commercial systems are available. These instruments 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. Automated cell finding and scoring systems are discussed in detail in Section 13.

### **9.5. 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 (see Section 13.4). However, many 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. Electronic systems can have the data stored and displayed in a variety of ways to suit the laboratory. It is important that the primary data comprising the observations made on every cell can be retrieved so that later on all possible compilations and aggregations of data can be made.

Table 6 illustrates a simple layout of a data sheet for recording aberrations.

TABLE 6. LAYOUT OF A DATA SHEET FOR RECORDING ABERRATIONS

Slide No:  
 Scorer:  
 Microscope No:  
 NDate:

Cell No.	Stage coordinates		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	46				
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	45				Chromatid exchange
9	126.8	1.7	46	2*			*= 1 tricentric
etc.							

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.

#### 9.6. 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.

## 9.7. DOSE ASSESSMENT

### 9.7.1. 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  $^{137}\text{Cs}$ . In general most research laboratories have more ready access to a  $^{60}\text{Co}$  source rather than  $^{137}\text{Cs}$ . 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 one calibration curve produced with a fission spectrum would suffice.

In industrial radiography  $^{192}\text{Ir}$  is commonly used and its gamma energy is on average 400 keV. Few laboratories have access to this isotope to produce a calibration curve which should lie somewhere between the X and  $^{60}\text{Co}$  /  $^{137}\text{Cs}$  gamma ray curves. However, it is generally considered to lie closer to the latter, and so it is recommended that the gamma ray curve be used.

### 9.7.2. 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 exposure with a dose of several Gy and higher, 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. Consideration of the dose uncertainty versus number of cells scored is important when deploying the dicentric assay as a triage tool for rapid assessment after a mass casualties event. This topic is considered more fully later (Section 14).

For lower doses, where the number of available cells is not the limiting factor, a dose estimate could be based on about 500 cells. This may require 2–3 person-days at a conventional microscope, although in an emergency several people can collaborate in scoring replicate slides. For a low or zero dicentric yield, the confidence limits resulting from 500 scored cells 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 justifying an extended analysis, 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. However, as a general rule it is suggested that 500 cells or 100 dicentrics should be scored in order to give a reasonably accurate estimate of dose.

Table 7 shows the limits calculated using this method for several dose estimates up to 1.0 Gy.

TABLE 7. THE EFFECT OF INCREASING THE NUMBER OF CELLS EXAMINED ON THE LOWER AND UPPER 95% CONFIDENCE LIMITS FOR FOUR ESTIMATES OF ACUTE GAMMA DOSE  
(based on the curve shown in Fig. 24)

Dose estimate, mGy	Confidence limits	No. of cells examined	
		500	1000
100	Upper	320	245
	Lower	< 0	16
250	Upper	448	380
	Lower	111	141
500	Upper	677	627
	Lower	333	383
1000	Upper	1178	1127
	Lower	830	881

A simple method for calculating confidence limits on dose estimates is discussed in the following section, 9.7.3.

### 9.7.3. Uncertainty on dose estimates

While there is no difficulty in deriving a dose from a measured yield of dicentrics, there are a number of different ways in which the uncertainty on the yield can be derived. The aim is to express uncertainty in terms of a confidence interval and it is standard practice to calculate 95% limits. 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 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 been discussed in the literature by Savage et al. [131], Merkle [105] and more recently, Szłuińska et al. [132]. The simplest solution was proposed by Merkle, it allows both the Poisson error on the yield and the errors on the calibration curve to be taken into account.

Merkle's approach, illustrated in Fig. 24, involves the following steps:

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

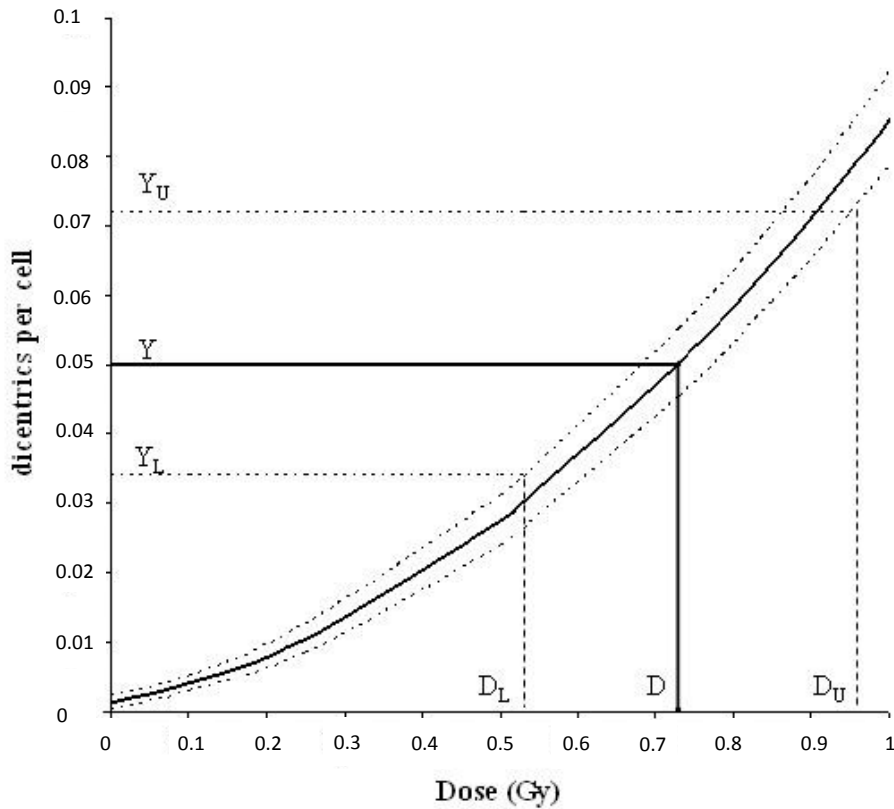


FIG. 24. A dose–response calibration curve with its 95% confidence limits, used to estimate uncertainties.

*Example:* Five hundred cells were analysed and 25 of them were observed each to contain one dicentric. This gives a yield ( $Y$ ) of 0.05 dicentrics / cell and the dispersion index and the  $u$  test of 0.95 and -0.78 respectively. Dose was estimated using the dose-effect curve for  $^{60}\text{Co}$  shown in Fig. 23 for which coefficients, variances and covariances are listed below.

$$\begin{aligned}
 C &= 1.28\text{E-}3 \\
 \alpha &= 2.10\text{E-}2 \\
 \beta &= 6.31\text{E-}2 \\
 \text{var } C &= 2.22\text{E-}07 \\
 \text{var } \alpha &= 2.66\text{E-}05 \\
 \text{var } \beta &= 1.61\text{E-}05 \\
 \text{covar } (C, \alpha) &= -9.95\text{E-}07 \\
 \text{covar } (C, \beta) &= 4.38\text{E-}07 \\
 \text{covar } (\alpha, \beta) &= -1.512\text{E-}05
 \end{aligned}$$

- (1) Since the dose-effect curve is linear-quadratic ( $Y = C + \alpha D + \beta D^2$ ), the estimated dose which is 0.73 Gy is obtained by solving the equation:

$$D = \frac{-\alpha + \sqrt{\alpha^2 + 4\beta(Y - C)}}{2\beta} \quad (6)$$

- (2)  $Y_L$  and  $Y_U$  are obtained from standard statistical tables of confidence limits for the expectation of a Poisson variable [133]. Table 8 shows the 95% limits for values of

observed dicentrics from 0 to 103. For the 25 dicentrics observed in this example the  $Y_L$  is  $16.768/500 = 0.034$  and  $Y_U$  is  $36.03/500 = 0.072$ .

TABLE 8. THE POISSON UPPER AND LOWER 95% CONFIDENCE LIMITS ON OBSERVED NUMBERS (X) OF DICENTRICS  
(adapted from [133])

X	Lower	Upper	X	Lower	Upper	X	Lower	Upper	X	Lower	Upper
0	0	3.285	26	16.77	37.67	52	38.165	66.76	78	61.9	96.06
1	0.051	5.323	27	17.63	38.165	53	39.76	68.1	79	62.81	97.545
2	0.355	6.686	28	19.05	39.76	54	4.094	69.62	80	62.81	99.17
3	0.818	8.102	29	19.05	40.94	55	40.94	71.09	81	63.49	99.17
4	1.366	9.598	30	20.335	41.75	56	41.75	71.28	82	64.95	100.32
5	1.97	11.177	31	21.36	43.45	57	43.45	72.66	83	66.76	101.71
6	2.613	12.817	32	21.36	44.26	58	44.26	74.22	84	66.76	103.315
7	3.285	13.765	33	22.945	45.28	59	44.26	75.49	85	66.76	104.4
8	3.285	14.921	34	23.76	47.025	60	45.28	75.785	86	68.1	104.58
9	4.46	16.768	35	23.76	47.69	61	47.025	77.16	87	69.62	105.905
10	5.323	17.633	36	25.4	48.74	62	47.69	78.73	88	71.02	107.32
11	5.323	19.05	37	26.31	50.42	63	47.69	79.98	89	71.09	109.11
12	6.686	20.335	38	26.31	51.29	64	48.74	80.25	90	71.28	109.61
13	6.686	21.364	39	27.735	52.15	65	50.42	81.61	91	72.66	110.11
14	8.102	22.945	40	28.97	53.72	66	51.29	83.14	92	74.22	111.44
15	8.102	23.762	41	28.97	54.99	67	51.29	84.57	93	75.49	112.87
16	9.598	25.4	42	30.02	55.51	68	52.15	84.67	94	75.49	114.84
17	9.598	26.306	43	31.675	56.99	69	53.72	86.01	95	75.785	114.84
18	11.177	27.735	44	31.675	58.72	70	54.99	87.48	96	77.16	115.605
19	11.177	28.966	45	32.28	58.84	71	54.99	89.23	97	78.73	116.93
20	12.817	30.017	46	34.05	60.24	72	55.51	89.23	98	79.98	118.35
21	12.817	31.675	47	34.665	61.9	73	56.99	90.37	99	79.98	120.36
22	13.765	32.277	48	34.665	62.81	74	58.72	91.78	100	80.25	120.36
23	14.921	34.048	49	36.03	63.49	75	58.72	93.48	101	81.61	121.06
24	14.921	34.665	50	37.67	64.95	76	58.84	94.23	102	83.14	122.57
25	16.768	36.03	51	37.67	66.76	77	60.24	94.705	103	84.57	123.77

(3) The lower and upper 95% confidence limits of the curve can be calculated by the equation:

$$Y = C + \alpha D + \beta D^2 \pm R \sqrt{\text{var}C + \text{var}\alpha D^2 + \text{var}\beta D^4 + 2\text{covar}(C, \alpha)D + 2\text{covar}(C, \beta)D^2 + 2\text{covar}(\alpha, \beta)D^3} \quad (7)$$

where:

$R^2$  is the regression confidence factor, and is the 95% confidence limit of a chi-square distribution,  $\chi^2(\text{df}, 95\%)$ , with 2 or 3 degrees of freedom (df).

For a linear-quadratic curve (df = 3)  $R^2$  is 7.81, and for a linear curve is 5.99. In Eq. (7) a value of 2.79 should be used, in the case of a linear curve the value used should be 2.45. Because in both, dicentric yield observation and calibration curve the 95% confidence limits

are considered, some authors have proposed to use an 83% confidence limit of the regression curve instead of 95%, in order to reduce a possible overestimation of the uncertainty [134, 135]. In this case  $R^2$  will be 5.02 for the linear-quadratic curve and 3.54 for the linear curve.

- (4) The calculation of the point where  $Y_L$  intercepts the upper confidence curve, which is the lower 95% confidence limit of the dose estimated ( $D_L$ ), can be done by iteration. The Excel program contains a tool ‘Solver’ that can be used. In the same way the point where  $Y_U$  intercepts the lower confidence curve ( $D_U$ ) can be obtained. Using the present example  $D_L$  and  $D_U$  are 0.51 and 0.97 Gy respectively.

If covariances are not available, the confidence limits can be approximated by Eq. (8). This equation is valid as the contribution to uncertainty from the covariances is comparatively small.

$$Y = C + \alpha D + \beta D^2 \pm R \sqrt{\text{var}C + \text{var}\alpha D^2 + \text{var}\beta D^4} \quad (8)$$

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, by considering where  $Y_L$  and  $Y_U$  cross the solid line in Fig. 25.

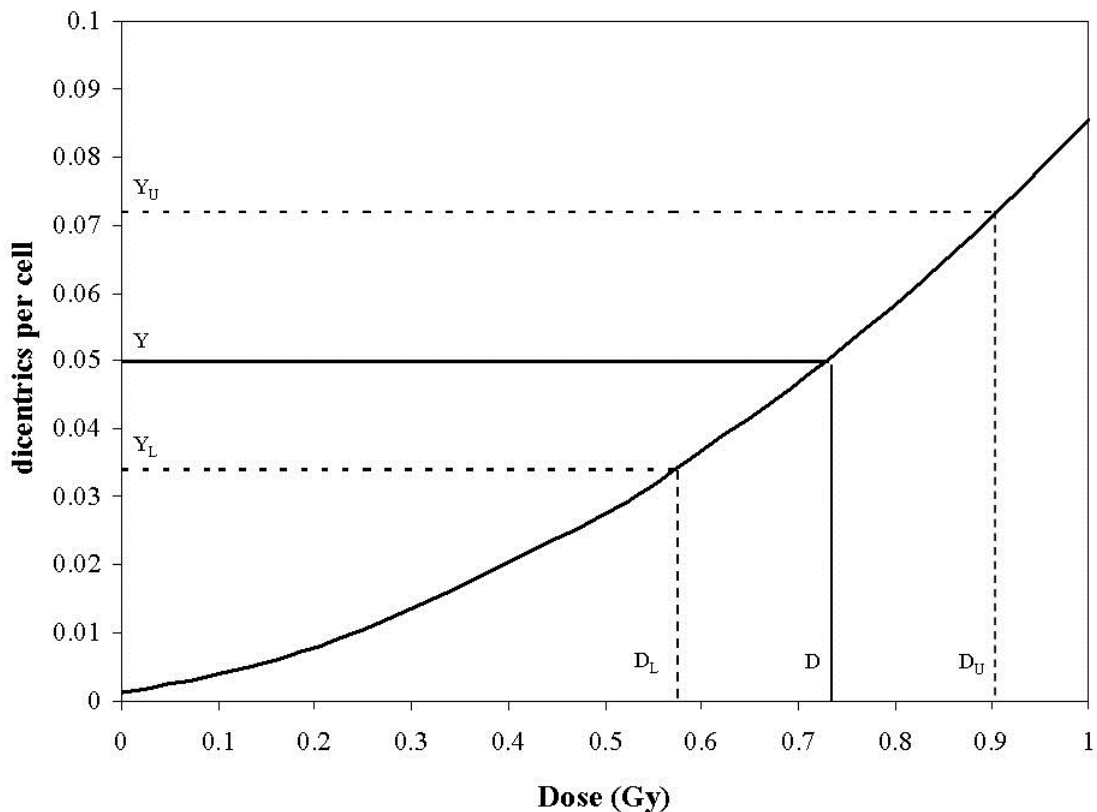


FIG. 25. A dose–response calibration curve used to estimate uncertainties ignoring the error due to the curve.

In the present example at 0.73 Gy the error associated with the curve is 0.002; this value is obtained by inserting 0.73 Gy for D in the last term of Eq. (7). The value obtained is smaller than the SE associated with the observed yield of dicentrics  $(25)^{1/2}/500$  which is equal to 0.01. With this approach  $D_L$  and  $D_U$  are 0.57 and 0.91.

If the u test statistic is higher than 1.96,  $Y_U$  and  $Y_L$  should be corrected to consider the overdispersion by multiplying by the factor indicated below, where CL is the Poisson confidence limit indicated in standard tables, X the number of dicentrics observed and  $\sigma^2/y$  the observed dispersion index:

$$Factor = \left( \frac{CL}{X} \right)^{\sqrt{\sigma^2/y}} \quad (9)$$

Using the above example, if instead of 25 cells with one dicentric, 19 cells with one dicentric and three cells with two were observed then the  $\sigma^2/y$  will be 1.19, and the u value 3.19. In this case  $Y_U$  and  $Y_L$  are:

$$Y_U = \frac{36.03}{500} \times \left( \frac{36.03}{25} \right)^{\sqrt{1.19}} = 0.1074 \quad (10)$$

$$Y_L = \frac{16.77}{500} \times \left( \frac{16.77}{25} \right)^{\sqrt{1.19}} = 0.0217 \quad (11)$$

Using these values  $D_L$  and  $D_U$  are 0.39 and 1.19 Gy respectively.

#### 9.7.4. Extension of dose calculations for more complex exposure scenarios

The previous section applies to cases where a large 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 assessed against an appropriate acute *in vitro* dose response curve provides a reliable estimate of the average whole body absorbed dose. In practice, however, such ideal circumstances rarely occur and protracted or fractionated irradiations are common. It is more usual for accidental 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 weakly penetrating radiation, but for more strongly penetrating 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 (see Section 3).



Delays in blood sampling will influence the aberration yield, as cells containing unstable aberrations are lost from the circulation and replaced by newly produced, cells that contain no dicentrics.

In this Section it is intended 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. Reporting on emergencies involving very low doses that, because of statistical limitations, may be difficult to distinguish from zero dose is also considered.

The following Section then contains worked examples for each of the emergency exposure situations.

#### *9.7.4.1. Criticality Accident*

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, to estimate the separate neutron and gamma ray doses by iteration. The iteration process is implemented as follows:

- (1) Assume that all the aberrations are attributable to neutrons, and from the measured yield of dicentrics estimate a dose from the neutron curve;
- (2) Use the estimated neutron dose and the supplied neutron to gamma ray ratio to estimate the gamma ray dose;
- (3) Use the gamma ray dose to estimate the yield of dicentrics due to gamma rays;
- (4) Subtract this calculated gamma ray yield of dicentrics from the measured yield to give a new value for the neutron yield;
- (5) Repeat steps 1 to 4 until self-consistent estimates are obtained.

In the case where a physical estimate of the ratio of neutron: gamma dose is not available, the above method is not possible. One approach would be to express the dose in Gy-Eq as was done for the Tokai-mura accident victims. However, Brame and Groer [136] have described a Bayesian approach to dose estimation in a criticality accident which allows estimation in the absence of the ratio estimate. The Bayesian method was found to give very similar results to the classical iterative approach in a simulated accident situation [137].

#### *9.7.4.2. 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 for the general population is on the order of  $\sim 0.5$ – $1/1000$  cells) and the limit on the number of metaphases that can realistically be scored. Dose estimates at low doses therefore carry large statistical uncertainties. As discussed, these come mainly from the Poisson error in the yield but with a small contribution from the SE on the coefficients of the dose–response curve, of which  $\alpha$  is the most important at low doses. For practical purposes the latter can be ignored unless the calibration data at low doses are sparse.

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.

When reporting results, experience has shown that lay persons rarely understand the concept of uncertainty. There are a number of approaches that can be used to aid interpretation of results. Firstly, although it is not strictly statistically accurate, it can be explained that there is only a 2.5% chance of the dose being greater than the upper 95% confidence limit. Additionally, the lower confidence limit can be used to define the ‘detection limit’ of the assay: using the figures in Table 9, a dose statistically greater than zero Gy would only be indicated by 4 or more dicentrics in 1000 cells, i.e. when the lower confidence limit is greater than zero Gy.

TABLE 9. 95% DOSE CONFIDENCE LIMITS ON VARIOUS LOW YIELDS OF DICENTRICS IN 1000 CELLS AND THE ODDS RATIOS SHOWING THE LIKELIHOODS OF ZERO DOSE OR 0.25 Gy  
(doses calculated using  $Y = 0.0010 + 0.0164D + 0.0492D^2$ )

Observation (dicentrics)	Dose (Gy)			Odds ratio p(0 Gy):p(0.25 Gy)
	Lower confidence limit	Mean	Upper confidence limit	
0	—	0	0.12	1306 : 1
1	0	0	0.18	160 : 1
2	0	0.05	0.23	20 : 1
3	0	0.09	0.26	2 : 1
4	0.01	0.13	0.30	1 : 3
5	0.03	0.16	0.33	1 : 28
6	0.06	0.19	0.36	1 : 229
7	0.09	0.22	0.38	1 : 1868

Alternatively, if one considers just two possible scenarios: zero dose or the suspected (e.g. badge) dose, the relative probabilities of each can be used to calculate the odds ratio for the two doses. The chances are derived from the Poisson distribution, as follows. If the dose was zero, then from the dose-response curve, 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. From the Poisson distribution, the relative chance of seeing no dicentrics when 1 and 8.2 are expected is  $e^{-1}/e^{-8.2}$ , which is 0.36788/0.00027 which is approximately 1300. The values of mean, lower and upper confidence intervals on dose, plus the odds ratio for zero dose: badge dose, are shown in the top line of Table 9, 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.

#### 9.7.4.3. Partial body exposure

The cytogenetic indication of a partial body exposure is a non-Poisson distribution of dicentrics among the patient’s scored metaphases. Therefore the first step is to calculate the ratio of variance to mean ( $\sigma^2/\bar{y}$ ) and then use the u-test to determine if the ratio deviates significantly from unity (see Section 8.3). If the data are consistent with a Poisson distribution

the recommendation is to report an averaged whole body dose estimate. If the data are non-Poisson two methods, see below, have been proposed whereby an estimate of partial body dose may be derived rather than simply quoting the averaged whole body value. The incentive for determining an estimate of partial body dose may also be based on information about the circumstances of the overdose event. The u-test is the recommended method to assess Poisson distribution of data but many consider that this test is not very robust, especially in cases when low number of metaphase spreads are scored. Hence it is routine practice in many laboratories to also compare dose estimates for whole body as well as partial body using either of the two methods described below to data. If the whole body dose estimate is significantly different than the partial-body dose estimate, then the laboratory should consider the case as potentially a partial-body exposure scenario. In cases of uncertainties in whether there are significant differences between the whole body and partial body dose estimates (Section 9.7.3), then the recommendation is to use the two methods described here only when the data are significantly non-Poisson.

### **Method 1**

This method was first proposed by Dolphin [138] and is termed the contaminated Poisson method. It considers the overdispersed distribution of dicentrics 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. Undamaged cells will comprise two subpopulations: those from the unexposed fraction and irradiated cells which received no damage (representing the first term ( $e^{-Y}$ ) of the Poisson series). Eq. (12) describes the distribution of the damage in the cells:

$$\frac{Y_F}{1 - e^{-Y}} = \frac{X}{N - n_0} \quad (12)$$

where:

$Y_F$  is the mean yield of dicentrics in the irradiated fraction,  
 $e^{-Y}$  represents the number of undamaged cells in the irradiated fraction,  
 $X$  is the the number of dicentrics observed,  
 $N$  is the total number of cells, and  
 $n_0$  is the number of cells free of dicentrics.

Eq. (12) can be solved by iteration to find the maximum likelihood estimate of the yield, and  $Y_F$  can then be used to calculate the fraction,  $f$ , of cells scored which were irradiated, using Eq. (13):

$$Y_F f = \frac{X}{N} \quad (13)$$

The dose to the irradiated fraction can then be calculated using  $Y_F$  and the appropriate calibration 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 hours in culture. If the fraction of irradiated cells which reach metaphase was  $p$ , the fraction of the body irradiated,  $F$ , is given by

$$F = \frac{f / p}{1 - f + f / p} \quad (14)$$

The p value is estimated by the equation

$$P = \exp(-D/D_o) \quad (15)$$

where:

$D$  is the estimated dose, and there is experimental evidence of  $D_o$  values between 2.7 and 3.5 [139, 140].

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 [141].

### **Method 2**

This approach has been proposed by Sasaki and Miyata [142] 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 among the damaged cells,  $N_U$ , and is given by

$$Qdr = \frac{X}{N_U} = \frac{Y_1}{1 - e^{Y_1 - Y_2}} \quad (16)$$

where:

$X$  is the number of dicentrics and rings, and  $Y_1$  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 also are several limitations with this method:

- (1) It assumes, as does method 1, that the exposure to the irradiated fraction is uniform, but according to Sasaki and Miyata [142] 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 (13) and (14).
- (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 dicentric and rings in those damaged cells that contain just dicentric and rings. Eq. (16) would now reduce to

$$Qdr = \frac{X}{N_U} = \frac{Y_1}{1 - e^{-Y_1}} \quad (17)$$

which is identical with Eq. (12). 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.

#### 9.7.4.4. *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 apparent following large doses that are sufficiently high to cause early deterministic reactions such as the depression of white blood cell counts. For lower doses, below the threshold for deterministic effect, 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 deduced. 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 [106]. 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 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. [63, 143, 144] 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, lasting 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 (Section 9.7.4.3 above) that the Qdr method considers the yield of dicentric 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 [145] 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 10.

#### 9.7.4.5. 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 effect of dose protraction is to reduce the dose squared coefficient,  $\beta$ , in the yield Eq. (2). 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 2 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 Catchside [146] to enable modification of the dose squared coefficient and thus allow for the effects of dose protraction. The linear quadratic Eq. (2) may be modified, as shown in Eq. (18):

$$Y = C + \alpha D + \beta G(x) D^2 \quad (18)$$

where

$$G(x) = \frac{2}{x^2} [x - 1 + e^{-x}] \quad (19)$$

and

$$x = \frac{t}{t_0} \quad (20)$$

where:

$t$  is the time over which the irradiation occurred, and

$t_0$  is the mean lifetime of the breaks, which has been shown to be on the order of  $\sim 2$  hours [96, 147].

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,  $G(x)$  in Eq. (18) can be replaced by  $\exp(-t_1/t_0)$ , where  $t_1$  is the time between fractions. Experimental evidence which supports the G function hypothesis has been presented by Lloyd et al. [96] and Bauchinger et al. [147].

#### 9.7.4.6. 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. [148] 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 days and 90% with 100 days.  $^{137}\text{Cs}$  was the nuclide released into the community in the Goiânia accident [149, 150] and was one of the major contributors to dose from environmental contamination at Chernobyl [151]. 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 days 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. [152] have demonstrated an RBE of 1.13 at low doses or dose rates for tritium with respect to 250 kVp X rays.

### 9.7.5. Examples of dose estimations

#### 9.7.5.1. Acute whole body exposure

Brewen et al. [91] and Preston et al. [153] 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 event. 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 an *in vitro*, gamma ray dose–response curve, where the dose D was expressed in roentgen (R):

$$Y = 3.93 \times 10^{-4} D + 8.16 \times 10^{-6} D^2 \quad (21)$$

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 event using a phantom. The general haematological changes noted were also consistent with an exposure of about 150 R.

#### 9.7.5.2. Criticality Accident

Consider a criticality accident in which 100 cells are scored and 120 dicentrics observed, i.e. 1.2 dicentrics 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 calibration curves for 0.7 MeV fission spectrum neutrons and <sup>60</sup>Co gamma rays. The yield equations for these curves are:

$$\text{Neutrons: } Y = 0.0005 + 8.32 \times 10^{-1} D \quad (22)$$

$$\text{Gamma rays: } Y = 0.0005 + 1.64 \times 10^{-2} D + 4.92 \times 10^{-2} D^2 \quad (23)$$

Following the steps listed in Section 9.7.4.1:

- (1) 1.20 dicentrics 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 dicentrics per cell;
- (4)  $1.20 - 0.266 = 0.934$ , which is the dicentric yield attributable to neutrons;
- (5) 0.934 dicentrics per cell is equivalent to 1.12 Gy neutrons.

Repeating step 2,  $1.12 \times 3/2 = 1.683$  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 10.

An *in vitro* validation of this approach has been described where very good estimates of actual neutron and gamma doses were obtained in international exercises to compare criticality accident dosimetry [137, 154]

TABLE 10. SEQUENCE OF STEPS USED IN MAKING DOSE ESTIMATES FOR MIXED GAMMA AND NEUTRON IRRADIATION

Steps 1 and 5	Step 2	Step 3	Step 4
Neutron dose	Gamma ray dose	Gamma ray yield	Neutron yield
(Gy)	(Gy)	(dicentrics per cell)	(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

### 9.7.5.3. Low dose overexposure

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  $Y = 0.001 + 0.0164D + 0.0492D^2$ , zero carried an upper 95% confidence limit of 0.12 Gy. 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. Using the odds ratio approach, described in Section 9.7.4.2 the odds in favour of zero come out at approximately 1300:1.

### 9.7.5.4. Acute non-uniform exposure

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 [155]. 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:

TABLE 11. DISTRIBUTION OF DICENTRICS AFTER ACUTE NON-UNIFORM EXPOSURE

	Dicentric 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:

$$Y_{dicentric} = 1.57 \times 10^{-2} D + 5.00 \times 10^{-2} D^2 \quad (24)$$

$$Y_{acentric} = 2.30 \times 10^{-2} D + 3.90 \times 10^{-2} D^2 \quad (25)$$

Using the contaminated Poisson method, (Section 9.7.4.3, Method 1) the maximum likelihood estimate for the yield of dicentrics,  $Y_F$ , in the irradiated cells is given by substituting data from the example into Eq. (12). By iteration,  $Y_F = 0.489$  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. (13), 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. (14), 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 [147] 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 of the irradiated cells ( $p$  in Eq. (14)) survived to be analysed. The fraction originally exposed,  $F$ , is equal to 0.393 and is obtained by solving Eq. (14). In round terms, therefore, the irradiated fraction of the body is about 40%, with an average dose of about 3.0 Gy.

In the Qdr method, (Section 9.7.4.3) 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, ie. Qd. 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. (16) and omitting centric rings gives

$$Qd = \frac{86}{99} = \frac{1.57 \times 10^{-2} D + 5.00 \times 10^{-2} D^2}{1 - e^{-3.87 \times 10^{-2} D - 8.90 \times 10^{-2} D^2}} \quad (26)$$

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 the contaminated Poisson method.

#### 9.7.5.5. Delayed blood sampling

Below are presented two examples of dose calculation in cases of delayed blood sampling.

##### *Adjusting the dicentric yield*

Stephan et al. [156] 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:

$$Y = 3.00 \times 10^{-4} D + 5.00 \times 10^{-6} D^2 \quad (27)$$

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. [91] and Preston et al. [153] from the accidental whole body irradiation described in Section 9.7.5.1. 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 spondylitis [143], 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 spondylitis' effect lasting up to 20 weeks, the delay of 103 days would require no correction.

##### *Using the Qdr method*

Ishihara et al. [157] have described a serious accident 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 whole-body

dose, 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 days, respectively, when further study ceased.

#### 9.7.5.6. Protracted and fractionated exposure

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 [158]. Ten persons were irradiated, mostly during one day, with exposure times ranging from 2 to 7 hours [159]. One of the subjects is used here as an example. His exposure was for 7 hours, 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

$$Y = 0.001 + 0.003D + 0.060D^2 \quad (28)$$

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

$$x = \frac{t}{t_0} = \frac{7}{2} = 3.5 \quad (29)$$

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

$$Y = 0.001 + 0.003D + 0.025D^2 \quad (30)$$

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

#### 9.7.5.7. Internal incorporation of radionuclides

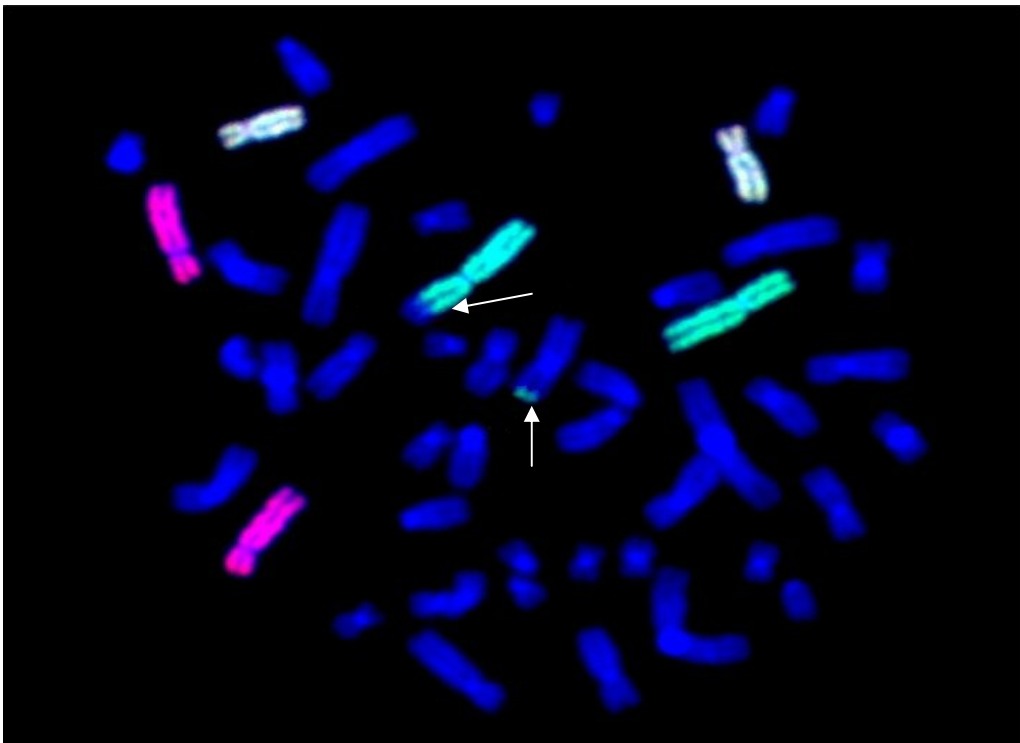
An accidental inhalation of about 35 GBq ( $\sim 1$  Ci) of tritiated water droplets by a factory worker is described by Lloyd et al. [160]. Removal of tritium from her body was speeded up by forced diuresis. A committed dose to soft tissue was obtained from measuring the concentration and rate of excretion of tritium in her urine. Dicentric yields were measured in blood samples taken at various times after the event, and data from 40–50 days 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 [160].



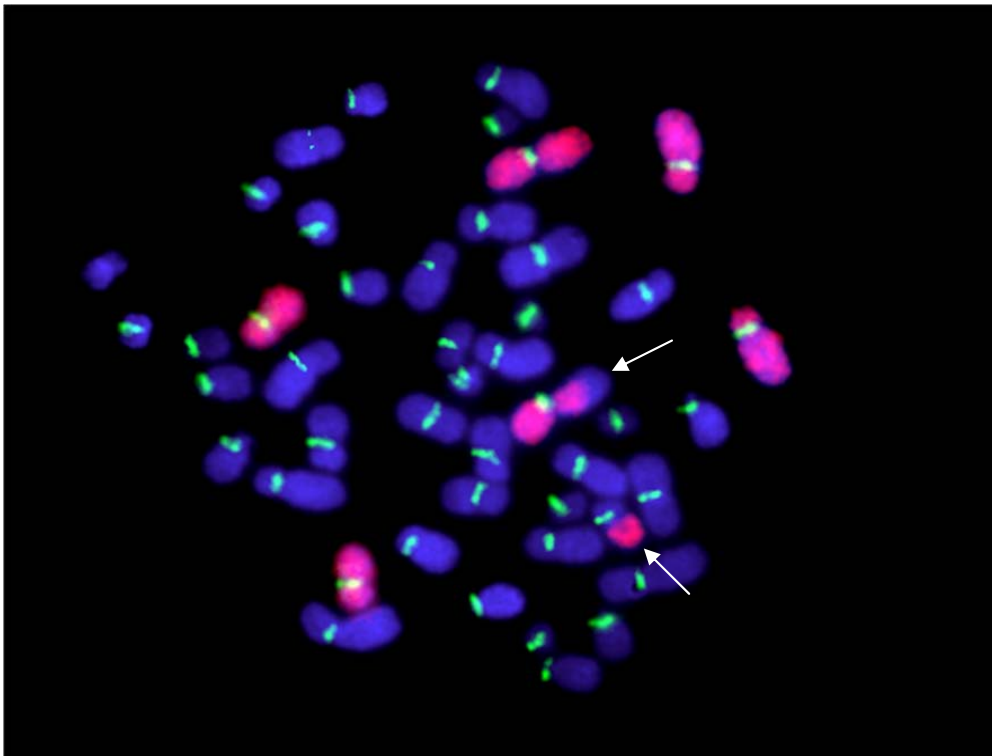
## 10. TRANSLOCATION ANALYSIS

A recognized drawback of the dicentric and cytokinesis-block micronucleus (CBMN) 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 4). It has long been recognized that analysis for more persistent types of damage, e.g. stable translocations, is needed to address biological dosimetry for old or long term exposures. Translocations are detectable by karyotyping, which is, however, too laborious to be applied routinely in biological dosimetry. The introduction of FISH [161] 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 (shown in Figs 26 and 27).

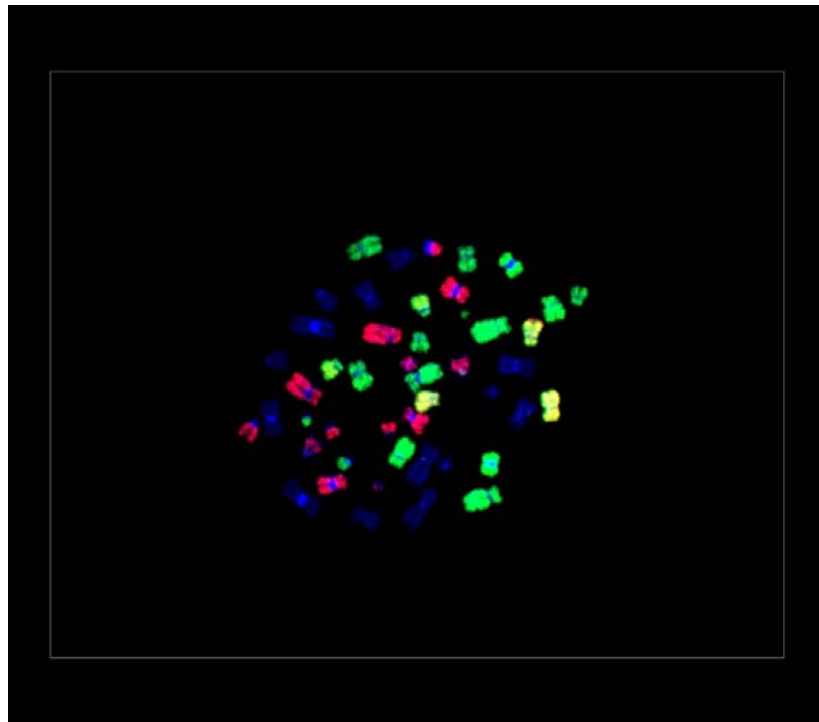


*FIG. 26. Human metaphase with coloured painted chromosomes #2 (FITC, green), #4 (Texas Red) and #8 (FITC+Texas Red, yellow), and the rest counterstained with DAPI. An apparently simple translocation, or two-way translocation [t(Ba),t(Ab)] involving chromosome # 2 is observed.*

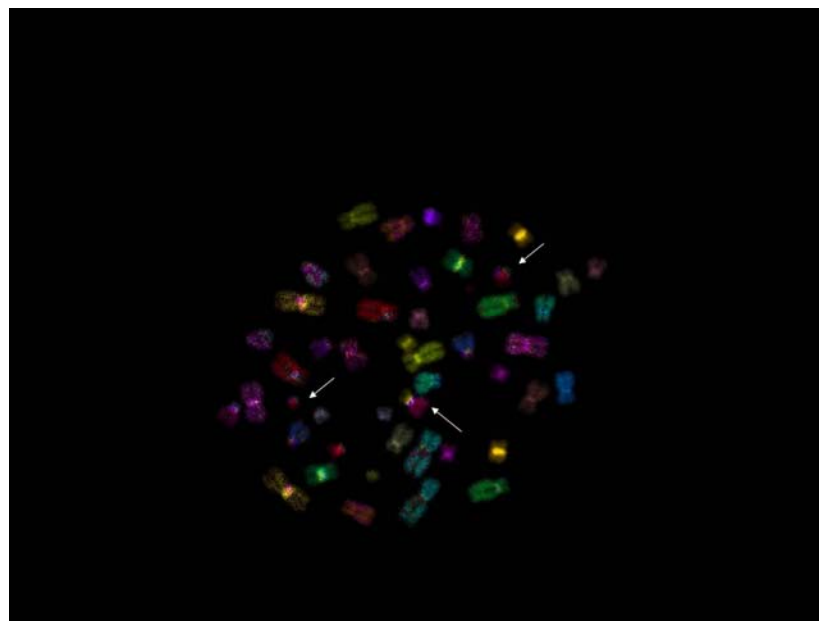


*FIG. 27. Human metaphase with monocoloured painted chromosomes #1, #4 and #11 labelled with Cy3 (red), centromeres highlighted with a pancetromeric probe labelled with FITC (green), and the rest counterstained with DAPI. An apparently simple translocation or two-way translocation  $[t(Ba),t(Ab)]$  involving chromosome # 1 is observed.*

FISH has many applications in medicine and in fundamental cytogenetics. In this publication, however, only its application to biological dosimetry will be addressed. A large variety of probes are now available so that one may selectively paint whole or limited regions of each of the human chromosomes. By attaching fluorochromes in varying ratios to specific sites it is possible to highlight different regions concurrently with a wide range of colours. One drawback in using many colours such as the multicolour FISH (mFISH) procedure is that the shade differences may be too subtle for discrimination by the human eye. Therefore electronic systems are required to capture images and display them with applied false colours (Figs 28 and 29).



*FIG. 28. View of a metaphase stained by mFISH. A: in RGB colours as taken by the camera.*



*FIG. 29. View of a metaphase stained by mFISH. B: the same metaphase but where a pseudo colour has been associated to each pair of chromosome.*

### 10.1. 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 (Sections 9.1 and 9.2). Although translocations are stable through mitosis, it is still good practice to carry out the analysis on M1 metaphases. This is particularly important because the mitotic loss of cells containing unstable aberrations could distort the mean frequency of translocations. Moreover there may be occasions when both stable translocation and unstable dicentric frequencies are required

from the same specimen. For FISH analysis it is better to store refrigerated fixed cell suspensions. Cells dispensed onto microscope slides can be stored. They should be kept at -20°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.

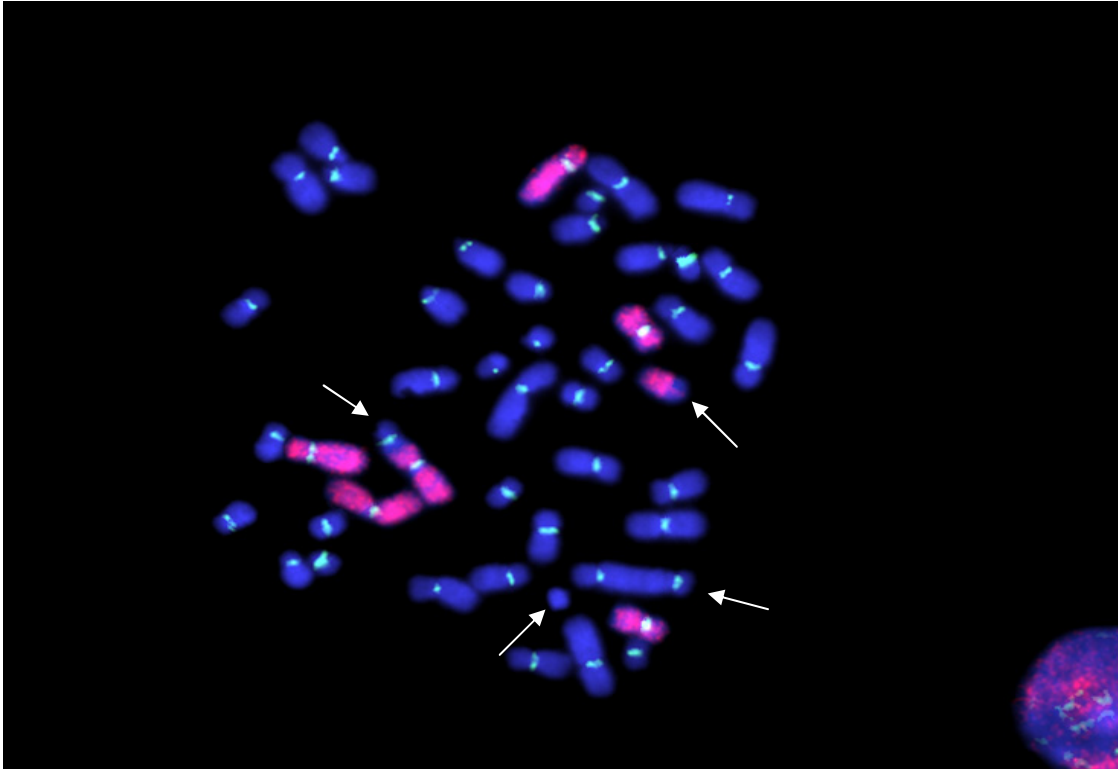
## 10.2. PAINTING THE CHROMOSOMES

With the range of probes and fluorochrome combinations now commercially available it is possible to highlight all chromosomes by the method known as multicolour FISH (mFISH) [162]. This permits full karyotyping and thus scoring all inter-chromosomal translocations. The centromeres and telomeres of all chromosomes can be separately highlighted too.

Intrachromosomal exchanges such as pericentric inversions may be detected by selectively painting the p and q arms of a chromosome in different colours [163], and rearrangements within a single arm may be detected by mBAND where multi-coloured banding is produced along a chromosome [164, 165]. An increased frequency of intrachanges with respect to interchanges has been proposed as a 'fingerprint' of the effect of high LET radiations [166] and therefore these methods have particular applications for researching radiation quality effects.

For most retrospective biological dosimetry applications it is sufficient to detect just inter-chromosomal translocations and ideally mFISH can provide the maximum information from each metaphase. It can also be extended so that individual chromosome arms can be highlighted in different colours (pq-mFISH) [167] but is an expensive, time consuming procedure and highly demanding of sophisticated image capture and manipulation systems. Therefore the practice has evolved for painting a limited number of chromosome pairs either with the same or in separate colours and counterstaining the remaining chromosomes. Application of a pan-centromeric probe [168] simultaneously with whole chromosome paints is recommended to distinguish between dicentrics and translocations more accurately (Fig. 30).





*FIG. 30. Human metaphase with monocoloured painted chromosomes #1, #4 and #11 labelled with Cy3 (red), centromeres highlighted with a pancetromeric probe labelled with FITC (green), and the rest counterstained with DAPI. An apparently simple dicentric [dic(BA),ace(ab)] involving chromosome # 1 is observed. A dicentric plus an acentric involving the counterstained chromosomes is also present.*

Generally, painting three of the larger chromosomes (i.e. #1 to #12 — see Fig. 7), representing about 20% of the genome (see Tables 2 and 3), leads to about 33% efficiency in detecting translocations when a single colour is used. The percentage of the genome that each cocktail ‘paints’ relative to the total genome is estimated from the physical lengths of chromosomes [169]. The total genomic translocation frequencies may be estimated according to a standard formula proposed by Lucas et al. [170], which applies with the assumption of simple pair-wise exchanges. 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 [171, 172].

For retrospective biological dosimetry a single colour FISH for a triple cocktail of target chromosomes appears to be sufficient. Multiple colour painting of the triplet increases the detection efficiency (if chromosomes #1, #4 and #12 are highlighted from about 31% to about 34%) and gives a better detection of complex translocations that can be encountered following high dose recent exposures. The equations, given below in Section 10.5 for converting to full genome equivalence can be applied to both single and multiple colour painting.

### 10.3. SCORING CRITERIA

#### 10.3.1. Selection of scorable cells

Although there is no firm consensus on which metaphases should be scored, well-spread metaphase cells are considered suitable for scoring if the cells appear to be intact, the centromeres are morphologically detectable and present in all the painted chromosomes, and the fluorochrome labelling is sufficiently bright to detect exchanges between chromosomes labelled in different colours [107].

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.

Some researchers consider, that all the painted material present should be scored although this involves a certain degree of judgement because the limits of resolution with current FISH technology are about 11–15 Mbp [173]. In consequence, some symmetrical translocations look like apparently incomplete exchanges, but investigations with telomere probes have shown, that a large portion of apparently incomplete translocations are complete ones [62]. Those cells that are obviously deficient in a large portion of painted material or labelled centromeres should be excluded from the scoring.

For retrospective dosimetry, it was shown, that the frequencies of translocations in the stable cells, defined as cells without dicentrics, centric rings or acentrics, are constant with time [64, 174]. Therefore it is recommended to record whether each translocation occurs in a stable or unstable cell.

#### 10.3.2. Nomenclature and recording data

To describe the chromosome aberrations detected by painting two specific nomenclature systems were developed independently, and descriptions based on the conventional terminology of routine cytogenetic scoring were also used [173, 175–178]. 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) A system with the acronym PAINT was developed to be purely descriptive of each aberrant painted object in the metaphase [175]. 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. [175] 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 relatable to dose has no practical application to retrospective dosimetry.
- (ii) Savage and Simpson (S&S) [176, 177] 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 [131]. This nomenclature has considerable uses in mechanistic studies, particularly, for example, in understanding complex rearrangements.

A more conventional terminology may be employed that names translocations as reciprocal, terminal or interstitial [179]. The first two have also been called complete or two-way and incomplete or one-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 two- or one-way, purely on the basis of their visual appearance and with no mechanistic implications. Indeed mechanistic studies have shown that one-way patterns do not provide a reliable estimate of exchange incompleteness [180]. 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 [177].

The nomenclatures described above are not mutually exclusive but rather complementary and comparisons between nomenclatures have been applied to a common data set [175].

Nowadays the most widely used method for recording data is to describe each abnormal metaphase as a unit using the PAINT nomenclature [175] but in a slightly modified way [178] that considers the underlying mechanisms of the formation of aberrations. The abbreviations of the PAINT system are used but a note is made of the associations between objects in the metaphase, thereby incorporating aspects of the conventional terminology too. Chromosome aberrations are classified as simples or complexes, the latter ones considered when three or more breaks in two or more chromosomes are needed to produce the observed abnormality. Aberrations are considered complete when all broken pieces are rejoined and as incomplete when one or more pieces appeared unrejoined.

For example, t(Ba) seen with t(Ab) is regarded as a simple complete or two-way translocation, and either pattern alone is regarded as a simple incomplete or one-way translocation when seen alone in a metaphase, sometimes with an associated painted acentric, t(Ba) plus ace(b). Complexes are recorded as such and described either as insertions, e.g. ins(Aba), or as the more complicated rearrangements like a t(Ba) with ace(ab) or dic(BA) with a t(Ab).

Note that painting a concrete set of chromosomes exchange aberrations like a t(Ba) plus a t(Ab) are considered as ‘apparently’ simple aberrations because they can arise from undetectable complex aberrations, that are only detectable with mFISH [182, 182].

#### 10.4. DATA HANDLING

Lucas et al. [170] derived the equations for calculating genome equivalence, and these have been further summarized by Lucas and Deng [183].

The genomic translocation frequency is usually calculated by using the formula for the painted fractions of the genome [32] as follows:

$$F_G = \frac{F_p}{2.05 f_p (1 - f_p)} \quad (31)$$

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 [170], taking into account the gender of the subjects.

This is more fully elaborated below in Section 10.5.1.

The conversion of data to full genome equivalence is a recommended procedure to use when data are to be combined or interlaboratory comparisons 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. This issue has been intensively investigated [184–186] and in essence it is accepted that this assumption gives a reasonable approximation. However, there is a consensus that using the (DNA content) in the Lucas formula, larger chromosomes may tend to be overestimated in their participation in simple exchange aberrations compared to the smaller ones [187, 188]. Therefore, the use in the Lucas formula of the (DNA content)<sup>2/3</sup> rather than the (DNA content) gives more accurate results. Some authors have argued that this kind of proportionality could be symptomatic of interchanges involving primarily chromatin near the boundary of chromosome territories [186–188]. The best data on relative DNA contents of the human chromosomes are given by Morton [32] and the values shown in Tables 2 and 3 have been calculated from data given in his Table 4, column 2.

#### 10.4.1. Single colour painting

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

*Note:*  $f_p$  in Eq. (31) above has, for simplicity, here been shortened to  $f$ .

There will be	$f^2$	green–green exchanges
	$(1 - f)^2$	blue–blue exchanges
	$\frac{2f(1 - f)}{}$	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. [170]). Hence, the fraction of all translocations that are blue–green translocations is given by Eq. (32):

$$\frac{F_P}{F_G} = \frac{2f(1 - f)}{0.974} = 2.05f(1 - f) \quad (32)$$

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 2 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.

#### 10.4.2. 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
	$f_3^2$	blue–blue exchanges
	$2f_1f_2$	red–green exchanges
	$2f_1f_3$	red–blue exchanges
	$2f_2f_3$	green–blue exchanges

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

$$\frac{2(f_1f_2 + f_1f_3 + f_2f_3)}{0.974} - 2.05[f_1(1 - f_1) + f_2(1 - f_2) + f_3(1 - f_3)] \quad (33)$$

#### 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 2 are  $f_1 = 0.227$  and  $f_2 = 0.186$ :

$$\frac{F_P}{F_G} = 2.05(0.175 + 0.151 - 0.042) = 2.05(0.284) = 0.582 \quad (34)$$

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

#### 10.4.3. More than two colours

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

$$\frac{F_P}{F_G} = 2.05 \left[ \sum_i f_i(1 - f_i) - \sum_{i < j} f_i f_j \right] \quad (35)$$

All the calculations detailed in this section are available as part of the Dose Estimate software, mentioned in Section 8.3.

## 10.5 THE CONTROL LEVEL OF TRANSLOCATIONS

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 important to take the translocation background into account, particularly after low doses, when attempting retrospective biological dosimetry.

Of course, a pre-exposure control blood sample from the accidentally irradiated subject or from a population study group is unavailable, and therefore an assumed value based on generic survey data has to be used. Ideally a laboratory should develop its own control database but this is an extensive undertaking given that it would have to cover a number of confounders and especially a wide span of age groups. A comprehensive meta-analysis published by Sigurdson et al. [107] currently provides the best international database, broken down by age, gender, race and smoking habits. It incorporates data from an earlier study that combined results from some European laboratories [189].

From both studies, it appears clearly that age is the major factor that determines the background frequency of translocations which rises substantially above the age of 60 years (Fig. 31).

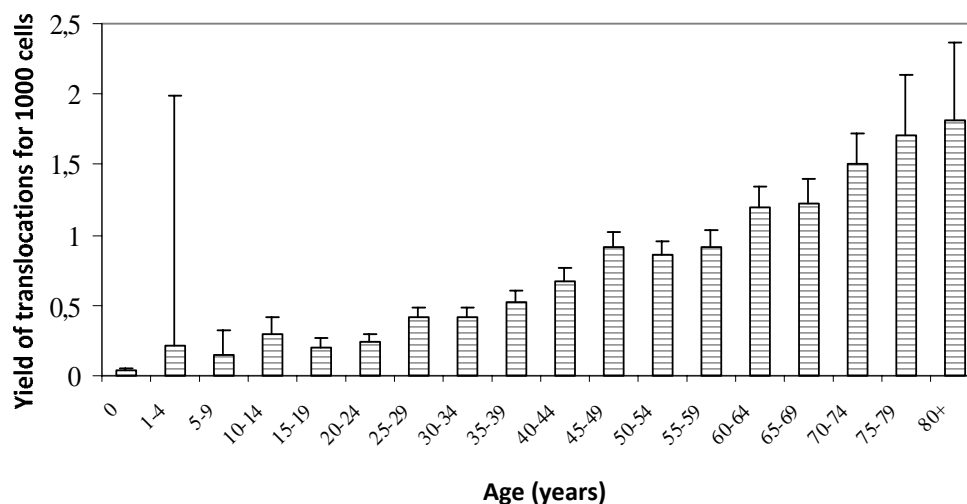


FIG. 31. Number of translocations as a function of age [107].

It is important to take the background into account and to subtract from the number of translocations observed in an individual's lymphocytes the translocation rate expected given his or her age. In addition to a confirmed laboratory effect, the study by Sigurdson et al. [107] showed a significant variation between the four principal geographic areas. On the other hand, no effect was observed for ethnicity or gender. It thus appears essential to compare only data from the same laboratory for studying factors that influence the translocation rate.

Among the few studies that report the effect of gender on the translocation rate [107, 189, 190], only that by Whitehouse et al. [189] shows a higher translocation rate in men than women for the 20–29-year-age group, significantly different from the 30–39-year-age group ( $p < 0.05$ ). For the other age groups, women had a higher rate than men, although this difference was not significant. The other studies examining the effect of gender found no evidence of any difference [107, 190, 191], even by age group.

While the studies unanimously agree that the translocation rate increases with age, the results of this literature review show that this trend is not as clear for some other factors (Table 12).

TABLE 12. SUMMARY OF THE EFFECTS OF THE FACTORS STUDIED ON THE PRODUCTION OF TRANSLOCATIONS AND DICENTRICS

Confounding factor	Significant studies and associated references	Additional references
Alcohol	Badr and Hussain [192]	
	Burim et al. [193]	
Tobacco	Ramsey et al. [191]	Tawn and Cartmel [195]
	Bothwell et al. [194]	Van Diemen et al. [196]
	Sigurdson et al. [107]	Pressl et al. [190]
		Tucker et al. [197]
		Whitehouse et al. [189]
		Maeng et al. [198]
Pesticides	Steenland et al. (applicators) [201]	Tucker [197]
	Smith et al. (>31 ppm) [203]	Kim et al. [202]
Benzene		Smith et al. (< 31 ppm) [203]
		Zhang et al. [204]
Industrial pollution	Beskid et al. [199]	Beskid et al. [205]
	Sram et al. [206]	
Heavy metals	Maeng et al. (Chromium, smokers only) [198]	Dulout et al. (Arsenic) [208]
	Doherty et al. (Chromium) [207]	Maeng et al. (Chromium, Non-smokers only) [198]

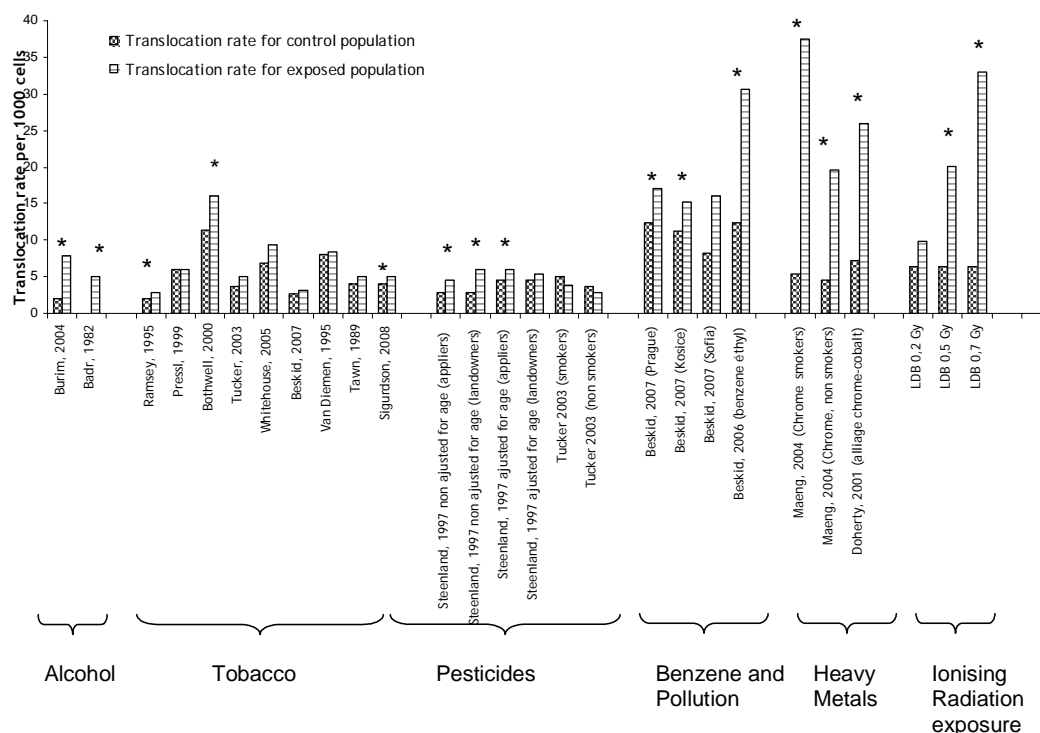
Only alcohol tended to create an excess of translocations in both of the studies that considered it. This trend was not observed for smoking (8/11 studies) or pesticides (1/2 studies). The case of benzene is particular because the analyses were performed for the chromosomes involved in diseases specifically related to exposure. This means that only exchanges between two chromosomes (# 8 and 21 or # 14 and 18) were recorded in some studies [202–204]. All three reported a significant effect when they studied only the t (8; 21) and t (14;18) translocation rates. However, 3 studies were identified in which the translocations between those chromosomes and all the others were examined, and none showed significant results.

Substances that are used and abused by choice, such as tobacco, drugs, and alcohol, affect translocations only when their consumption is heavy and chronic. Nonetheless, they often

induce a significant increase in translocation rates when they are combined or associated with other types of agents (metals and mutagenic chemicals) [192]. The synergistic effects of abusable substances reported in some studies suggest that smoking increases the translocation rate generated by an occupational exposure. This is the case for people exposed to ionizing radiation [194], to pesticides [197], and to chromium [198]. The synergistic effect of smoking is all the more evident in that most studies of the effects of smoking alone do not show any significant increase in translocation rates. On the other hand, for alcoholism, the synergistic effect is more difficult to show because alcoholics rarely have only a single addiction, or at least because the number who do not have other addictions is too low for a comparative analysis with a control group. A study of the impact of alcoholism alone on the translocation rate would thus be instructive.

The effect of toxic agents used in the workplace (pesticides, benzene, and metals) on the number of translocations is often proportional to dose and duration of exposure. It has also been shown that exposure to a mixture of products is more harmful than exposure to a single chemical element. Finally, the efficacy of individual protection (gloves, masks, jumpsuits/coveralls) was shown by the diminution in the translocation rate in the population exposed to these types of mutagenic substances.

Fig. 32 illustrates the impact of the factors studied on the rate of translocations as a function of the type of factor, of the exposure, and of the studies.



LDB : Translocation rate obtained in the Laboratoire de Dosimetrie Biologique for given radiation dose (Cobalt exposure, dose rate of 0.5 Gy/min).

\* Studies where the difference between the exposed and the control group is significant

FIG. 32. Comparison between the translocation rates generated by different agents and the translocation rate generated by *in vitro* irradiation.

For each study the translocation rate per 1000 cells in the control population and in the exposed population are indicated. In addition, the translocation rate obtained after *in vitro* irradiation of blood samples to doses of 0.2, 0.5, and 0.7 Gy are also presented in this figure,



to compare the induction rates. However, it is necessary to note that the irradiations were performed in a short time period and were acute exposures whereas, exposures to alcohol, smoking, pesticides, or benzene are chronic. Exposures are designated as chronic when the individual is exposed to the genotoxic agent for all or a significant part of their life span (lifestyle or occupational exposure).

Fig. 32 also shows that the translocation rates encountered in populations exposed to alcohol, smoking, and pesticides are considerably lower than the rates measured for benzene and especially chromium exposure. Moreover, the values for the control groups are relatively similar to those observed in the groups exposed to alcohol, smoking, and pesticides. In comparison with a group exposed to ionizing radiation at a dose of 0.5 Gy, the translocation rate for people exposed to alcohol, smoking, and pesticides is low. One can therefore conclude that if a high translocation rate is observed it can be attributed to the irradiation. On the other hand, during a retrospective study of exposure to ionizing radiation among people highly exposed to chromium or benzene, it will not be possible to differentiate the radiological from the environmental translocation rates. Nevertheless, exposure to benzene or chromium is not common and it should be possible to identify it by an appropriate questionnaire covering past and present occupational activities.

#### 10.6. PERSISTENCE OF TRANSLOCATIONS

The greatest disadvantage of the dicentric method is that the aberration yield in exposed people decreases with time after exposure. Dicentrics induced in the peripheral circulating lymphocyte pool will be removed by cell death and diluted by renewal of blood cells. They are mitotically unstable aberrations unable to pass through repeated cell divisions. By contrast, translocations are generally mitotically stable and, provided that the cell as a whole is stable ( $C_s$ ), translocations induced in stem cells can pass through to mature circulating lymphocytes. Initially translocations are induced at a frequency similar to that of dicentrics but it is their post-irradiation stability that makes them more suited for retrospective biological dosimetry.

The persistence of the translocation frequency has long been a matter of discussion. Several years after the Goiânia accident in Brazil, the frequency of translocations was found to be lower than the frequency of dicentrics observed just after the exposure [209, 210]. However, the initial translocation yield was not available in either of these studies. In a retrospective study of Mayak (Southern Urals) nuclear-industrial personnel, the estimated doses were mainly lower than would be predicted by the calibration curve for transmissible apparently simple translocations [211]. Other follow-up studies of accidentally irradiated persons showed that the frequency of translocations remained relatively constant with time. No substantial change in the translocation frequencies was observed in Chernobyl irradiated subjects from 5 to 8 years after the accident [212]. In whole-body irradiated individuals from an accident in Estonia, the frequencies of translocations remained fairly constant for two years, except for one individual exposed to protracted whole-body but also to non-uniform irradiation [213]. However, four years after the same accident, the frequency of all translocations in cells containing only simple rearrangements fell on average to 65% of their initial value, but two-way translocations were slightly more persistent than total translocations [214]. The reduction in the frequency of translocations with time was attributed to the partial-body irradiation and agrees with the idea of a coincident distribution of dicentrics and translocations in such exposures [215, 216]. From this it would follow that, at long times after partial-body exposures, the estimation of the whole-body dose will tend to be lower as the dose increases [215].

A more marked decrease with time post-irradiation in the frequency of translocations as the dose increased has been described in other cases of accidental exposures of humans; the frequency of translocations persisted with time for doses below 1–3 Gy but a decrease was observed after higher doses [217, 218]. In cancer patients treated with radiotherapy, a clear reduction of the yield of translocations with time post irradiation was observed, which was more pronounced in persons with a higher initial frequency of translocations. [219–222]. A possible explanation for these observations in radiotherapy patients is the lethal dose to the stem cells and the repopulation of this area by unirradiated cells from outside the irradiated part of the body. Overall, these results indicate that at high doses the initial yield of translocations may not always be constant with time post-irradiation.

Completeness and complexity are factors that can influence the disappearance of translocations with time post-irradiation. The majority of complex aberrations are not transmissible, and as a consequence cells carrying such aberrations will disappear with time post irradiation [213, 215, 223–226].

Another factor contributing to the persistence of translocations is the co-occurrence of translocations and unstable aberrations in the same cell. The procedure to consider stable cells instead of total cells for retrospective dose-estimation has therefore been proposed and is under consideration. In a follow-up study on victims of the Estonia accident the initial yield of translocations, when all cells are considered, decreased to about 70% after 2 years [214]. However, a further study in the same group, in which digitalized images of damaged cells were re-analysed to select those without unstable aberrations, indicated that after 7 years the yield of translocations was similar to that in the first 2 years. This study however was limited because it had to rely on cell images that were retained because they contained damage in the painted chromosomes. No correction was possible for those cells originally considered as ‘normal’ regarding the painted material because they had not been digitized. Nevertheless, this study taken with the theoretical likelihood that after a long period of time only stable cells will remain, suggests that to consider the yield of translocations in stable cells is more appropriate than the yield of translocations in all cells. This can only be finally resolved by good follow-up studies with FISH and unstable aberration analyses run in parallel on irradiated accident victims starting promptly after their exposure.

## 10.7. CALIBRATION CURVES

For dose estimations with translocations detected by FISH, each laboratory needs to establish its own curves. The mathematics of curve fitting is just the same as for dicentric chromosomes which has been described earlier (Section 8.3). The curve should be made with the same FISH probe cocktail that is routinely used for case investigations. Doing this prevents the need to convert to genome equivalence which could introduce some extra uncertainty.

For low-LET radiations, when calibration curves for translocations have been constructed taking into account stable or total cells, there were no differences in the fitted coefficients if only apparently simple translocations were considered [227, 228]. However, during microscope analysis, it is recommended to score all aberrations detected in the entire chromosome set, not only those affecting the painted material [64]. This will give the opportunity to establish for certain if the restriction to stable cells only gives more realistic dose-estimations.

FISH dose estimations generally will be undertaken for cases where doses were high, but protracted or after low radiation exposure a long time ago, revealing no medical symptoms. In contrast to acute exposure dosimetry, where the linear quadratic curve will be used, here the linear  $\alpha$  term of the dose response curve is crucially important. The F-test indicated in Section 8.3 can be used to ascertain the reliability of the linear coefficient. Few, if any,

published calibration curves have enough scoring in the low dose range to have obtained a sufficiently reliable linear coefficient with a small confidence range [64]. To construct a linear dose relationship, several dose points below 1 Gy each with a large number of cells need to be scored and this comprises a considerable workload. In the interim, one may make a number of reasonable assumptions to resolve this problem. The ratio of formation of dicentrics and translocations is about 1:1 [229, 230] and therefore similar dose response coefficients can be expected. Evidence from various published data from dicentrics suggest a linear calibration coefficient of about 15–20 translocations per 1000 genome equivalent cells per Gy for high energy gamma rays [51]. It is further known, that the linear term of the dose effect curves is mainly influenced by the linear energy transfer of the radiation quality, whereas the curvature due to the  $\beta$  coefficient is dose rate dependent (as described for the G-term correction in Section 9.7.4.5). There are indications that the linear coefficient obtained using *in vitro* chronic exposure, provided it is made at body temperature, is not different from that reported for the linear term of the acute dose response [233]. Thus until reliable linear coefficients for FISH translocations have been obtained by *in vitro* low dose-response calibration experiments, it is reasonable to use the linear term obtained with the same radiation quality for acute dicentric calibration curves.

With dose reconstruction a long time after exposure, the dose estimation will be based on the assumption that lymphocytes irradiated *in vitro* and blood stem cells irradiated *in vivo* will show similar frequencies of translocations. It is not sure whether the radiosensitivity of stem cells and of mature lymphocytes are identical or whether there might be an impact of the intervening cell divisions where cells containing unstable aberrations will be eliminated. Retrospective dosimetry assumes that these are not major confounders and the recent literature suggests that this is of no practical importance [64].

In conclusion, dose reconstruction on the basis of translocations in stable cells is an established method [174] but has limitations. It seems to be a good tool after protracted and low dose exposure where the linear term of the calibration curve predominates. As an interim measure the term from dicentric dose-response curves could be assumed. After high, especially acute, dose exposure, restricting to stable cells may underestimate the dose because the number of cells with translocations in unstable cells will increase together with the number of complex aberrations. Moreover there is a limit on the upper dose to which one may calibrate as cells free from unstable damage become increasingly rare.

## 10.8. EXAMPLES OF FISH BEING USED FOR RETROSPECTIVE BIOLOGICAL DOSIMETRY

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 contaminated areas; (3) Chernobyl cleanup workers; and (4) individuals or groups of persons accidentally exposed.

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

In order to perform retrospective estimations of radiation doses, the frequency of chromosomal aberrations was determined in 15 individuals known to be severely exposed as a

result of Chernobyl accident, 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 [212, 233, 234]. 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 [234]. 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 [212].

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.

#### **10.8.2. 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 (Dosimetry System 1986) estimated doses compared with an *in vitro* dose-response curve [145]. 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) Y-12 accident in 1959, where some years later the translocation frequencies were substantially below expected values [170].

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 [235]. In another set of investigations, 52 cleanup workers were studied with FISH painting [236]. 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 [235], and this is supported by the study of the Estonian cleanup workers [92, 237].

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 [238]. 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 [235, 238].

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 [239].

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 [240].

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 [241]. The Hiroshima atom bomb survivor 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.

### **10.8.3. 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 9.7.5.7) was also examined retrospectively by FISH [242]. 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 one- and two-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 radiation accident (Brazil, 1987) a large number of persons were exposed when a spent  $^{137}\text{Cs}$  radiotherapy source was broken open [149, 150]. 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. Twenty-nine persons had an estimated dose in the range of 0.3 to 5.9 Gy [243]. 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 [209].

Data on translocation frequencies (using various probe 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 [209]. The frequencies of translocations 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 one- and two-way translocations during the follow-up was found [209], which is similar to the Chernobyl studies. Straume et al. [244] 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 [245] undertaken 11 years after an accident involving 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 two-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 [213, 246]. In the follow-up studies, two-way translocations remained relatively stable in all five subjects, and in one person a significant decrease in one-way translocations was observed. Dicentrics decreased in all subjects to about 50% of the initial frequencies by 12 months post-exposure [246]. A further follow-up study spanning the 7 years after the accident indicated that scoring translocations in stable cells appeared to abolish the decline in translocations observed in all cells. In stable cells, the yield of translocations was independent of time during the first years of follow-up [174].

#### *Istanbul accident*

In Section 9.7.5.6, a case is described where several persons were irradiated by an unshielded <sup>60</sup>Co source mixed with scrap metal. One month elapsed between the accident 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 9.7.4.4, 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 [158], and the resultant dose estimates were based on the combined yields of one- and two-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.

#### *Georgian accident*

Eleven young frontier guards were accidentally exposed to one or several  $^{137}\text{Cs}$  sources with activity not exceeding 150 GBq, at the Lilo military training centre. The sources were intended for training and instrument calibration purposes. The victims were irradiated for approximately one year, from mid-1996 to April 1997 [247].

Four most exposed persons were hospitalized in France where cytogenetics was done in November 1997 [248] (Table 13).

TABLE 13. NUMBERS AND, IN BRACKETS, FREQUENCIES PER CELL OF UNSTABLE CHROMOSOME ABERRATIONS<sup>a</sup>

Patient	Scored cells	Dic	Rc	Ace	u test	Dose Dic	Dose, FISH
						Gy [95% CI]	Gy [95% CI]
1	500	14 (0.03)	0 (0.000)	11 (0.022)	-0,43	0.4 [0.2–0.6]	0.7[0.4–1.0]
2	500	19 (0.04)	1 (0.002)	15 (0.030)	-0,59	0.5[0.4–0.7]	0.4[0.1–0.7]
3	502	55 (0.11)	4 (0.008)	24 (0.048)	4,68	1.1[0.9–1.3]	0.8[0.6–1.1]
4	518	80 (0.15)	4 (0.008)	25 (0.048)	3,61	1.3[1.1–1.5]	1.7[1.4–1.9]

<sup>a</sup>The u-test indicates significant overdispersion,  $u > 1.96$ ; or underdispersion,  $u < 1.96$ . Dose estimates are derived from dicentrics and two-way translocations.

An acute ( $0.5 \text{ Gy} \cdot \text{min}^{-1}$ )  $^{60}\text{Co}$  *in vitro* calibration curve for dicentrics and rings was used to estimate the doses. For persons 1 and 2 the physical dose reconstruction suggested a highly localized irradiation, which was not confirmed by the distribution of dicentrics per cell ( $u < 1.96$ , Table 13). Therefore, the doses which were calculated assuming an acute homogeneous exposure are far below the dose estimates for persons 3 and 4 where the aberration distributions were overdispersed ( $u > 1.96$ ) suggesting a partial-body exposure. This is in agreement with the circumstances of exposure as reconstructed by physical dosimetry.

All four patients probably suffered from lymphopenia before their arrival in France and therefore, based on unstable aberrations (Section 9.7.7.4), the averaged whole-body dose estimates may be underestimates. Therefore the FISH translocations assay was also carried out, considering all cells and not confined to stable cells only. Three pairs of chromosome (# 2, 4 and 12) painted together with a pan-centromeric probe. For person 2 no difference was seen in the doses estimated by using dicentrics or translocations yields (Table 13). For persons 1 and 4, the FISH values were higher than for dicentrics, but the differences were not statistically significant. For person 3 however, a higher dose was estimated using dicentrics. These differences can probably be explained by the heterogeneity and fractionation of exposures, which differed markedly from one patient to another, and therefore modified the distributions of translocations in unstable cells and consequently the relative disappearance of dicentrics compared to translocations.

Further follow-up cytogenetics were undertaken (Figs 33 and 34) although samples were not available for each person on each occasion.

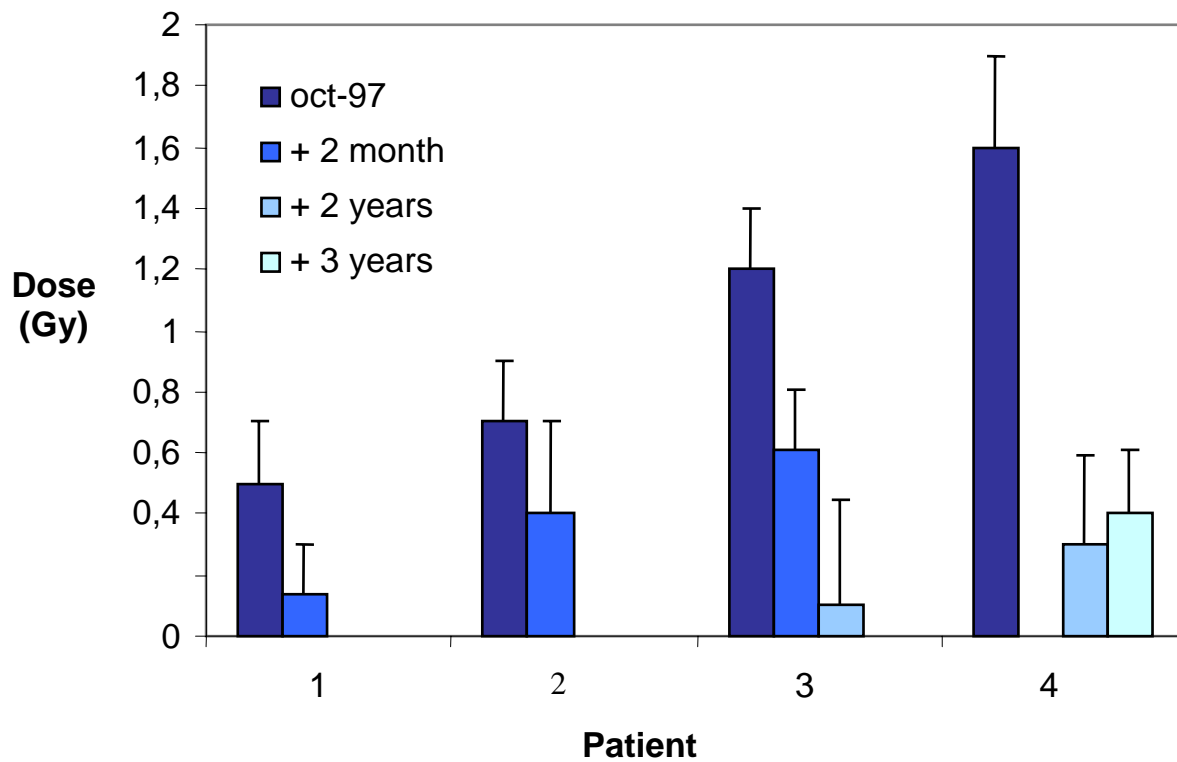


FIG. 33. Changes in dicentric derived dose estimates with time after irradiation.

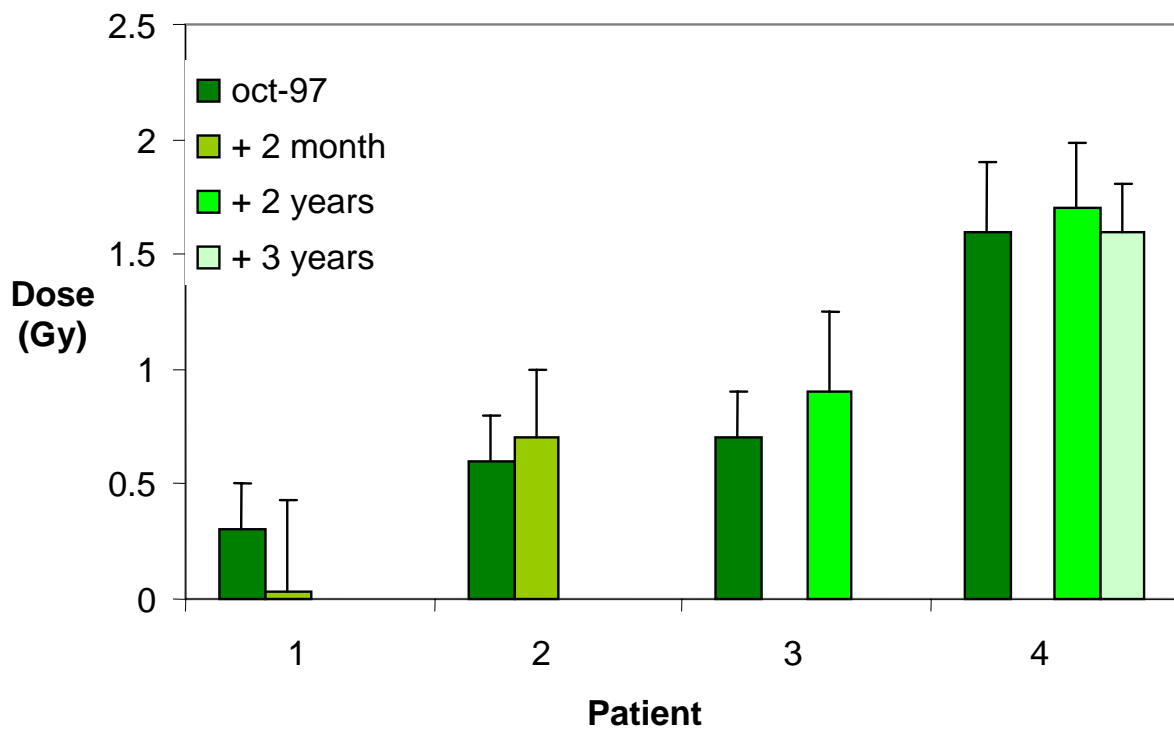


FIG. 34. Changes in two-way translocation derived dose estimates with time after irradiation.



As expected, a decrease in the dicentric yield was observed over time in all patients. By contrast, no decline in the frequency of two-way translocations was observed for three of the four patients. For person 1 the frequency of translocations decreased two months after the first blood sample was taken but, given the uncertainties, is not statistically significant. The general stability of the later translocations data probably reflects the rapid lymphocyte turnover associated with their lymphopenia and rapid elimination of unstable aberrations. The later FISH data are possibly indicating the dose received by the bone marrow stem cells.



## 11. PREMATURE CHROMOSOME CONDENSATION (PCC) ANALYSIS

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 especially after high doses, which can cause considerable underestimation of the radiation exposure dose [250]. This section describes techniques for inducing the chromosomes to condense prematurely, i.e. at some time before the first mitosis and so reduce or eliminate the culture time and hence the opportunity for mitotic delay or death to occur.

### 11.1. PCC BY MITOTIC FUSION

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 need for any mitogen stimulation or culturing [66]. The use of this 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 [67, 72, 75, 250]. The 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 [75]. Since with this 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 assay similarly could also be better able to quantify small localized burns from partial body exposures.

#### 11.1.1. Cell culture and cell fusion conditions

##### 11.1.1.1. 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 hours. CHO cells can be grown in 750 mL tissue culture flasks or roller bottles, in complete medium (composed of F-10, 15% newborn calf serum and antibiotics). Mitotic cells can be obtained by adding Colcemid (at a final concentration of 0.1 µg/mL) for 4 to 6 hours when cultures are half full, followed by mitotic shake-off. 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°C before use.

##### 11.1.1.2. Isolating lymphocytes

Generally, Ficoll Hypaque should be used for isolating lymphocytes as described in the earlier Section 9.1.5.2. This has the advantage that when enough lymphocytes are isolated, a part can be used immediately and the rest frozen at -80 to -120°C for future use, if found necessary.

##### 11.1.1.3. Fusing agent

Generally, polyethylene glycol (PEG) of molecular weight 1450 should be used, and the desired concentration for fusion is 40% to 50% w/v (in F-10 medium without serum, PBS, or preferably in RPMI-1640 medium with HEPES without serum).

#### *11.1.1.4. 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 F-10 should be added gradually). This is followed by a one hour incubation in complete medium with Colcemid at 37°C [67, 72, 251].

#### *11.1.1.5. Fixation procedures*

In principle, this is similar to the method used for metaphases (Section 9.2) 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 water bath (at 37°C) for 3 to 4 min, and, following centrifugation, cells can be fixed in a mixture of acetic acid:methanol (1:3).

Slide preparation is performed by using the standard technique that is similar to other assays described earlier (see Section 9.2).

#### *11.1.1.6. Staining procedures*

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

(1) Standard chromosome breaks analysis.

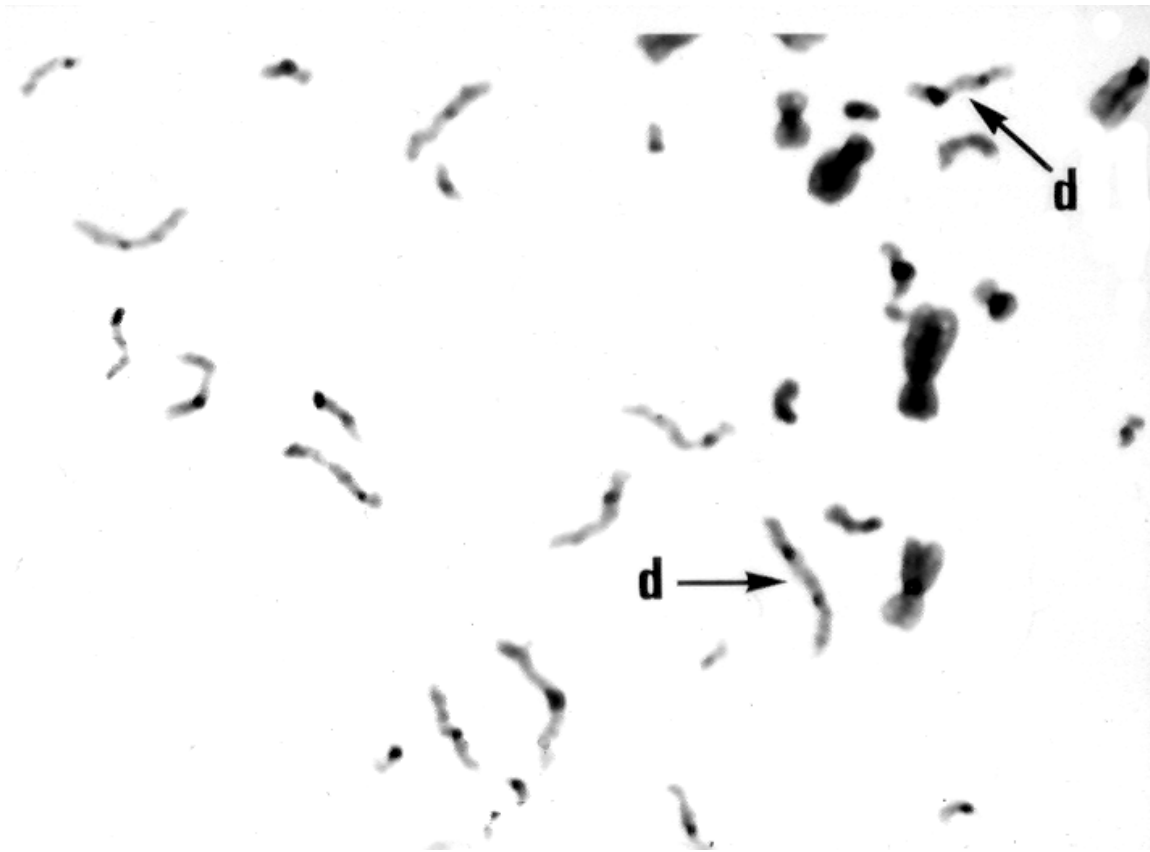
For the purpose of analysing chromosomal aberrations as radiation induced chromosome breaks, slides can be stained with conventional Giemsa (Gurr improved R66) or the FPG technique as was already described in Section 9.3.2 (Fig. 35) [67, 252]. The protocol using FPG was developed for PCC with cells where the two fused chromosome complements are completely intermingled. It is probably not necessary for the lymphocyte technique described here because the two sets of chromosomes tend to remain in two groups as shown in Fig. 35 where it can be seen that the single stranded human chromosomes are clearly distinguishable. A disadvantage of FPG staining is that chromosomes tend to swell and this may hinder accurate scoring of PCC fragments as small adjacent swollen objects could touch and appear to be a single structure.



*FIG. 35. Human  $G_0$  PCC with some fragments arrowed produced by the mitotic fusion method.*

(2) Dicentric analysis using C banding.

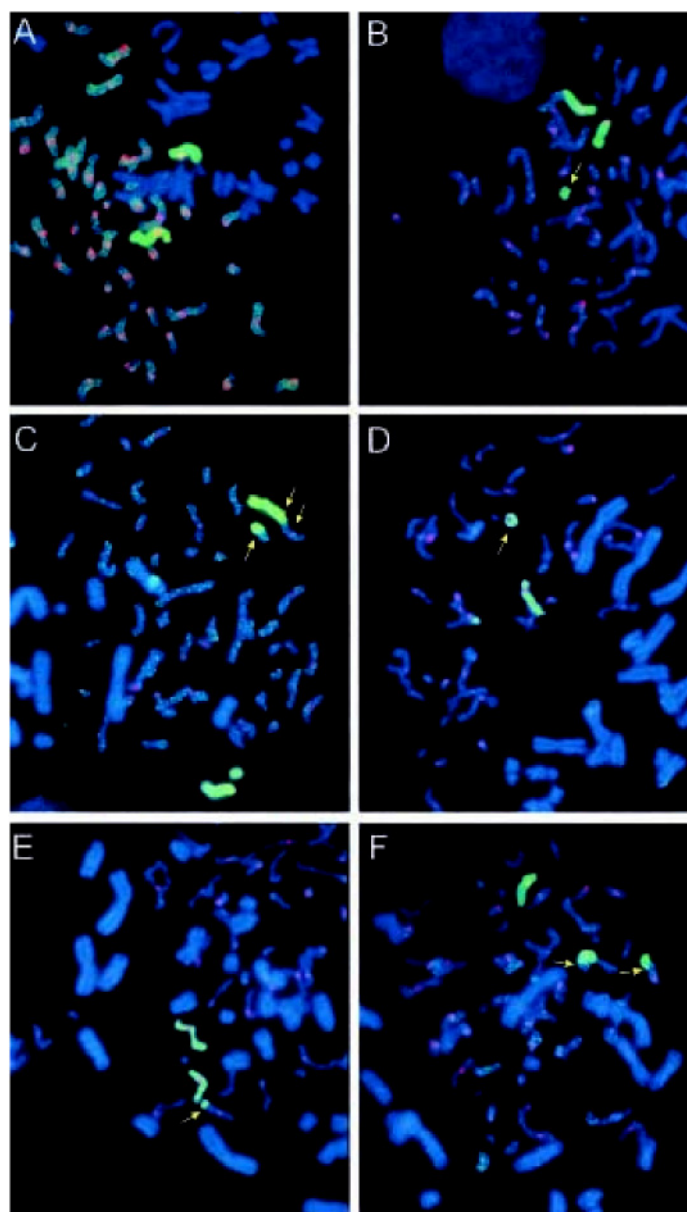
In addition to chromosome breaks, dicentrics can also be visualized. For this, slides should be pretreated with barium hydroxide and salt solution, (Section 9.3.3) followed by Giemsa staining, which highlights the centromeric region of all chromosomes so that dicentric chromosomes can be easily distinguished from monocentrics (Fig. 36) [70, 79].



*FIG. 36. PCC stained by the C-banding method showing two dicentrics (d).*

(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 (Fig. 37). The latter gives more accurate discrimination between translocations and dicentrics [72].



*FIG. 37. PCC 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  $t(AB)$  [8].*

### 11.1.2. Analysis

Criteria for analysis of slides are similar in part to those described in Section 9.4 (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 (Section 13.3) [75]. 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 microscope attachment (camera lucida) 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 coordinates 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. 22). When the FISH assay is used, cot-1 hamster DNA can be used to mask all signals in the CHO chromosomes so that only the appropriate human PCC are highlighted (Fig. 37).

### **11.1.3. 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<sub>1</sub>, S and G<sub>2</sub> appear as single chromatid, pulverized chromosomes and having two chromatids, respectively. For biological dosimetry with Giemsa stained preparations, one scores only the spreads comprising single chromatids, i.e. human lymphocytes that were treated in G<sub>0</sub>/G<sub>1</sub>, and each element represents one human chromosome (Fig. 35).

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 [75]. This method is identical in concept to the Qdr method introduced by Sasaki and Miyata [142] (see Section 9.7.4.3). 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. 37), recorded and analysed, as described in Sections 9.4 and 10.4.

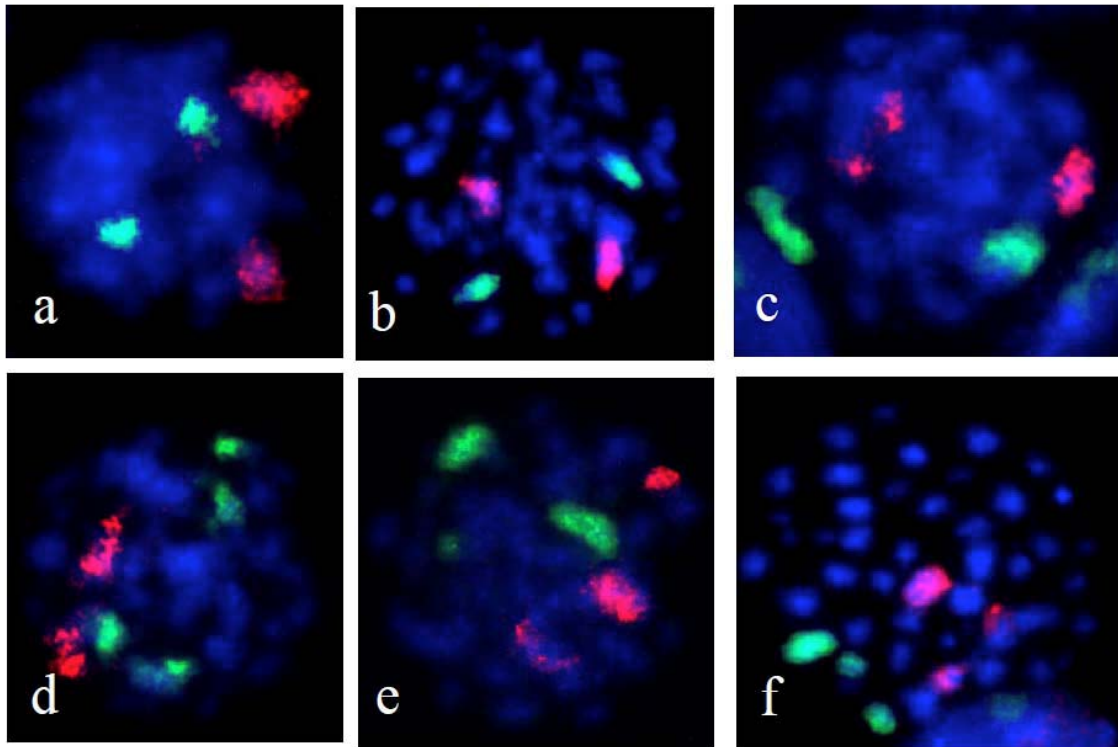
## **11.2. PCC BY CHEMICAL INDUCTION**

### **11.2.1. The rapid interphase chromosome assay (RICA)**

This assay also removes the need for prolonged culturing. Lymphocytes isolated from blood by the Ficoll Hypaque method (Section 9.1.5.2) are placed in culture medium containing a phosphatase inhibitor such as okadaic acid or calyculin A, adenosine triphosphate and p34<sup>cdc2</sup>/cyclin B kinase and incubated at 37°C for just 3 hours. The full protocol is described by Prasanna et al. [76]. Fixation and spread preparation (hypotonic potassium chloride; 3:1 methanol: acetic acid; dropping onto cleaned slides) follows the normal procedures used for metaphases.

Radiation induced damage is then analysed after in situ hybridization and chromosome painting by fluorescence microscopy (Fig. 38).





*FIG. 38. Photomicrographs showing FISH painted human chromosome 1 (red) and 2 (green) in interphase lymphocytes irradiated by  $^{60}\text{Co}$   $\gamma$ -rays and visualized by the RICA assay [78]. Normal cell producing two red spots and two green spots (a & b), aberrant chromosome 1 producing more than two red spots (c), aberrant chromosome 2 producing more than two green spots (d), cells with more than two green and red spots (e & f) (courtesy Pathak and Prasanna, AFRRRI, USA).*

Normal cells display two fluorescent spots per chromosome, whereas cells with structural aberrations (breaks and exchanges) involving specific chromosome(s) corresponding to painted probe(s), may show more than two spots. Using a single large chromosome probe is adequate for biological dosimetry [76]. However, using more than one chromosome probe improves sensitivity [78].

### 11.2.2. The PCC ring assay

Among the chemically-induced PCC methods for biological dosimetry, a simple and useful procedure is the scoring of rings in Giemsa-stained chromosomes. This technique still requires the lymphocytes to be cultured and the method, described by Kanda et al. [68] recommends 48 hours cultures. It therefore does not save time but the PCC-rings assay is particularly applicable to high overdoses in the range where the dose response for the conventional dicentric assay shows signs of saturation. It has been calibrated and used for doses up to 20 Gy equivalent to X rays. At such a dose the number of induced dicentrics and fragments is too large for reliable scoring. However in lymphocytes rings are induced at a much lower frequency, often ~10% of that of dicentrics, and this makes ring scoring a feasible endpoint after a very high dose.

#### *11.2.2.1. Cell culture, chemical treatment and slide preparation*

This is described in a step by step detailed protocol in Annex III.

##### *Preparation of chemicals*

Inhibitors of DNA phosphorylation such as okadaic acid or calyculin A should be prepared. These chemicals are carcinogenic and therefore should be handled with appropriate safety precautions. Calyculin A can induce PCC about 20 times more effectively than okadaic acid, although their mechanisms of PCC induction are probably similar as judged by the dose-dependence and the resulting chromosome morphology. Okadaic acid or calyculin A is dissolved in dimethylsulphoxide (DMSO), diluted with medium and stored at -20°C as a stock solution (e.g. 5–10 µM).

##### *Culture*

Chemically-induced PCC in lymphocytes generally requires the cells to be cycling. Therefore the procedure is to PHA stimulate and culture the cells for 48 hours using a method similar to that described in Section 9.1 for obtaining metaphases. Although PCC can be induced in whole blood cultures, using isolating lymphocytes produces cleaner preparations with a lot of cells (described in Section 9.1.5.2). Thus, especially in the case of very high-dose exposure, isolating lymphocytes is strongly recommended.

The standard protocol for PCC induction is that okadaic acid (500 nM) or calyculin A (20–50 nM) is added to the cultures during the final hour and this will therefore produce a mixture of PCC cells in all stages of the first cell cycle. However, the effectiveness of the chemicals might be dependent on the culture condition and drug quality. The concentration and period of optimal treatment should be determined based on the incidence of cells exhibiting PCC and the quality of chromosome morphology in each laboratory. Insufficient treatment results in a lack of analysable cells, whereas overtreatment results in fuzzy and too condensed chromosomes. The fixation, slide preparation and Giemsa staining procedures are similar to the methods used for metaphases.

#### *11.2.2.2. Scoring criteria*

In 48 hours cultures of highly irradiated lymphocytes most analysable cells are between the late G<sub>2</sub> phase and metaphase. With low-dose exposures there may be contamination with cells at anaphase. Compared with the appearance of ring chromosomes in metaphase spreads (Fig. 11), PCC rings in late G<sub>2</sub> and anaphase (Fig. 39A) cells are narrow which makes their identification easy. Therefore, these cells are preferred for scoring PCC rings. Cells at the late G<sub>2</sub> phase and those at anaphase can be distinguished by having attached or separated sister chromatids, respectively (Fig. 39B).

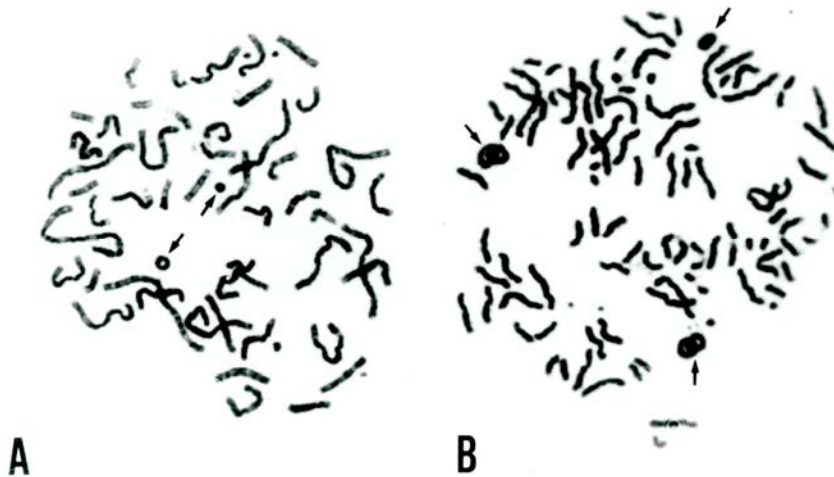


FIG. 39. Examples of okadaic acid induced PCC of irradiated lymphocytes at different cell cycle phases. (A) G<sub>2</sub>/M-PCC cells, (B) M/A-PCC cell exhibiting separated sister chromatids. Arrows indicate ring chromosomes [68].

The frequencies of PCC rings are not significantly different between late G<sub>2</sub> cells and anaphase cells, and these data can be pooled.

A circular shaped chromosome is scored as a PCC ring. The centromeres are not clearly visualized in PCC cells stained just with Giemsa so that PCC rings are not classified into centric or acentric forms.

Just as with dicentrics, (Section 9.7.4.3) the analysis of the intercellular distribution of PCC rings compared with the Poisson distribution provides some information regarding uniformity of exposure to low LET radiation or the radiation quality involved in an accident. In situations e.g. delayed discovery cases, where there is a time gap between irradiation and blood sampling it should be possible to use a half-life calculation to adjust the observed yield to obtain an estimate of the original PCC ring frequency. At present there are little firm data to support this. However the cytogenetic follow-up study of one survivor of the Tokai-mura accident reported a half-life of about 8.7 months [253].

### 11.3. A RADIATION ACCIDENT INVESTIGATED BY THE PCC RINGS METHOD

Soon after the PCC rings technique, calibrated *in vitro* with 200 kV X rays, was published [68], the opportunity arose to examine a serious radiation accident using the method where PCC was induced by okadaic acid. Biological dosimetry was performed on three seriously exposed victims of the Tokai-mura criticality accident in Japan in 1999 [254].

The frequencies of PCC rings per 100 cells from samples obtained 9 hours 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. One should bear in mind that the exposures were to a mixed field of gamma and neutron radiation, and the equivalent dose measured in 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. The organ RBE-weighted dose was specially defined for characterizing high dose exposure as a product of absorbed organ dose and RBE in order to evaluate onset of deterministic health effects [15]. The RBE of 200 kV X rays is set to 1. The unit of RBE-weighted dose is J kg<sup>-1</sup> and is called in [15] the gray-equivalent (Gy-Eq). For the most highly irradiated person the dose could only be approximated to >20 Gy-Eq because the

published *in vitro* calibration [68], showed a levelling off (saturation) in the linear quadratic dose–response for whole body RBE-weighted dose approaching 20 Gy-Eq of 200 kV X rays (or 20 Gy of whole body absorbed dose since RBE is 1).

Parallel analyses of the blood samples were also made by conventionally scoring dicentrics and rings (dic+rc) 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 [123]. This method concentrates the lymphocytes using a Ficoll Hypaque column and is similar to that described in Section 9.1.5.2. This yielded, for the most heavily irradiated patient, 715 dicentrics and 188 centric + acentric rings in 78 cells where every metaphase was damaged. The corresponding yields for the other two persons were 479 dicentrics and 55 rings in 175 cells and 191 dic + rc in 300 cells. Table 14 taken from [255] summarizes the resultant estimates of doses by the cytogenetic methods and also by physical measurements using sodium activation analysis.

TABLE 14. COMPARISON OF THE DOSES ESTIMATED BY VARIOUS INDICATORS

Patient	Estimated Whole Body RBE-weighted Dose (Gy-Eq) <sup>a</sup> by			
	PCC-Ring	Dic	Dic+R/Rc	<sup>24</sup> Na <sup>b</sup>
A	>20	22.6	24.5	17–24
B	7.4(6.5–8.2)	8.3	8.3	8.7–13
C	2.3(1.8–2.8)	-	3.0(2.8–3.2)	2.5–3.6

<sup>a</sup> RBE set 1 for X- (patients A and B) or  $\gamma$ -rays (patient C).

<sup>b</sup> Ishigure et al. [255], where the neutron RBE is assessed at 1.5–2.0.

## 12. THE CYTOKINESIS-BLOCK MICRONUCLEUS (CBMN) ASSAY

### 12.1. BACKGROUND

Ionizing radiation induces the formation of acentric chromosome fragments and to a small extent 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 based on MN expression in short term culture of lymphocytes was first described by Countryman and Heddle [82]. However, in this original method no attempt was made to determine whether the cells scored had actually completed nuclear division *in vitro* which made the assay unreliable because chromosome damage in cells can only be expressed as micronuclei if cells divide. A more reliable approach was eventually developed based on the use of the cytokinesis inhibitor, cytochalasin-B. Using cytochalasin-B, Fenech and Morley were able to demonstrate in 1985 [83, 84] that cells that had completed one nuclear division could be accumulated and recognized as binucleated (BN) cells. MN could then be specifically and efficiently scored in these BN cells while excluding non-dividing mononuclear cells that were unable to express MN *in vitro* (Fig. 19). Consequently, the results obtained with the MN assay are not 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 [84, 256, 258]. The resulting cytokinesis-block 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 shown that scoring MN in mononucleated lymphocytes in conventional blood smears could be particularly useful for monitoring genetic damage in chronically exposed populations [259–263]. Furthermore, scoring of MN in mononucleated cells could also be used as an interesting additional parameter in the CBMN assay [262, 263].

In the 1990s the CBMN-centromere assay was developed, using FISH and a pan-centromeric probe to visualize centromeres. This method allows discrimination between MN containing acentric fragments and whole chromosomes [69, 85, 263–267]. Applying this method, the sensitivity of the CBMN assay can be substantially increased in the low dose range [85, 266, 267] (see Section 12.4.2).

More recently a more comprehensive version of the CBMN assay known as the Cytokinesis-Block Micronucleus Cytome (CBMN Cyt) assay has been developed and validated [86] which, apart from MN in binucleated and mononucleated cells, also includes measurement of nucleoplasmic bridges (NPB, Fig. 19C) and nuclear buds in binucleated cells which are biomarkers of dicentric chromosomes [89] and gene amplification [86] respectively. Furthermore, in the CBMN Cyt assay the proportion of mono-nucleated, binucleated and multinucleated cells as well as necrotic and apoptotic cells is scored which provides measures of cellular proliferation and cell death that can also be informative in biological dosimetry [88, 268].

It is also possible to score micronuclei in erythrocytes as a biomarker of chromosome damage noting that the method has an upper limit of detection of 1 Gy and samples need to be collected as soon as possible after exposure due to inhibition of erythropoiesis. Recently the flow cytometric *in vivo* MN assay in immature mouse erythrocytes has been adapted for use in humans, by restricting MN scoring to the transferrin receptor positive reticulocytes

(Tf-Ret; CD71) [269]. Evaluation of the reticulocyte assay in patients treated with radioiodine for thyroid cancer shows that the method may be of use for monitoring individuals after suspected accidental radiation exposure [270, 271].

## 12.2. LYMPHOCYTE CULTURE FOR CBMN ASSAY

The lymphocyte culture method is similar to that described in Section 9.1 for obtaining metaphases. The main differences, however, are that (i) Cyt-B is added to the cultures at 24 or 44 hours (24 hours is preferable for radiation biological dosimetry to ensure only first division cells are captured), (ii) bromodeoxyuridine and Colcemid are not used, (iii) the culture time is extended to 72 hours, and hypotonic treatment, fixation and centrifugation are modified to preserve the cell cytoplasm so that binucleated cells are easily identified. The preparations are either conventionally stained with Giemsa for light microscopy or with a fluorescent dye such as acridine orange for fluorescence microscopy. The preparations can also be further processed to highlight centromeres using FISH and a pan-centromeric FISH probe. Detailed protocols are given in Annex IV.

## 12.3. CBMN ASSAY SCORING CRITERIA

Detailed scoring criteria for all the biomarkers in the CBMN Cyt assay have been published [86]. In this section only scoring criteria for MN and NPB in binucleated cells are provided because these are the best validated biomarkers for biological dosimetry of ionizing radiation exposure.

### 12.3.1. Criteria for selecting binucleated cells which can be scored for micronucleus frequency

The cytokinesis-block cells that may be scored for MN frequency should have the following characteristics (Fig. 19):

- (a) The cells should be binucleated (BN).
- (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.

### 12.3.2. Criteria for scoring micronuclei

MN are morphologically identical to but smaller than the main nuclei (Fig. 19). 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.

Table 15 illustrates a simple layout for a data sheet for recording MN.

**TABLE 15. LAYOUT OF A MICRONUCLEUS SCORING RESULTS SHEET FOR DUPLICATE CULTURES (1 & 2) FROM A SINGLE BLOOD SAMPLE**

Sample No:  
 Scorer:  
 Date:

Slide No.	Micronucleus distribution in BN cells							Total No. of BN cells	Total No. of Micronuclei
	0 MN	1 MN	2 MN	3 MN	4 MN	5 MN	> 5 MN		
1								500	
2								500	
1 + 2								1000	
Remarks:									

### 12.3.3. Criteria for scoring nucleoplasmic bridges

A nucleoplasmic bridge (NPB) is a continuous DNA-containing structure linking the nuclei in a binucleated cell. NPB originate from dicentric chromosomes (resulting from misrepaired DNA breaks or telomere end fusions) in which the centromeres are pulled to opposite poles during anaphase (Fig. 19A and C). They have the following characteristics:

- (a) The width of a NPB may vary considerably but usually does not exceed 1/4th of the diameter of the nuclei within the cell.
- (b) NPB should also have the same staining characteristics as the main nuclei.
- (c) On rare occasions more than one NPB may be observed within one binucleated cell.
- (d) A binucleated cell with a NPB may contain one or more MN.
- (e) BN cells with one or more NPB and no MN may also be observed.

Table 16 illustrates a simple layout for a data sheet for recording NPB.

TABLE 16. LAYOUT OF A NUCLEOPLASMIC BRIDGE SCORING RESULT SHEET FOR DUPLICATE CULTURES (1 & 2) FROM A SINGLE BLOOD SAMPLE

Sample No:

Scorer:

Date:

Slide No.	NPB distribution in BN cells							Total No. of BN cells	Total No. of NPB
	0 NPB	1 NPB	2 NPB	3 NPB	4 NPB	5 NPB	> 5 NPB		
1								500	
2								500	
1 + 2								1000	
Remarks:									

It may be more difficult to score NPB in BN cells with touching nuclei, and it is therefore reasonable to specify whether NPB were scored in all BN cells regardless of proximity of nuclei within a BN cell or whether they were scored separately in those BN cells in which nuclei were clearly separated and those BN cells with touching nuclei. There is not enough evidence yet to recommend scoring NPB only in BN cells in which nuclei do not touch.

#### 12.4. CBMN ASSAY DATA HANDLING

##### 12.4.1. Dose–response

The procedures for producing *in vitro* dose–response calibration curves are as previously described in Section 8. Many studies have shown that the number of radiation induced micronuclei is strongly correlated with radiation dose and quality [87, 272–275]. As there are however interlaboratory differences in MN dose response, due to the use of different protocols, scoring criteria, etc., just as for the other assays described in this publication, any laboratory intending to carry out biological dosimetry, should make its own *in vitro* dose response calibration curves. Ideally, at least 8 doses should be used in the range up to 5 Gy. Curve fitting by linear (high LET) and linear-quadratic (low LET) models follow the procedures described in Section 8. A typical example of a MN dose response curve for low LET radiation ( $^{60}\text{Co}$   $\gamma$ -rays, dose rate 0.5 Gy/min) is shown in Fig. 40.



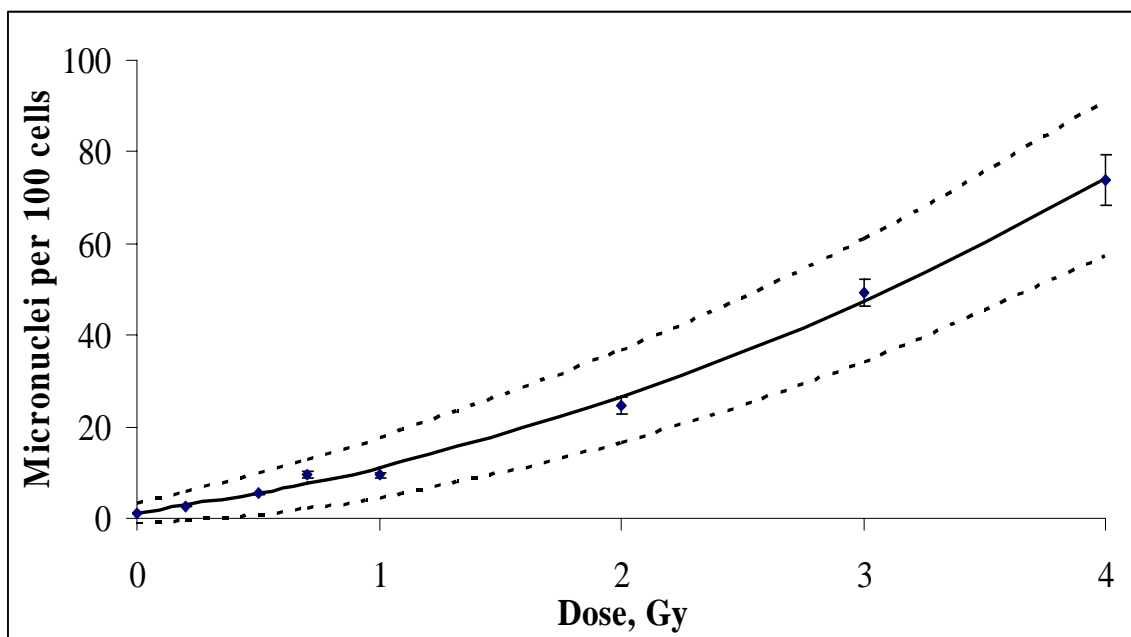


FIG. 40. Typical linear-quadratic MN dose response curve for  $^{60}\text{Co}$   $\gamma$ -rays. Solid curve: pooled data from 47 donors; broken curves: the upper and lower 95% confidence intervals.

#### 12.4.2. Background frequency

The background frequency of MN is reported to be quite variable; values ranging from 0 to 40 per 1000 BN cells have been recorded [257–286]. The two most important factors influencing MN background frequency, besides dietary factors [276] and exposure to a wide range of environmental clastogens and aneugens, are age and gender [84, 277].

Large scale biomonitoring studies have shown that the spontaneous micronucleus yield increases systematically with age. For a male control population values of 0.35 MN/1000 BN cells/year and 0.44 MN/1000/year were obtained respectively in a study of nuclear power plant and hospital workers [278–280]. These values are in agreement with the large scale study of Fenech [108] of variables influencing baseline micronucleus frequencies: 0.31 MN/1000/year. For a female control population a more prominent increase of 0.58 MN/1000/year was found [279], again in agreement with Fenech [108]: 0.52 MN//1000/year. Analysis of the MN for the presence of centromeres, by using a pan-centromeric FISH probe (Fig. 41), showed that the age increase of baseline MN frequencies can be attributed almost totally to centromere-positive MN, reflecting an increased chromosome loss with age [266, 279, 280].

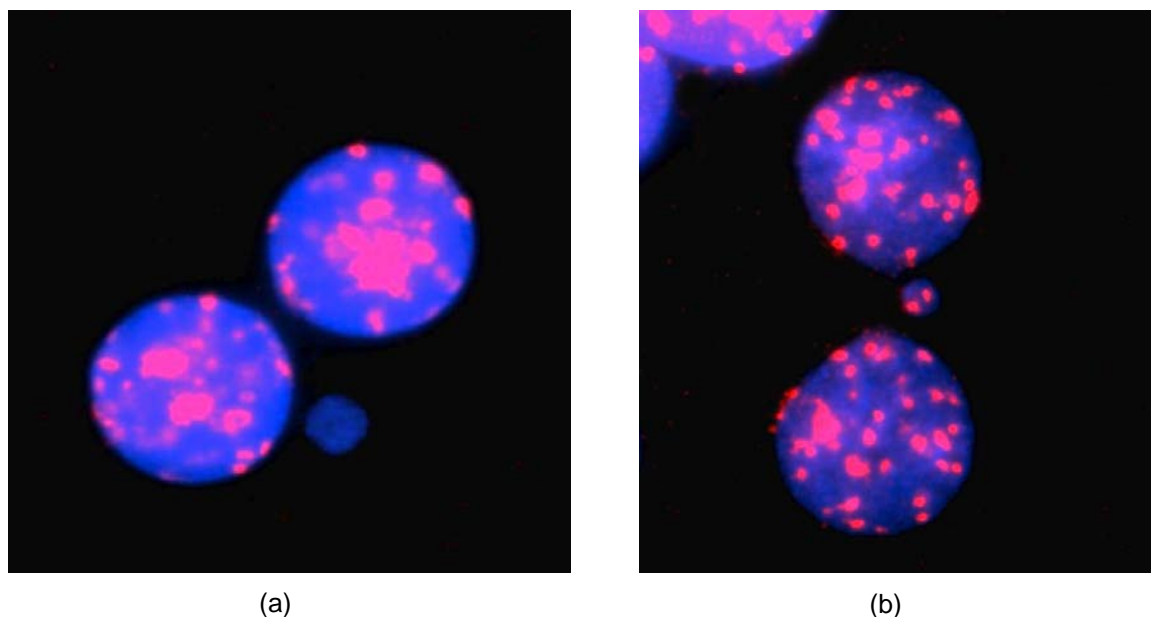


FIG. 41. Binucleated cells showing a centromere negative MN (a) and a centromere positive MN (b). Centromeres are stained with a pan-centromeric probe (spectrum orange) and nuclei and MN are counterstained with DAPI.

The X-chromosome is almost completely responsible for this spontaneously occurring chromosome loss [281, 282]. This explains also the gender difference in spontaneous MN frequencies where for a population with mean age 41.4 and 41.8 years the mean spontaneous MN frequencies were 16.4 for males and 23.5 per 1000 BN cells for females respectively; by contrast, the difference in centromere-negative MN is not significant: 6.7 versus 7.7 [279].

This background variability clearly poses limitations on using MN as a biological dosimeter for low doses, where pre-existing individual background frequencies are not known. Estimates have been made, suggesting that the CBMN assay in its basic form could only detect *in vivo* exposures in excess of 0.2–0.3 Gy X rays [87, 266, 283].

As it has been shown that most of the radiation induced MN originate primarily from acentric fragments while spontaneous MN contain especially whole chromosomes [85, 264–267] the use of the CBMN-centromere assay substantially increases the sensitivity of the CBMN assay in the low dose range [85, 266]. In both studies [85, 266], using a pan-centromeric probe, the majority of spontaneous MN were centromere positive (MNCM<sup>+ve</sup>) (respectively 73 and 71%) while most radiation induced MN were centromere negative (MNCM<sup>-ve</sup>). The number of MNCM<sup>+ve</sup> only showed a very small increase with dose (respectively 3.7 and 5.3 MNCM<sup>+ve</sup> per Gy per 1000 BN cells). By manual scoring of MNCM<sup>-ve</sup> in 2000 BN cells a detection limit, at the 95% confidence limit, of 0.1 Gy can be achieved [266, 267].

#### 12.4.3. Nuclear division index (NDI)

When scoring cytokinesis-block (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.

This is referred to as the NDI [284]. The index is in itself not sufficiently robust for direct application as a biodosimeter. Nevertheless the assay is frequently employed as a useful research tool for understanding the cell cycling kinetics of the cultures. It will indicate perturbations that may be caused by exposure to a mutagen such as radiation. The data arise

directly from the CBMN assay, without any additional laboratory effort, and therefore the method is included in this publication.

#### 12.4.3.1. Criteria for scoring viable mono-nucleated, binucleated and multinucleated cells

These cell types have the following characteristics:

- Mono-, bi- and multi-nucleated cells are viable cells with an intact cytoplasm and normal nucleus morphology containing one, two, three or more nuclei respectively.
- They may or may not contain one or more MN or nuclear buds (NBUD) and in the case of bi- and multi-nucleated cells they may or may not contain one or more NPB.

Necrotic and apoptotic cells should not be included amongst the viable cells scored.

On rare occasions multinucleated cells with more than four nuclei are observed if the cell cycle time is much shorter than normal or the cytokinesis-blocking time is too long.

#### 12.4.3.2. Calculating the NDI

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 (36):

$$NDI = \frac{M_1 + 2M_2 + 3M_3 + 4M_4}{N} \quad (36)$$

where:

$M_1$  to  $M_4$  represent the number of cells with one to four nuclei, and  $N$  is the total number of viable cells scored.

The published methods for calculating NDI [284] did not consider its uncertainty. Indeed, a method for deriving the uncertainty does not seem to have been published subsequently. It is therefore described here and due to its complexity, a fully worked example is shown in Annex IV-4.

As the values  $M_1$  to  $M_4$  are correlated, the uncertainties on the NDI cannot be calculated using standard error analysis. Instead, the covariance, which measures how the variables are dependent on each other, must be taken into account. The values  $M_1$  to  $M_4$  in the NDI can be assumed to form a multinomial distribution, which means that there is a fixed number (three or more) of possible outcomes for numbers of nuclei in a cell — i.e. 1, 2, 3 or 4 in this case. The variance (var) and covariance (covar) of each variable,  $M_1$  to  $M_4$  can then be calculated using Eqs (37) to (39):

$$\text{var}(M_i) = np_i(1 - p_i) \quad (37)$$

$$\text{covar}(M_i, M_j) = -np_i p_j \quad (38)$$

for  $i$  and  $j = 1, 2, 3$  or  $4$

where:

$M_1$ ,  $M_2$ ,  $M_3$  or  $M_4$  are the values of  $1 \times M_1$ ,  $2 \times M_2$ ,  $3 \times M_3$  and  $4 \times M_4$ ,

$n$  is the sum of the total number of cells times their respective numbers of micronuclei (equivalent to the numerator of the NDI equation), and  $p_i$  and  $p_j$  are the probabilities of  $M_i$  and  $M_j$  which are equal to  $M_i$  or  $M_j$  divided by  $n$ .

Using the definition of covariance, it can then be shown that the variance of NDI is dependent on the variances and covariances calculated using Eqs 37 and 38:

$$\text{var}(NDI) = \sum_{i=1}^4 M_i^2 \text{var}(M_i) + 2 \sum_{i=1}^4 \sum_{j=i+1}^4 M_i M_j \text{cov}(M_i, M_j) \quad (39)$$

This calculation is relatively complex but can be easily carried out using one of the widely available statistical packages or the Dose Estimate program mentioned in Section 8.3.

## 12.5. APPLICATION OF THE CBMN ASSAY FOR BIOLOGICAL DOSIMETRY

### 12.5.1. Patient studies

To verify the applicability of the CBMN method in biological dosimetry, MN yields were measured in peripheral blood lymphocytes of 1) different groups of cancer patients receiving fractionated partial body radiotherapy, e.g. prostate, cervix, Hodgkin's disease [285–288] and 2) thyroid cancer patients undergoing radioiodine treatment [289–291].

These studies showed that the doses estimated by MN agreed quite well with averaged whole body doses calculated from the radiation treatment plans plus cumulative dose–volume histograms [285–287, 292].

A meta-analysis, focused on thyroid cancer patients [289], showed that the post-radiation MN induction increased more than three times in comparison with the pre-irradiation frequency, demonstrating that the CBMN assay is sensitive enough to detect the genetic damage in circulating lymphocytes from exposure to low averaged whole body dose from internally incorporated radiation sources.

#### 12.5.1.1. Radioiodine case study

The CBMN test was used to study the response of lymphocytes of a 34-year male following treatment with  $^{131}\text{I}$  ablative radiation therapy after a total thyroidectomy for cancer [291]. Fortuitously, several months before diagnosis the patient had volunteered a blood sample for an *in vitro* study of micronucleus expression following external exposure to graded doses of X rays (198 mGy/min). The background frequency (pre-treatment baseline) in the unexposed culture showed a mean frequency of 6.0 MN per 1,000 binucleated (BN) cells while mean values of 18.5, 29.0, 41.0, 61.0 and 75.5 MN per 1,000 BN cells were found following X ray doses of 50, 100, 150, 200 and 250 mGy, respectively. The data were found to fit a non-threshold, linear dose-response function ( $Y = 3.714 + 2.783D$ ;  $r=0.99$ ) as shown in Fig. 42.

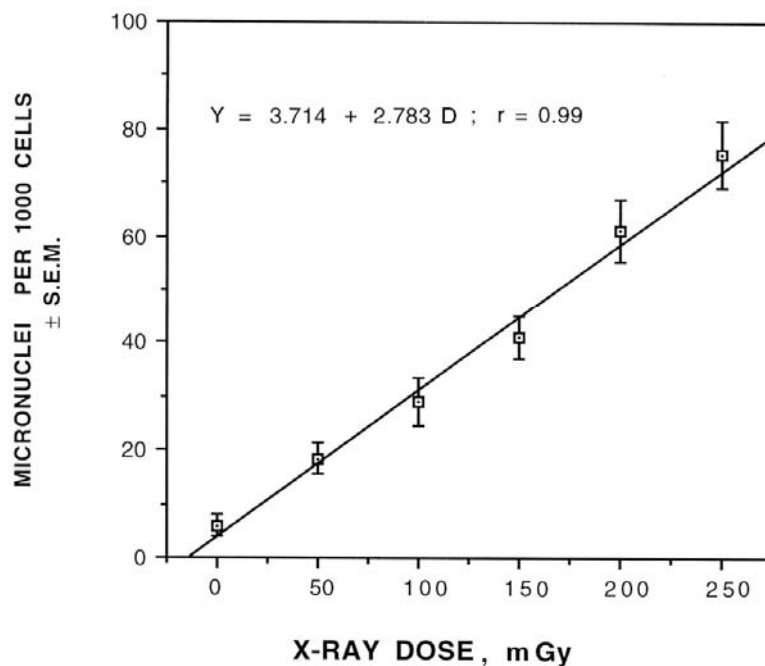


FIG. 42. *In vitro* MN dose response for low doses of X rays to the patient's lymphocytes before diagnosis and after  $^{131}\text{I}$  therapy (courtesy Livingston, REAC/TS, USA).

Blood was taken 11 days after the first *in vivo*  $^{131}\text{I}$  treatment with 48 mCi (1.78 GBq) and at monthly intervals thereafter and eventually at quarterly intervals out to five years. The first post-treatment sample showed 35.5 MN per 1,000 BN cells and the six-fold increase above the pre-treatment baseline suggests a dose to the peripheral blood of about 110 mGy. Twenty-six months after the first  $^{131}\text{I}$  treatment a second treatment of 390 mCi (14.46 GBq) was administered to the patient which resulted in a further increase in micronuclei. The micronuclei count fluctuated widely over time and was about 10-fold higher than the pre-treatment baseline value after 5 years of follow-up (Fig. 43).

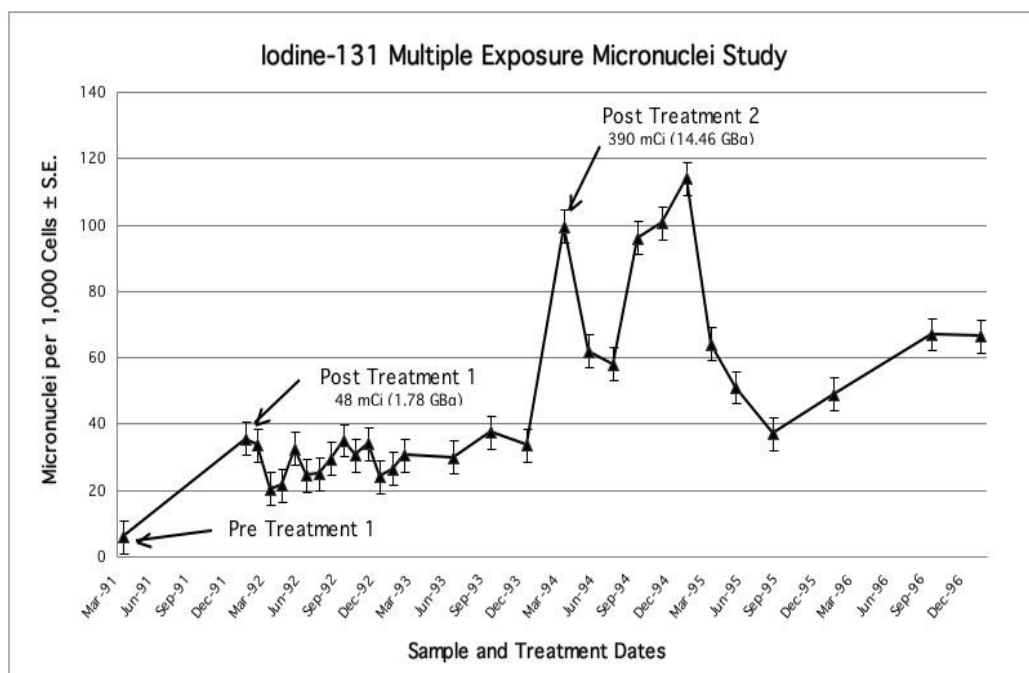


FIG. 43. A five year MN follow up of the patient before, during and after  $^{131}\text{I}$  therapy treatments (courtesy Livingston, REAC/TS, USA).

More than 15 years after the second treatment the patient was cancer-free and healthy. Results of this study support the conclusion that the CBMN test is a rapid, sensitive and quantitative biomarker of radiation exposure. However such studies are not able to determine local dose to the target tissue which in this case was any residual thyroid cells plus metastases of thyroidal origin.

### 12.5.2. Biomonitoring studies

After having been validated as an *in vivo* biomonitor in several patient studies, the CBMN assay as well as the CBMN-centromere assay have been applied for large scale biomonitoring of occupationally exposed radiation workers, e.g. nuclear power plant and hospital staff [266, 278–280, 293–295]. These biomonitoring studies showed the dependence of MN on the accumulated dose received over the years preceding the venipuncture. In the study of Thierens et al. [280] a linear regression of the individual micronucleus frequencies, corrected for the age effect (see Section 12.4.2.), showed an increase of 0.0175 MN per 1000 BN cells/mSv with a Pearson correlation coefficient value of 0.10. Application of the CBMN-centromere assay in a second study of radiation workers by Thierens et al. [295] resulted in almost the same increase of MN with dose, 0.025 MN per 1000 BN cells/mGy and demonstrated that this dose dependence is completely due to MNCM<sup>ve</sup> pointing to the clastogenic action of ionizing radiation. Dose dependence of MN in an occupational exposure setting was also found in a study by Vaglenov et al. [296]. They reported an increase of 0.03 MN per 1000 BN cells/mGy. Large scale biomonitoring studies show that the micronucleus assay is able to demonstrate genetic damage at the population level for accumulated doses received occupationally exceeding 50 mGy.

### 12.5.3. Accident studies

#### 12.5.3.1. Chernobyl accidental

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 [297]. 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 ( $R = 0.71$ ).

#### 12.5.3.2. 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 [158, 159] previously described in Sections 9.7.5.6 and 10.9.3 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 with the CBMN assay as well as for dicentrics 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 10.9.3 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, especially after high doses.

#### 12.5.3.3. Semipalatinsk Nuclear Test Site

The Semipalatinsk nuclear test site area has been highly contaminated with radioactive fallout during 40 years of continual weapons testing (1949–1989). Individuals living near the site have been exposed to both internal and external radiation. Dicentric and MN analysis was performed in people living in different contaminated villages and one control village. A higher incidence of dicentrics as well as micronuclei was found in residents of the contaminated areas and this higher incidence seems to be mainly caused by their internally incorporated radionuclides [298].

#### 12.5.3.4. Accident with a 50 kV contact radiotherapy X ray device

In 2003, the CBMN assay was applied for retrospective assessment of the dose received by a hospital worker, who was exposed accidentally by a 50 kV contact radiotherapy X ray device during maintenance [299]. A dose estimate of 0.73 Gy was obtained with 95 % confidence limits of 0.54–0.96 Gy. Dicentric scoring resulted in a dose estimate of 0.62 Gy (range 0.45–0.90 Gy), in very good agreement with the CBMN assay dose. A skin injury on the back of the worker indicated that the overexposure was a partial body irradiation. From the overdispersion of the dicentrics data it was deduced that a fraction of 49 % of the body was irradiated. It was not possible to apply this type of analysis to the MN data as MN invariably exhibit overdispersion, even in the case of a total body irradiation. A second blood sample, taken 1 year later, showed that the MN yield decreased with time post-exposure. The

disappearance half-time was 342 days; very close to a value of 377 days obtained from dicentric. This result is in agreement with the decline in the micronucleus frequency with post-irradiation time down to about 60 % at 1y post-treatment, observed in radiotherapy patients [292].

#### *12.5.3.5. Large scale radiation accidents*

In case of large scale radiation accidents, when hundreds of people may be exposed, it is important to distinguish the severely exposed individuals ( $\geq 1$  Gy), who require early medical treatment, from those less exposed. For this purpose, a rapid biological dosimetry assay is needed. In a recent study [87] the efficacy of automated MN scoring has been confirmed for fast population triage in a multicentre setting. More detailed information is given in Section 13.3.3.



## 13. AUTOMATION OF CHROMOSOMAL ASSAYS

For efficient preparedness for response to radiation events involving mass casualties, it has become imperative to automate cytogenetic dose assessment methods to increase throughput as they are time-consuming and laborious. Moreover, automation also improves quality control and assurance. Furthermore, it also enhances safety of laboratory personnel as the protocol involves processing of blood, which is an occupational biohazard. Cytogenetic laboratory automation involves: (i) automation of sample preparation, (ii) automation of analysis, and (iii) laboratory information management system for sample tracking and data handling [300].

### 13.1. AUTOMATED SAMPLE PROCESSING

Automated sample-processing in a cytogenetic laboratory may consist of any or all of the following equipment stations: (i) a robotic blood handler, (ii) a biosafety hood, (iii) incubators, (iv) metaphase harvester, (v) metaphase spreader, and (vi) slide stainer.

#### 13.1.1. Robotic blood handler

A customized automated liquid-handling robot for high-throughput processing of blood samples and isolation of lymphocytes from peripheral whole blood can eliminate an important rate-limiting bottleneck in sample processing for cytogenetic dose assessment [300]. The commercially available liquid-handling robots capable of dispensing, diluting, and aspirating blood samples, specifically for blood banking applications [301] may be customized and used for the desired purpose. These systems are precise, accurate, and do not cross contaminate specimens [302]. A customized robotic blood handling station may be equipped with a large customized work deck, a bar-code reader for maintaining chain-of-custody of samples, robotic arms for liquid-handling and transporting of vacutainers, centrifuge tubes, and a wash station for pipette tips. The robot can also be integrated with both a cell viability analyser for correcting for lymphocyte density while setting up cultures and a swinging-bucket automated centrifuge for density gradient isolation of lymphocytes for setting up isolated lymphocyte cultures. However, all equipment must be enclosed in an engineered Biosafety Level 2 environment to ensure sterility of the samples and occupational safety of laboratory personnel. The system must provide a positive chain-of-custody [301].

#### 13.1.2. Metaphase harvester

To obtain consistently and reliably high quality metaphase spreads, customized commercially available metaphase harvesters can be used for harvesting spreads from a blood culture. These devices eliminate the labour-intensive process by performing repetitious tasks involved in metaphase harvesting from cultures such as centrifugation of cell suspensions, aspiration and safe disposal of supernatant, treatment with hypotonic solution, and fixation of cells with acetic acid:methanol. These steps are carried out under controlled environmental conditions in a one-step protocol without user interaction thereby enhancing the quality and reproducibility of the process [300].

#### 13.1.3. Metaphase spreader

Metaphase spreading onto glass slides is influenced by temperature and humidity [303]. An automated system provides optimal environmental conditions of temperature and humidity during spreading of the cell suspension onto glass slides with a greater throughput than can be achieved manually. The spreader may be fitted with a microprocessor to precisely balance and control temperature, humidity and drying time. These controls, in conjunction with its functionally derived shape allow different users to obtain consistent results for both human and animal cells. A built-in sealable pipette guide provides consistent sample spreading and helps in preventing sample cross over.

#### **13.1.4. Slide auto-stainer**

This provides a rapid and consistent method of staining slides with Giemsa with minimal operator involvement. Currently available autostainers allow intelligent and flexible sample scheduling from 1 to 520 slides to be stained and rinsed unattended with identical or varied protocols. A sample priority assignment feature allows specific sample batches to be queued and processed ahead of others with no user involvement. The built-in battery backup can ensure continuation of ongoing sample processing by providing up to 40 min of run time in case of an electrical power outage.

### **13.2. AUTOMATED IMAGE ANALYSIS**

Automated analysis of images captured in a microscope is not yet routinely used by many laboratories for biological dosimetry, although it is likely to increase as systems improve. Attempts have been made to automate scoring for all four assays described in this publication.

#### **13.2.1. Metaphase finding and image capture**

While automated analysis stations with walk away reliability for scoring cytogenetic damage is still under development, high-throughput metaphase finders and satellite-scoring stations for computer assisted manual analysis can significantly improve the throughput of a technician. Metaphase-finders assist in locating metaphase spreads on slides and present them in focus, at high magnification ready for analysis [304, 305].

A traditional image-analysis based metaphase finding system may consist of a computer, a high resolution digital camera, a high-quality microscope, an automated stage with autofocus, and a robotic slide-feeder. The computer is loaded with automated metaphase-finding software and interactive automated scoring and annotation software for chromosome aberration analysis. Such metaphase finders can scan up to 150 slides per run for metaphase spreads [300]. As it scans, the results (images and locations of potential spreads) are stored on the centralized server for subsequent automatic relocation at multiple satellite scoring stations for chromosome analysis. Alternatively, virtual high-resolution images of metaphase spreads acquired by metaphase finders can be digitally encrypted and transferred via a virtual private network for downstream remote analysis and assessment. This kind of 'telescoring' needs harmonized scoring criteria to be established to ensure comparable results.

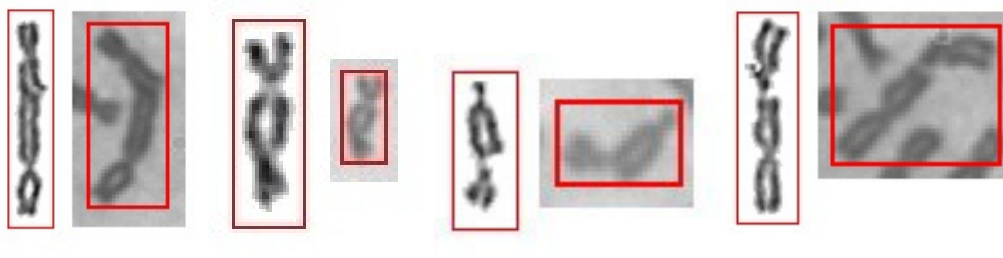
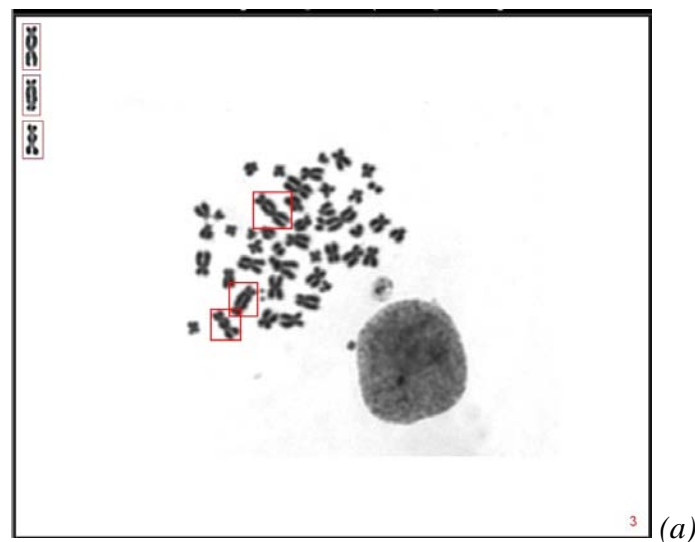
#### **13.2.2. Automation of the dicentric assay**

Microscope analysis of dicentric chromosomes is a time consuming procedure, performed in biological dosimetry laboratories routinely by well trained and experienced scorers, who need to analyse a few hundred cells per day. At low doses a large number of metaphases must be analysed, and therefore, the primary strategy to improve the method is the automation of dicentric scoring to save time, particularly for assessing exposure to low radiation doses.

Several attempts were started in the 1980s to develop automatic scoring systems [306–308]. In the meantime, several commercial systems became available and the corresponding software modules of metaphase finding and karyotyping are now well established in many cytogenetic laboratories. This kind of computer assisted microscopy facilitates the work enormously. At first, a slide will be scanned at low magnification, the metaphases detected and their coordinates stored in an unsupervised operation. During this procedure, a gallery of the detected metaphases can be generated. As the cells are relocated and analysed manually, the individualized electronic scoring sheets are easily maintained, printed and archived as files. In rapid response to a radiation emergency, the observed aberrant cells can be captured manually, digitized and archived immediately. In total, using a metaphase finder the time of scoring might be reduced by a factor of 2 [304].

It has long been realized that the automatic metaphase finding, image capture and other processes should then be followed by electronic image analysis to realize automatic chromosome analysis, including dicentric scoring. There are several steps on the way to perform automatic dicentric scoring. At first, a slide will be scanned by a metaphase finder. In a second step, the detected metaphases will be captured automatically and digitized at a high resolution. Then, the metaphase images will be segmented, to identify the chromosomes and candidate dicentrics. In the 1990s, only the images of the dicentric candidates and their coordinates were stored. Nowadays, with the progress in digital imaging, this procedure became much faster and more efficient. Furthermore the advances in hard disk technology make it possible now to store all the cells of one slide in a high resolution mode.

The experience with dicentric scoring software shows, that it is very difficult, to develop hierarchical multistep algorithms, which allow the segmentation of a complete cell, resulting in 46 chromosomes [308, 309]. In general some chromosomes will not be detected, because they were overlapping or lying close together as chromosome clusters. In consequence, some dicentrics will be missed (false negatives). Also some dicentrics may be systematically ignored, because they are smaller than an X chromosome, which might be the case in less than 8%. The automatically detected candidate dicentrics have to be validated by a trained scorer, but this is a much faster and easier process than manual scoring. The dicentric candidates are displayed marked on the screen (Fig. 44a) and this allows fast evaluation. Most false positives (i.e. artefacts, overlapping chromosomes, see Fig. 44b) can easily be rejected.



*FIG. 44. a) Automatically detected dicentric candidates are identified, which makes evaluation easier and faster. b) False positive dicentric candidates can easily be recognized (i.e. overlapping chromosomes, twisted chromatids or not segmented objects) and rejected.*

Because of the incomplete analysis of the cells (the manual standard is to score only complete cells with 46 centromeres) and the resulting uncertainty, automated dicentric scoring

has not yet been established as a routine method. Furthermore data are needed to decide if the detected frequency of dicentrics should be related to the number of detected chromosomes, or if it may be counted as dicentrics per cell bearing in mind that it is not a constant number of chromosomes being evaluated in every cell. This raises questions about using the dicentric overdispersion index (Section 9.7.4.3) and thus the potential of this method to detect partial body irradiations. Another aspect of interest might be the influence of the preparation quality of the slides. Good metaphase spreads increase the number of detected chromosomes. Here more investigation is needed and may be optimized by software training of the dicentric classifier.

The automatic dicentric detection gives very reproducible results. Comparisons between dose effect curves, established by manual and semiautomatic scoring demonstrate a very good correlation between both methods. The detection efficiency of dicentrics by automated systems has been reported to be about 50–70% [310, 311].

The automation of dicentric scoring has the potential to improve the dicentric assay as a helpful tool to screen large numbers of blood samples in case of a large scale radiation emergency. One workstation can be supplemented by satellite stations, where the cells and dicentrics will be evaluated, which increase the capacity and throughput of the system. For triage mode the automation of dicentric scoring significantly reduces the time for analysis and results correlate well with manual scoring [300, 310, 311].

### **13.2.3. Automated scoring of micronuclei**

Several algorithms for automated image analysis of the CBMN assay were already developed in the 1990s [312, 313]. These systems however showed limitations such as a relative high inaccuracy in classification of the BN cells. More recently, new and better automated image analysis systems for the CBMN assay have been developed. The MN software module integrated in the metaphase finder system MSearch, developed and commercialized by Metasystems (a manufacturer of microscopic imaging systems) automatically identifies by morphological criteria BN cells by the occurrence of two adjacent similarly DAPI stained nuclei. In a second step, MN are counted automatically in a circular area defined around the two nuclei of the BN cell [314, 315] (see gallery of BN cells with MN, Fig. 45). A further evaluation of the detected yield of MN by a scorer is not necessary. It is important to note that, unlike visual scoring criteria for the CBMN assay, the Metasystems software does not use the cytoplasmic boundary to identify binucleated cells but simply assumes that close proximity of 2 nuclei (meeting specified parameters in the software pattern recognition classifiers) is sufficient to accurately identify a binucleated cell; if required the cytoplasmic boundary can be visualized using phase-contrast microscopy as recommended by Eastmond and Tucker (1989) [284] to verify the accuracy of binucleated cell detection.

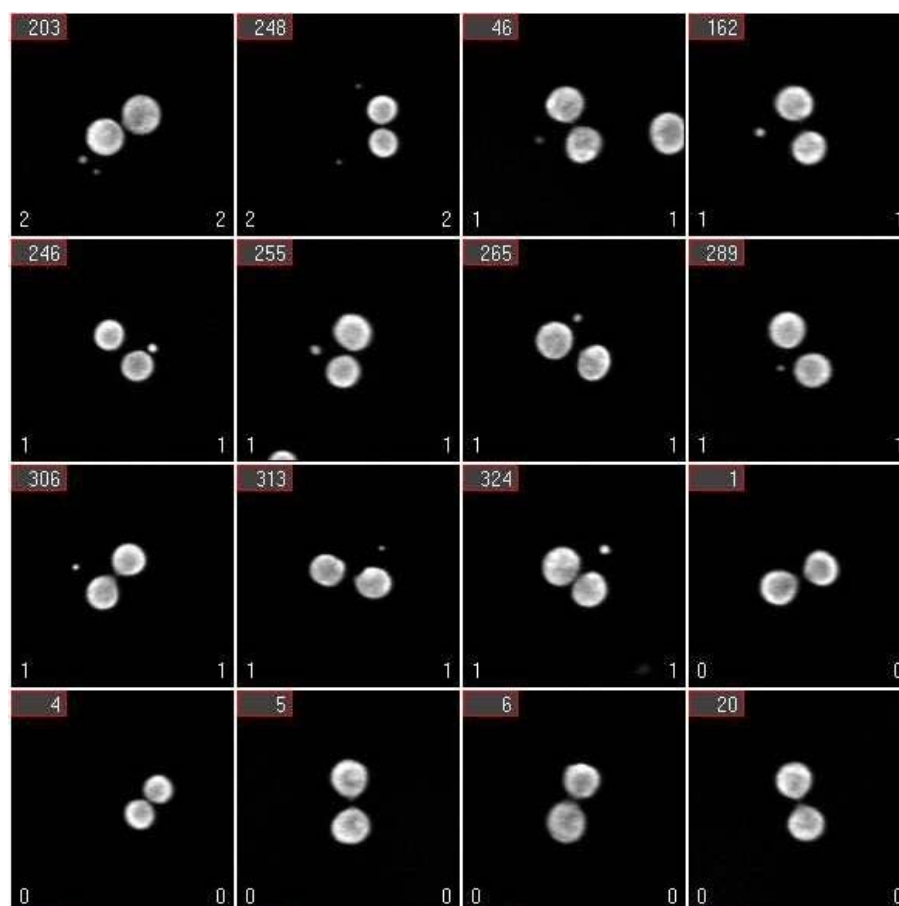


FIG. 45. Gallery of BN cells with and without MN captured by an automated system.

A system developed by Decordier et al. [316] for use in biomonitoring of *in vivo* exposure to mutagenic agents, uses a capture station and two MN analysis workstations. This system identifies firstly the cytoplasm of Giemsa stained cells, then detects the number of nuclei in the cell thus allowing identification of BN cells and in a third step it scores the MN.

A study performed by Willems et al. [87] demonstrated the suitability and advantage of automated MN scoring for population triage in case of large scale radiation emergencies, where it is important to distinguish severely exposed individuals ( $\geq 1$  Gy), who require early medical follow up and treatment, from those less exposed.

The fully automated MN scores obtained in the latter study were highly correlated with the manual MN scores ( $r^2 = 0.917$ ) and demonstrated that a visual validation was not needed [87]. The reference dose response curve obtained for automated MN scoring, based on MN data of 10 individuals, showed that the uncertainty on a dose determination of 1 Gy amounts to 0.2 Gy. The 95 % confidence intervals of the 0 Gy and 1 Gy doses did not overlap. Accurate dose estimations were also achieved at the higher doses of 2 and 3 Gy. Therefore, the MN scoring system is able to distinguish exposures with doses of 1, 2 or 3 Gy. In this study it was estimated that 2 scorers can process at least 60 blood samples (120 slides) in a 12 hours shift.

In general, the number of blood samples analysed can be increased extensively by using more automated working units. Here, a network of trained laboratories with similar equipment and MN classifiers, using standardized fixation protocols can give comparable results. By this means, the throughput of MN automated scoring can be increased to permit a rapid response to a large scale radiation emergency.

#### **13.2.4. Premature chromosome condensation assay**

The approach here is essentially similar to that for dicentric analysis by using automated metaphase finding on Giemsa stained preparations [75]. 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.

#### **13.2.5. FISH based translocation assay**

Some considerable success has been obtained by using FISH staining of 3 or 4 pairs of chromosomes [75]. 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 for candidate dicentrics on Giemsa stained slides [317]. A longer screening time was needed for fluorescence: 1 hour 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 [318]. 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 [319]. Piper et al. [320] 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 [321]. 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.

### **13.3. LABORATORY INFORMATION MANAGEMENT SYSTEM (LIMS)**

A customized, commercially available LIMS can be an indispensable tool for addressing challenges arising from increased sample preparation/analysis throughput. In addition, a LIMS can help to maintain general laboratory records regarding personnel training, instrument calibration and chemical inventory, etc. Electronic data management via LIMS

offers benefits that include the ability to link, search, and retrieve data and to rapidly report results after a radiation disaster. Several modules are available and a brief description follows:

- **Sample identification** — Samples assigned unique bar code identifier.
- **Sample transport** — Structured templates to input data about sample conditions during transport and on arrival (e.g. data from a temperature logger).
- **Test setup** — Assigning to suitable processing and cytogenetic tests for any given sample (e.g. whole blood culture or lymphocytes isolation; dicentric or CBMN assay).
- **Sample scheduling** — Prioritizing sample analysis depending on case urgency and logging cases to specific laboratory personnel.
- **Security** — Requirement of user authentication through passwords. Users may be assigned different privileges within the subsystem.
- **Auditing** — Records and modification are tracked.
- **Archiving** — Keeps the database working efficiently, helps maintain record integrity and ensures that scientific data are securely backed up.
- **Reporting** — Generating formatted individual case reports which can be communicated to the treating physician.
- **Instrument integration** — Data can be automatically collected and collated directly from the component modules reducing the risk of transcription errors. This helps to improve data accuracy and consistency, which are critical during response to a mass casualty event.

A schematic representation of a scalable and high-throughput automated cytogenetic laboratory is shown in Fig. 46 [301, 302].

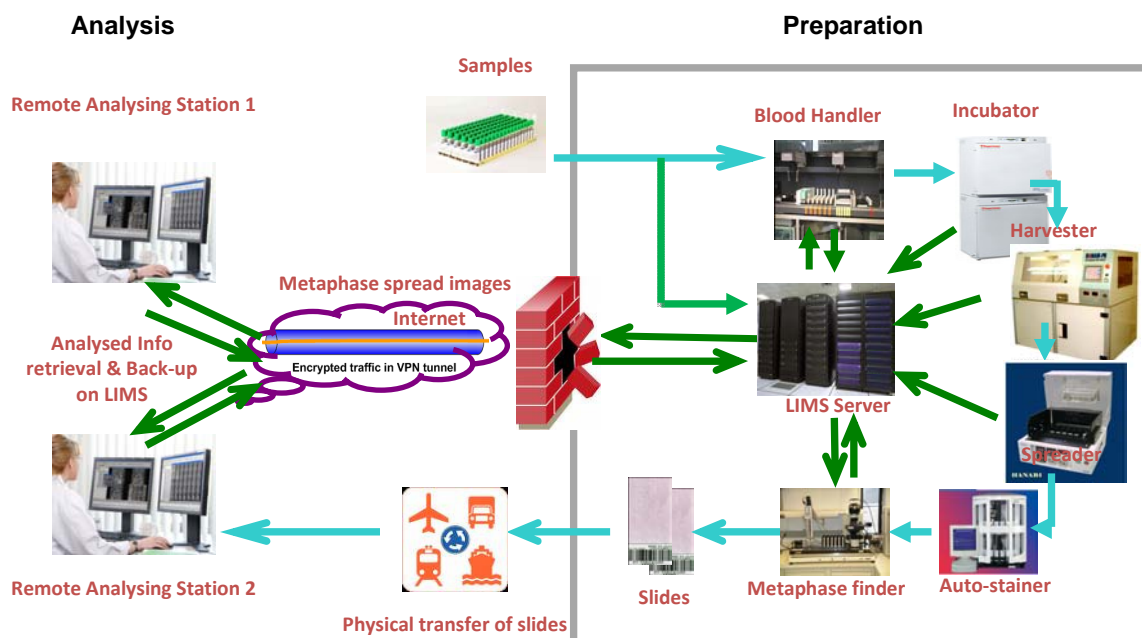


FIG. 46. Schematic representation of a high throughput cytogenetic laboratory and automation with LIMS network. The slides produced in the central laboratory can be physically transferred to scoring stations/laboratories or captured images securely transferred electronically to the receiving laboratories for analysis (courtesy Ramakuma and Prasanna, AFRRRI, USA).





## 14. MASS CASUALTY EVENTS

A mass casualty event is defined as one that involves injury to a sufficient number of individuals such that it exceeds the response capability of the local responders [322, 323]. When this type of event involves radiation, the result can be a large population, who may have received a range of radiation doses spanning from background levels to those large enough to cause medical consequences. These individuals need to be rapidly assessed for exposure levels to determine whether medical intervention is required [4, 322, 324–326].

Events involving radiation can result from accidents or malicious acts, both of which, if they were to happen, may cause casualties within the general public. Confounding factors such as conventional physical injuries could also be present and dealing with life threatening injuries takes precedence over dosimetric and other activities [327].

Planning and preparedness is critical for an effective response to a mass casualty event. In the case of a radiation emergency the generic accepted guidelines include: a) establishment and training of local and national response teams equipped with critical equipment and supplies, b) knowledge and application of appropriate and available diagnostic approach for assessing radiation injury and dose, and c) access to reach-back reference laboratories, including expert laboratories for dose assessment by cytogenetic biological dosimetry [322, 328, 329]. A critical component in the biological dosimetry ‘concept of operations’ is the process to prioritize the selection of samples for rapid cytogenetic triage-dose assessment that requires dynamic communication between the medical responders and reference cytogenetic biological dosimetry laboratory staff.

### 14.1. POTENTIAL RADIATION EXPOSURE SCENARIOS

#### 14.1.1. Malicious Events

A number of possible scenarios for malicious exposure to radiation have been identified and are listed here in three broad categories [322, 324, 326].

- (a) Radiological Exposure Devices (RED) involve sealed sources distributed in an environment but not presenting a contamination threat. Individuals who come close to these sources can receive significant localized doses but numbers of highly exposed individual are anticipated to be low.
- (b) Radiological Dispersal Devices (RDD) use explosive or mechanical devices to distribute radiological material resulting in radioactive contamination. A relatively small area would be affected and radiation exposures could take the form of both internal and external contamination, however exposures are expected to be lower than medically significant.
- (c) Improvised Nuclear Devices (IND) incorporate nuclear material that can produce nuclear explosions. This can cause extensive radiation and thermal injuries with large numbers of fatalities and casualties with high doses of radiation. The result of such an event would be catastrophic.

#### 14.1.2. Accidental Events

Radiation exposures could result from several scenarios including but not limited to [326]:

- (a) Reactor emergencies with a breach of irradiated fuel elements during loss of coolant. These emergencies may result in high doses to workers and general public near the

site and contamination leading to low doses to the general public in the vicinity (e.g. Chernobyl).

- (b) Criticality accidents may occur when sufficient quantities of special nuclear material are inadvertently allowed to undergo fission. This results in high levels of exposure to persons in close proximity (e.g. Tokai-mura).
- (c) Emergencies involving lost or stolen ‘orphan’ sources can result in several exposure scenarios depending on the activity, length of time of exposure and distribution of the source. Such emergencies can result in high doses to the whole body or partial body exposures as well as internal or external contamination (e.g. Goiânia).

## 14.2. HISTORICAL EXPERIENCE

There have been several examples in the recent past where cytogenetic biological dosimetry has been used to assess exposures to radiation after accidental events involving multiple casualties (Table 17).

TABLE 17. SELECTED EXAMPLES FOR USE OF CYTOGENETIC BIOLOGICAL DOSIMETRY IN RADIATION ACCIDENTS INVOLVING MULTIPLE CASUALTIES

Year of accident	Accident location	Number of people involved	Cytogenetic Assay Cases				References
			Dicentrics	PCC	FISH	CBMN	
		>100 000	436				[342, 343, 344]
1986	Chernobyl, Ukraine		1755		97		[235, 236, 347]
						140 <sup>a</sup>	[259, 260, 297]
1986–1987	Lilo, Georgia	11	11		4		[247]
							[248]
1995	Istanbul, Turkey	21	21	10			[349]
					5	10	[158]
1997	Goiânia, Brazil	250	129				[350]
1998	Matkhoji, Georgia	multiple	85				[333]
		43	43				[351]
1999	Tokai-mura, Japan			3			[253]
							[254]
2000	Bangkok, Thailand	multiple	28	28			[352]
2005	Concepción, Chile	233	45		1		[353]
2006	Dakar, Senegal	63	33				[354]

<sup>a</sup> Retrospective

Accidents can have different characteristics such as a sudden recognized event with many identified casualties in a short period of time (e.g. Chernobyl) or a more slowly evolving situation with delayed discovery of exposed individuals (e.g. Goiânia). An accident could

also involve just a few real cases but with tremendous public pressure to extend biological dosimetry to the surrounding community even though there was little to no physical evidence to justify this action (e.g. Tokai-mura). In this case, the cytogenetic biological dosimetry laboratory of the National Institute of Radiological Sciences (NIRS) was able to determine the doses to these 265 concerned individuals through surveys of their location during the event and assure them that no significant dose was received [330]. A cohort of 43 persons at a uranium processing facility, who were confirmed to have been exposed slightly on the basis of measurements of whole-body counting of  $^{24}\text{Na}$ , were also assessed for dose by chromosome aberrations analysis.

Historically, cytogenetic biological dosimetry using dicentric analysis, along with routine leukocyte counting, is used as an initial assay for dose estimates following accidental exposures involving multiple casualties (see Table 17). Other cytogenetic assays (FISH, PCC and CBMN) have been used to confirm dose estimates, however, often this has been performed from months to years after the accident.

### 14.3. ROLE OF BIOLOGICAL DOSIMETRY

#### 14.3.1. Radiation Exposure Assessment Methods

After a mass casualty radiation event, physicians are primarily concerned with preserving life and evaluating medical signs and symptoms for early treatment decisions. Several radiation exposure assessments, evaluated by an international consensus of experts, are applicable for early-phase acute radiation [6, 25, 322, 325–327, 329]. Depending on the radiation scenario and available resources, appropriate radiation assessment methods should be implemented in a mass casualty radiation emergency.

#### 14.3.2. Biological dosimetry concept-of-operations

Generic guidelines for the ‘concept of operations’ for first-responders in a mass casualty radiological incident are well described by IAEA resources [322, 323, 331]. The implementation of a multiparameter biological dosimetry assessment approach in a mass casualty radiation emergency, however, can be a significant confounder without access to expert teams [322, 323]. Fig. 47 illustrates the components of the REAC/TS and AFRR treatment strategy along with the concept of operations for use of multiparameter biological dosimetry [329].

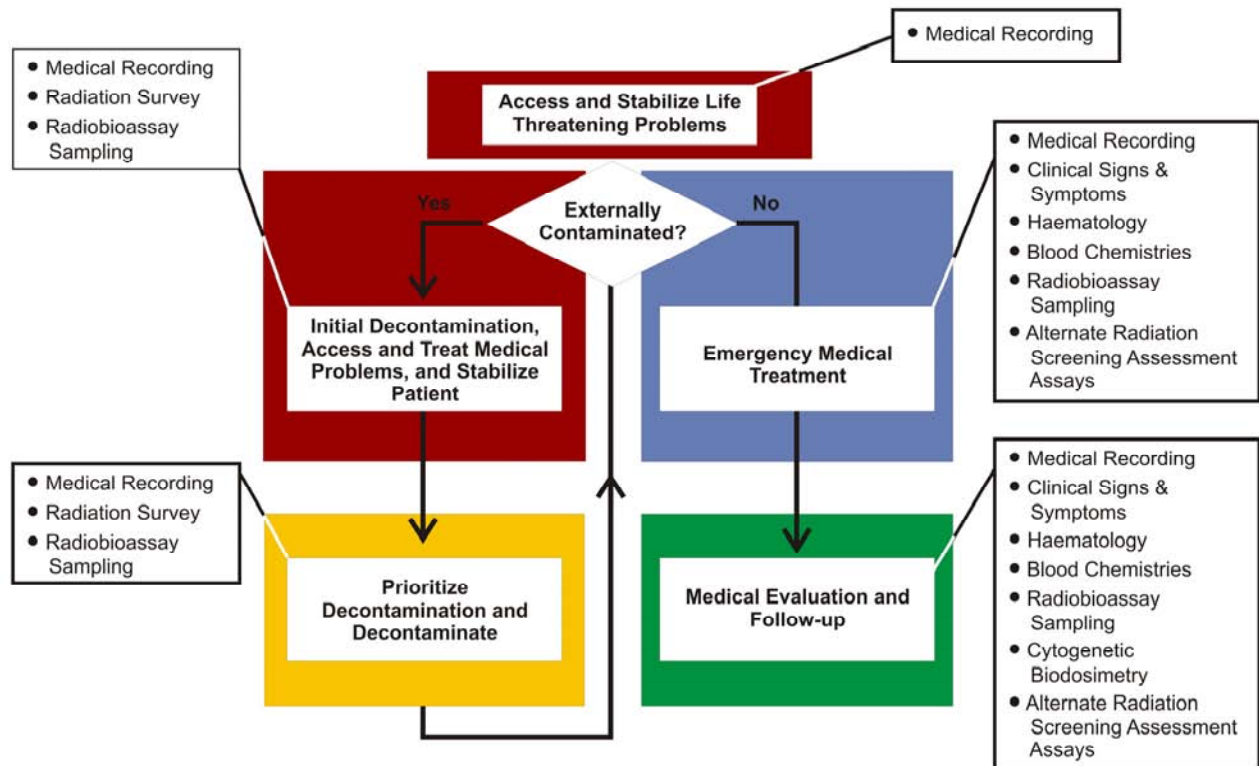


FIG. 47. Biological dosimetry concept of operations during management of radiation emergency with trauma or illness. Biological dosimetry functions are illustrated for the individual action steps of the REAC/TS and AFRRR 'Radiation Patient Treatment' algorithm [329].

Current radiation exposure assessment methods and emerging technologies can offer a potential to contribute in radiation injury and dose assessment response. Research and development are needed to establish a diagnostic triage concept to facilitate a functional biological dosimetry concept of operations in a mass casualty radiation emergency [329]. The initial screening radiation assay must be rapid (1 assay per min or less), use a hand-held device, and ideally involve a self-use test. Secondary and tertiary radiation assay may require more expertise and take longer (>1 day) for use but have higher radiation specificity.

Once identified as potentially exposed, patients may be recommended for biological dosimetry to provide confirmation of the suspected exposure and to determine a dose level. In the early-response phase of a radiation emergency, the initial purpose of cytogenetic triage is to rapidly estimate the dose for each referred patient to supplement this early clinical assessment. Although these first dose estimates may not be extremely accurate, the goal is to quickly place the patient into one of 4 dose ranges (1 Gy to 2 Gy, 2 Gy to 4 Gy, 4 Gy to 6 Gy and > 6 Gy) to provide timely information to the medical community that can be used for patient treatment [4]. At this stage it is also possible to refute false positive samples due to symptoms such as vomiting from other causes. Partial-body exposures may also be identified at this stage.

Once the initial urgency of the requirement for rapid triage dosimetry has passed, those patients identified as having received significant doses can be further analysed to provide more accurate dose estimates.

After the emergency has passed and more accurate dosimetry is complete on those identified as exposed, further follow up will continue on those individuals who received very

low or no doses, but still require reassurance. Also, follow-up epidemiological studies with other techniques such as FISH will be required.

### **14.3.3. Communication with the medical community**

Communication between the medical community and the biological dosimetry laboratories is essential. This should be done taking due regard for medical confidentiality [3, 4]. Any information from the medical community that can assist the biodosimetrists with sample prioritization is extremely helpful. Equally essential is the communication of the biological dosimetry laboratory back to the medical community in a timely manner that will assist them with making decisions on the treatment of the patients. This requirement of continuous communication highlights the importance of robust sample tracking during response to the event. It is essential to have a unique, well established, documented sample coding system (e.g. LIMS described in Section 13.4) such that samples can be tracked from collection, through processing, analysis and reporting back to the medical community. Cytogenetic laboratories work with blinded samples while medical professionals work with names. The laboratory needs to identify individuals who will be accessing information from LIMS or similar documents to communicate to the medical professionals. These individuals will have to break the coding to be able to communicate to the physicians and therefore should probably not be involved in sample scoring.

## **14.4. EXISTING MASS CASUALTY STRATEGIES**

### **14.4.1. Triage scoring**

Rapid triage scoring can be applied to several of the cytogenetic assays used for biological dosimetry. It has been determined that by scoring 50 cells (or 30 dicentrics) in the dicentric assay, dose estimates can be made with sufficient accuracy to provide useful dose estimates to the medical community. It has been shown that this method of scoring will deliver dose estimates within 1 Gy [332, 333]. Compared to full dicentric scoring of 500 or 1000 cells, this triage method increases throughput up to 20 times. To further increase scoring speed, a QuickScan method has been introduced by Flegal et al. in which only the damage in each cell is scored without the requirement of ensuring the presence of 46 centromeres, however, only cells which appear complete are scored. This method of scoring reduces the microscopy time by an additional factor of 6 [334].

For mass casualties, PCC is particularly useful for high dose exposures. The PCC-ring method has been shown to be useful for triage dosimetry of doses above 6 Gy, measuring 300 PCC cells or 50 rings [335]. This assay, however, has limitations for the low dose region.

Triage scoring can also be applied to the CBMN assay. For standard biological dosimetry, it is recommended that 1000 binucleated cells be scored. However, it has recently been demonstrated that scoring 200 BN cells allowed the identification of doses greater than 1 Gy [335]. The time required to score 200 BNC is approximately 15 min which is significantly faster than triage dicentric scoring and still slightly faster than QuickScan. Another advantage of this method is that the expertise and training required for scoring is much less than the dicentric assay so that scorers could be quickly trained in a mass casualty situation.

### **14.4.2. Automation**

Automation has been discussed in detail in Section 13. It is clear that automation will increase throughput and free up human resources for other tasks required during mass casualty events. This can include automation of the blood processing, metaphase harvesting, metaphase finding and dicentric or micronucleus scoring.

### 14.4.3. Networks

Many nations have established reference expert cytogenetic biological dosimetry laboratories. Recently some of these laboratories have established national and regional networks to enhance their capabilities [118, 337, 338]. Others have reviewed individual national resources and capabilities with a view to forming a regional network [339]. United Nations (UN) agencies (IAEA, WHO) that provide international cooperation in biological dosimetry have also established cytogenetic networks [340, 341] (Table 18). There are very few countries with more than one cytogenetics laboratory having the primary function of undertaking biological dosimetry. Nevertheless there may be a lot of cytogenetics expertise in other research institutes and particularly in hospitals' clinical genetics departments. National networks (e.g. France, Korea, Japan, Canada) have implemented arrangements, including training, whereby this expertise can be mobilized promptly under the leadership of the specialist reference biological dosimetry laboratory. The networking, whether national or international, requires a coordination of infrastructure of logistics, data management, and communications. These networks also afford an excellent platform for exercises and inter-comparison studies to ensure suitable performance of individual laboratories and the cytogenetic biological dosimetry networks. Use of cytogenetic networks enhances the capabilities for use of triage and reference dose assessment by cytogenetic analysis for mass casualty radiation events.

TABLE 18. SUMMARY OF EXISTING BIOLOGICAL DOSIMETRY NETWORKS

	Location	Name	Lead	Participants (number or name)	<sup>2</sup> Assays in use
International	Worldwide	Response and Assistance Network (RANET)	IAEA	Continue to change <sup>4</sup>	DCA, FISH, PCC, CBMN
	Worldwide	BioDoseNet	WHO	63	DCA, FISH, PCC, CBMN
	Europe	Tri-Partite	dependent on location of event	<sup>3</sup> UK, France, Germany	DCA, FISH, PCC, CBMN
	Latin America	Latin American Biological Dosimetry Network	<sup>1</sup> Argentina —Nuclear Regulatory Authority and Cuba — Centro de Protección e Higiene de las Radiaciones	Argentina (2), Brazil, Chile, Cuba, Mexico, Peru, Uruguay	<sup>2</sup> DCA, FISH, CBMN
National	Canada	Cytogenetic Emergency Network	Health Canada	4 reference 18 satellite	DCA, FISH, CBMN
	France	Réseau de dosimetry biologique	Institut de Radioprotection et de Sûreté Nucléaire (IRSN)	2 labs from CEA and one from the MNHN	DCA, FISH, PCC
	Japan	The Chromosome Network	National Institute of Radiological Sciences (NIRS)	7	DCA, PCC, FISH
	South Korea	Korean Radiation Biodosimetry Network	Korea Institute of Radiological & Medical Sciences	6	DCA, FISH, PCC, CBMN

<sup>1</sup> With rotating leadership among participant countries every 2 years.

<sup>2</sup> DCA is the technique applied for mutual cooperation purpose; PCC, FISH and CBMN are established in at least some of the network partners.

<sup>3</sup> Co-leads.

<sup>4</sup> Part of broader assistance and response network.





## 15. QUALITY PROGRAMMES AND THE ISO STANDARDS

### 15.1. THE RATIONALE FOR A QUALITY ASSURANCE AND QUALITY CONTROL PROGRAMME

This publication has demonstrated that there are no universally adopted procedures for the cytogenetic assays employed for biological dosimetry. In broad outline laboratories follow similar methods but when fine detail is considered some variations occur in methods which potentially can influence the quality of results. Therefore, it is reasonable to expect each service laboratory to develop a quality programme that ensures the robustness, accuracy and reproducibility of its procedures.

To ensure the quality of a biological dosimetry laboratory's output over extended periods of time, its production process must be solidly based on scientific principles, method validation, and product verification. A complete quality programme provides the strategy for safeguarding the quality of the laboratory's product, whether it is a measurement or a service. Furthermore, these capabilities require periodic comparison with those of other certified or suitably qualified cytogenetic biological dosimetry laboratories, continued stability of the laboratory process, and periodic evaluation of the final product to confirm that it meets predefined specifications.

Operating within the guidance of the documented criteria under an in-house quality assurance programme, periodic peer assessments and documented quality procedures assure stable operation between formal proficiency evaluations.

The in-house quality assurance programme must provide for programme assessments, adequate operational environment, personnel qualifications, procedure manual, instrumentation, calibration, data reduction, record system and data reporting. Control over the cytogenetic process between proficiency evaluations provides another assurance of end products with reproducible quality. Adoption of a total quality management approach would assure continued improvement of operations.

The proficiency tests periodically evaluate measurement consistency with other certified or suitably qualified cytogenetic biological dosimetry laboratories, (see Annex VII) and test the laboratory and its capabilities to verify their ability to produce high quality products and/or services, i.e. dose estimations. An essential element is successful completion of tests within specified limits of accuracy. In addition, this measurement process can be used to verify the quality of a laboratory's service/product output. For the specific area of biological dosimetry, two measurement proficiency testing strategies can be used: 1) samples exposed *in vitro* to a known radiation dose, dose rate, and quality of radiation, are sent to the service laboratory for analysis, and 2) the laboratory engages in an interlaboratory comparison study of samples sent to certified or suitability-qualified laboratories for analysis. In both cases, analyses are carried out and comparisons are made between the value obtained by the laboratory and that obtained by its testing laboratory. The laboratory is then notified of the percentage difference through a report. For direct testing, only the laboratory's measurement capabilities are being tested. On the other hand, when the laboratory assays its own product and also sends an aliquot to the testing laboratory for confirmational and explicit traceability measurements, both the laboratory's analytical processes and measurement capabilities are being tested.

Through the combination of all these quality assurance strategies, the quality and integrity of the laboratory's measurements or services can be assured. Of these strategies, a major emphasis should be placed on strong in-house quality assurance programmes, active and thorough on-site expert evaluations, strict adherence to the documented operational criteria, and laboratory evaluation by 'blind' testing. This combination of checks will assure that the analytical processes will remain in control within specified precision objectives. Although periodic end-product evaluation is a requirement (e.g. between 1 and 3 years), its frequency can be minimal when the analytical processes remain under control.

Quality assurance plans for service laboratories performing biological dosimetry should include the following elements:

- identification and preparation of samples
- validation of procedures or methods
- measurement
- data reduction
- documentation

Systematic actions should be included in the quality assurance plan to provide adequate confidence that a measurement or procedure will be performed satisfactorily.

The International Organization for Standardization (ISO) seemed an appropriate environment to define and write such a set of common rules. The general principles, by which standards are developed within the ISO, are voluntary, consensus and industry-wide. In addition, each standard draft is peer reviewed, by a specialist-working group followed by participating countries via the national representatives of ISO. After publication, each standard may be used directly, or implemented into national standards. The creation of a working group on the standardization of biological dosimetry was proposed in 1998 and accepted by ISO in 1999 within Technical Committee 85, Nuclear Energy, at the level of Subcommittee 2, Radiation Protection. The working group includes 13 specialists from 11 countries, plus a representative of IAEA. ISO 19238 standard, published in 2004, provides Standard Criteria for Service Laboratories Performing Biological Dosimetry by Cytogenetics [3].

## 15.2. CURRENT STRUCTURE OF THE ISO 19238 DOCUMENT [3]

In its current format, the document is divided into 11 chapters and 4 informative annexes.

The main features described in this document address:

- (a) The confidentiality of personal information with respect to:
  - (i) The transmission of confidential data concerning the patient or the overexposure circumstances, from the doctor representing the patient (or the patient him/herself) to the laboratory.
  - (ii) The anonymity of the blood sample and the confidentiality of the results and of the report.
  - (iii) The delegation of confidentiality within the laboratory.

- (b) The potential risks incurred by the laboratory staff during the processing of a potentially infective blood sample. While this problem is not specific to biological dosimetry *per se*, it appeared essential to emphasize, the minimal microbiological, chemical and optical safety requirements.
- (c) The establishment of at least one appropriate calibration curve within the service laboratory is an essential condition for dose estimation. In particular, this curve has to be produced with the same laboratory protocols as used by that laboratory for all its dose assessments. A report must include the experimental conditions of the calibration curve fit, e.g. nature of source and source physical calibration, dose ranges and minimum detection levels.
- (d) While the service laboratory does not control some conditions, such as the quality of blood sample taken and its despatch, the service laboratory must upon receipt provide sound processing of the sample, a dose estimate and, finally, a report that is reviewed and endorsed by a qualified expert.
- (e) Routinely, the laboratory report should reproduce any relevant information provided by the customer since this may influence the interpretation of the findings. All observed aberrations must be listed and interpreted according to the current understanding of mechanisms for radiation-induced chromosome aberration formation.
- (f) Quality assurance plans for service laboratories should comprise in-house procedures to ensure long term accuracy and stability of performance plus periodic peer assessment/cross-calibrations with an external reference programme. It addresses the following broad elements: identification and preparation of samples, validation of procedures or methods, measurement and instrumentation, data interpretation, record keeping and documentation.

### 15.3. APPLICATION TO POPULATION TRIAGE

As already discussed in Section 14 the potential for nuclear and radiological emergencies involving mass casualties from accidents or malicious acts is ever-present. After such event, individuals will be assessed clinically and categorized on the basis of any prodromal signs and symptoms of overexposure plus available information concerning their involvement in the emergency. In this early response phase of a radiation emergency, cytogenetic triage, i.e. the use of chromosome damage to evaluate approximately and rapidly radiation doses received by individuals, is also appropriate in order to supplement the early clinical categorization of casualties.

However as time progresses clinicians would request more accurate estimations of doses, both in the low-dose range on risks of late stochastic effects and also for higher doses for anticipating severe tissue reactions. A secondary cytogenetic inspection should achieve a quantitatively more precise estimate of dose, and also search for any evidence of heterogeneity of exposure.

However, this event can also exceed the resources of the locally involved biological dosimetry laboratory, requiring the intervention of other laboratories within the constitution of a network (see Section 14.5.3). Several biological dosimetry laboratories have independently and successfully performed rapid dose assessment in mass casualty emergencies or exercises. Their approach, essentially based on the

dicentric assay, included preplanning, reagent stockpiling, simplified sample processing, and automation, scoring criteria, and networking with other expert laboratories. Whilst following the principles of ISO 19238 [3], some departures from the exact protocol are needed in order to satisfy the requirement for rapid response and delivery of dose estimations.

Building upon this experience, a new ISO 21243 standard, published in 2008, defined the “Performance criteria for laboratories performing cytogenetic triage for assessment of mass casualties in radiological or nuclear emergencies. General principles and application to the dicentric assay” [4].

The standard is written in the form of procedures to be adopted for biological dosimetry triage where the criteria required for such measurements will usually depend upon the application of the results: medical management when appropriate, radiation protection management, record keeping and medical/legal requirements. For example, selected cases would be analysed to produce more accurate evaluation of high partial-body exposure; secondly, doses would be estimated for persons exposed below the threshold for deterministic effects, by using the ISO 19238 criteria.

The content of the ISO 21243 standard can be summarized as follows:

**(1) Before the event each laboratory is responsible for:**

- (a) Maintaining a stockpile of the required reagents and other laboratory consumables or must be able to immediately access them from a local, state or national stockpile or a commercial supplier.
- (b) Maintaining established communication links with the local/state/federal healthcare facilities.
- (c) Specifying and documenting the responsibilities, roles, and interrelations of all laboratory personnel whose functions affect the quality of emergency biological dosimetry response.
- (d) Knowing its maximum throughput capability for samples processing (time versus number).
- (e) Maintaining its own quality control and quality assurance programme.
- (f) Participating, as appropriate, in relevant educational, training and exercise programmes.
- (g) Participating in periodic interlaboratory comparison studies.

**(2) During the event:**

- (a) The reference laboratory responsible for the dose estimation calls for collaboration of network laboratories when the number of cases to be examined is above its own capacity.
- (b) When the decision to activate the network is made, the reference laboratory becomes the focus for communication between the network. The reference laboratory informs the partners of the circumstances of the incident, and together they establish the extent of cooperation needed.
- (c) Cytogenetic examination for dose estimation is performed at the request of physicians. Selection of cases to be examined is made by discussion between experts in cytogenetic dose estimation, scene managers, and physicians.

- (d) The reference laboratory and the network laboratories discuss the details of work sharing in biological dosimetry.
- (e) An informed consent in written form has to be submitted from each individual or a treating physician, as applicable, prior to blood taking. Special care has to be taken to protect privacy throughout the assignment.
- (f) The reference laboratory organizes the blood sampling and dispatching of specimens to the partners, or designates another suitable agency to take over.
- (g) The results of scoring (and sometimes dose estimation) are reviewed by more than one laboratory, and dose estimation for each person is made based on the reviewed results.
- (h) The associate laboratories send to the reference laboratory the raw data including the aberration distribution data. They also send the dose estimates, adjusted when necessary for dose protraction or heterogeneity, obtained from their own calibration curve most appropriate for the type of radiation involved.
- (i) The reference laboratory receives the results from the network partners and acts as the central point of communication/liaison with the physicians.
- (j) Following review with medical staff some patients may be selected for increased cell scoring in order to improve statistical uncertainties on dose estimates and better discrimination of inhomogeneous overexposure. Such further examination will be made according to the performance criteria described in the ISO standard 19238.

According to these different configurations, the flow chart (Fig. 48) describes the interactions between the reference laboratory, the network and the medical team.

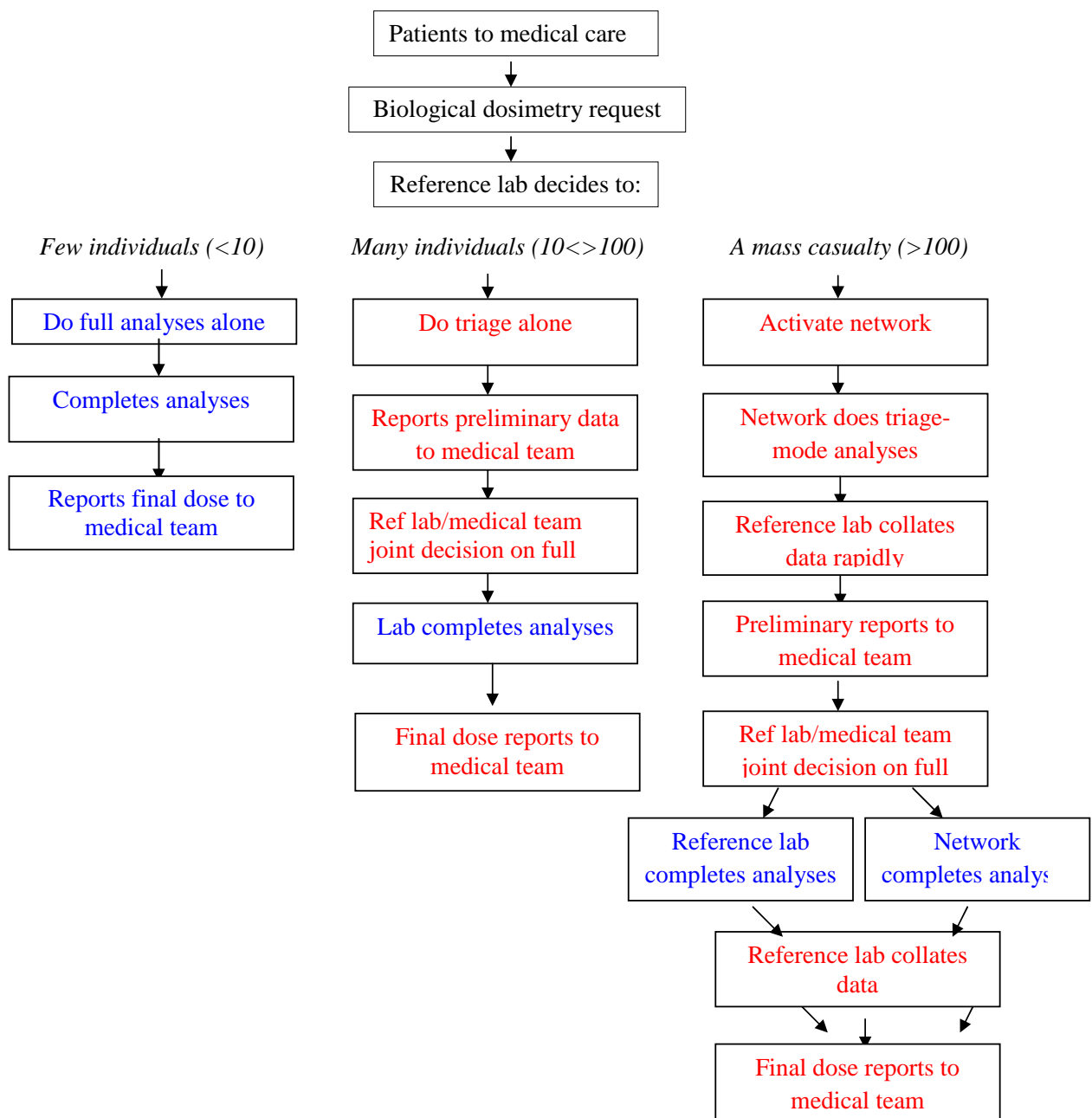


FIG. 48. A flow chart describing the interactions between the reference biological dosimetry laboratory, the network and the medical team, in the context of a dose assessment of few individuals (ISO standard 19238 (blue) [3]) or a mass casualty (ISO standard 21243 (red) [4]) (this figure is reproduced from the ISO standard ISO 21243:2008 with the permission of AFNOR on behalf of ISO. Copyright remains with ISO).

## 16. SAFETY OF LABORATORY STAFF

'Laboratory biosafety' is the term used to describe the containment principles, technologies and practices that are implemented to prevent unintentional exposure to pathogens and toxins, or their accidental release. Global events of the recent past have highlighted the need to protect laboratories and the materials they contain from being intentionally compromised in ways that may harm people, livestock, agriculture or the environment. It has thus become necessary to expand biosafety through the introduction of laboratory biosecurity measures.

'Laboratory biosecurity' describes the protection, control and accountability for valuable biological materials within laboratories, in order to prevent their unauthorized access, loss, theft, misuse, diversion or intentional release. [355]

Considering cytogenetic laboratories, biosafety and laboratory biosecurity are comprehensively presented in the WHO Laboratory Biosafety Manual and the accompanying document Biorisk Management: Laboratory biosecurity guidance, such as: laboratory design and facilities, equipment (i.e. biological safety cabinets), safe working practices, occupational health and medical surveillance, disinfection and sterilization, waste handling, chemical exposures, fire, electrical, radiation and equipment safety. This information is fully applicable and of particular use for operating a cytogenetics laboratory [355, 356].

In addition to guidance documents, staff should conform to their national and institutional legislation or regulations regarding safe and secure working practices in laboratories.

The following are some particular features concerning safety in cytogenetics laboratories that are worth highlighting.

### 16.1. INFECTION

Universal precautions should always be applied and adopted when handling human blood, and all specimens should be regarded as being potentially infectious. Specimens should be unpacked and manipulated in appropriately used, maintained and certified biological safety cabinets.

The use of sharps, e.g. hypodermic needles, should be limited to reduce risk of needle stick injuries. Contaminated sharps should always be collected in puncture-proof containers fitted with covers and treated as infectious waste. Suitable disinfectants should be available to deal with spills and to decontaminate work surfaces and equipment after specimens are processed. All biological waste and used disposable plastic ware should be sterilized, for example by autoclaving, before disposal. If infectious waste has to be removed from laboratories for decontamination and disposal, it must be transported in sealed, leak proof containers according to national and/or international regulations, as appropriate. [356]

If a particular vaccine or toxoid is locally licensed and available, it should be offered after an appropriate risk assessment of possible exposure and a clinical health assessment of the individual have been carried out [356].

### 16.2. OPTICAL

The use of Ultraviolet (UV) light may be necessary for certain procedures.

UV lights are not required in biosafety cabinets. If they are used, they must be cleaned frequently to remove any dust and dirt the germicidal effectiveness of the light. Where UV lights are in use, they must be turned off while the room is occupied, to protect eyes and skin from inadvertent exposure [356].

When UV lamps are used in exposing slides during the FPG staining procedure, shielding and working procedures should be in place to avoid direct irradiation of the skin or eyes of laboratory staff.

Fluorescence microscopes are generally engineered to be inherently safe during normal use.

### 16.3. CHEMICAL

Certain fine chemicals and pharmaceuticals are used routinely in the procedures covered in this publication. For more information about hazardous chemicals and chemical safety see the WHO Laboratory Biosafety Manual, part VI [356, part VI] with its thorough list of chemicals, detailing their hazards and precautions to be used.

When present in cultures or used in staining procedures, chemicals and pharmaceuticals 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 in Table 19.

Storage of chemicals in laboratories should be limited to amounts necessary for daily use. Bulk stocks should be kept in specially designated rooms or buildings.

Chemicals should not be stored in alphabetical order! [356]

**TABLE 19. MAIN REAGENTS OF CONCERN FOR BIOLOGICAL DOSIMETRY LABORATORY AND THEIR INTERNATIONALLY AGREED RISK PHRASES**

Reagent	Risk phrase (R number <sup>a</sup> )
Acetic acid	10; 25
Acridine orange	36; 37; 38
Barium hydroxide	20; 22; 34
Benzylpenicillin	42; 43
Bromodeoxyuridine	20; 21; 22; 46; 61
Calyculin A	23; 24; 25; 38
Colcemid	25; 63
Cytochalasin B	26; 27; 28; 63
DAPI (4',6-diamidino-2-phenylindole)	36; 37; 38
DMSO (Dimethyl sulphoxide)	36; 37; 38



Reagent	Risk phrase (R number <sup>a</sup> )
Formaldehyde	23; 24; 25; 34; 40; 43
Formamide	37; 38; 41; 61
Giemsa stain	20; 21; 22; 40; 41
Heparin	36; 37; 38
Hoechst stain	23; 24; 25; 36; 37; 38
Hydrochloric acid	34; 37
Hypaque	42; 43
Methanol	11; 23; 24; 25; 39
Okadaic acid	23; 24; 25; 38
Pepsin	36; 37; 38; 42
Phytohaemagglutinin	20; 21; 22; 43
Ribonuclease A	20; 21; 22; 38
Sodium hydroxide	35
Streptomycin sulphate	20; 21; 22; 61
Xylene	10; 20; 21; 38

- <sup>a</sup> R 10: flammable  
R 11: highly flammable  
R 20: harmful by inhalation  
R 21: harmful in contact with skin  
R 22: harmful if swallowed  
R 23: toxic by inhalation  
R 24: toxic in contact with skin  
R 25: toxic if swallowed  
R 26: very toxic by inhalation  
R 27: very toxic in contact with skin  
R 28: very toxic if swallowed  
R 34: causes burns  
R 35: causes severe burns  
R 36: irritating to eyes  
R 37: irritating to respiratory system  
R 38: irritating to skin  
R 39: danger of very serious irreversible effects  
R 40: possible risk of irreversible effects  
R 41: risk of serious damage to eyes  
R 42: may cause sensitization by inhalation  
R 43: may cause sensitization by skin contact  
R 46: may cause heritable genetic damage  
R 61: may cause harm to the unborn child  
R 63: possible risk of harm to the unborn child



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## Annex I

### DICENTRIC ASSAY

The variety of materials and methods for making and processing lymphocyte cultures which are commonly used by laboratories around the world have been described and discussed, and probably no two laboratories adopt precisely the same technique. This Annex provides a detailed, step by step description of a reliable method which could be of assistance for some laboratories.

#### 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 culture medium (MEM), commercially available: ready to use, x10 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.
  - (i) 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 100 IU/mL of benzylpenicillin and 100 µg/mL streptomycin sulphate and can be stored frozen.
- (4) 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 µM. The stock can be stored for one month in the dark at 4°C or for several months at -20°C.
- (5) Heat inactivated (56°C for 0.5 hour) foetal calf serum, commercially available and stored frozen.
- (6) Colcemid: stock solution of 10 µg/mL in sterile physiological saline. It can be stored at 4°C for 6 months.
- (7) Sterile culture vessels. There are various options, e.g. glass bacteriology bottles or disposable plastic containers. The volume should be 15 to 20 mL.
- (8) Cultures should be set up in a class 2 microbiological safety cabinet, under subdued lighting. Liquids can be transferred between vessels using sterile disposable syringes or 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 hours.
- (8) Add 50 $\mu\text{L}$  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 a constant rate with vigorous agitation, ideally using a vortex mixer, to prevent the cell button from becoming a solid clump. A further aid to preventing clumping is to use a latex rubber bulb on a Pasteur pipette to gently mix the cell button prior to adding the fixative.
- (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. Melt the frost on the slide with your breath.
- (15) Allow one or two drops of the cell suspension to drip onto the slide from a height of at least 10 cm.
- (16) Prepare at least two such slides from each culture.
- (17) Place the slides to dry in gentle heat over a hotplate.

## I-3. STAINING

### I-3.1. Materials

- (1) Hoechst 33258 stain. A 1000 x concentrated stock solution of 50  $\mu\text{g}/\text{mL}$  in pH 6.8 phosphate buffer can be stored at  $4^{\circ}\text{C}$  in the dark.

- (2) Giemsa stain.
- (3) Phosphate buffer (pH 6.8) made up from commercially available tablets.
- (4) 2 x 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 at room temperature 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. Alternatively, slides can be dried at 37°C and stained with FPG the following day.

#### *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 hour.
- (3) Carefully remove the coverslip.
- (4) Wash well with pH 6.8 buffer.
- (5) Place in 2 x 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

The procedure given here uses both directly and indirectly labelled (commercially available) probes and describes painting three pairs of chromosomes in different colours, all centromeres in a fourth colour and counterstaining the remaining chromosomes. Manufacturers do supply protocols which could be read in conjunction with the method below.

#### II-1.1. Pre-treatment

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-1.2. RNase and pepsin treatment

Mix 445  $\mu\text{L}$  water, 50  $\mu\text{L}$  20 x 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 x 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 water bath at  $37^{\circ}\text{C}$  and put 100  $\mu\text{L}$  onto each slide for 1 to 2 min. Wash with PBS for 5 min at room temperature.

Wash with 50 mM  $\text{MgCl}_2$ -PBS (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 PBS for 5 min at room temperature. Air dry in an ethanol series (70, 90 and 100%) 2 to 5 min each at room temperature.

#### II-1.3. 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 placed in an 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 water bath. Then put on ice for 2 to 3 min, and transfer to a water bath ( $37^{\circ}\text{C}$ ) and incubate for 60 min.

When 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  $85^{\circ}\text{C}$  for 10 min in a water bath, 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 painted: Chromosome #1 (biotin), #4 biotin/FITC, #8 FITC and CP FITC, they will generate red, yellow, green and green colour signal, respectively.

(b) *Pre-hybridization*

Pre-hybridization of slides should be started approximately 30 min before the end of probe competition. Put 100  $\mu\text{L}$  of 70% formamide in 2 x 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 x SSC). The formamide should be deionized shortly before its use.

Denature slides at  $70^{\circ}\text{C}$  for 2.5 min on a 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 air dry.

(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  $42^{\circ}\text{C}$ . This can be extended to two days.

Detection:

- (1) Prepare a wash solution (WS) of 4 x SSC containing 0.05% Tween 20.
- (2) Dilute blocking protein (BP) to 15% (v/v) in WS.
- (3) Use the diluted BP for diluting antibodies as follows:
  - 3.1.1 First layer B3 (1:500), Texas Red Avidin.
  - 3.1.2 Second layer B4 (1:250) biotinylated goat anti-avidin.
  - 3.1.3 F1 (1:200) rabbit anti-FITC.
  - 3.1.4 Third layer B3 (1:500) F2-FITC, goat anti-rabbit IgG.
  - 3.1.5 F2 (1:100).
- (4) Incubate in the dark for 10 min at room temperature, microcentrifuge at 11,000 g for 10 min, and use the supernatant.
- (5) Prewarm the following solutions to  $42^{\circ}\text{C}$ :
  - i) The wash solution.
  - ii) Some 2 x SSC.
  - iii) 50% formamide in 2 x SSC.
  - iv) 0.1% x SSC.
- (6) Carefully remove coverslips in a jar of warmed 2 x SSC.
- (7) Wash the slides in the warmed solutions as follows:
  - i) The wash solution.
  - ii) Some 2 x SSC.
  - iii) 50% formamide in 2 x SSC.
- (8) Put 100  $\mu\text{L}$  of the diluted blocking protein onto each side and overlay with a coverslip, incubate in a moist chamber for 15 to 20 min at  $37^{\circ}\text{C}$ .
- (9) Wash slides with 0.05% Tween 20 in 4 x SSC for 2 to 5 min at  $42^{\circ}\text{C}$ .

- (10) Put 100  $\mu\text{L}$  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.
- (11) Wash slides in 0.05% Tween 20 in 4 x SSC, three times, 5 min each at 42°C.
- (12) Put 100  $\mu\text{L}$  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.
- (13) Wash slides with 0.05% Tween 20 in 4 x SSC, three times, 5 min each at 42°C.
- (14) Put 100  $\mu\text{L}$  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.
- (15) Wash slides with 0.05% Tween 20 in 4 x SSC, three times, 5 min each at 42°C.
- (16) Repeat steps 11 to 14 once.
- (17) Dehydrate slides in an ethanol series of 70, 90 and 100%, 2 to 5 min each at room temperature.
- (18) Allow the slides to air dry.
- (19) Counterstain with DAPI (0.15  $\mu\text{g}/\text{mL}$  in Vectashield mountant), 25  $\mu\text{L}$  per slide under a coverslip.

If all the painting signals are insufficiently bright one may interpose after step 14 another round of the second and third layers. Alternatively if just one of the colours is feint then one may repeat the steps B3 / wash / B4 for Texas Red or F1 / wash / F2 for FITC.





## Annex III

### PREMATURE CHROMOSOME CONDENSATION

#### III-1. PCC BY MITOTIC FUSION

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-1.1. Isolation of human peripheral blood lymphocytes

For the separation of mononuclear cells from anti-coagulated whole blood, a LeucoPREP or, a Ficoll-Hypaque cell separation tube can be used.

##### A. LeucoPREP

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.

- (1) Store LeucoPREP tubes (10 mL) upright at room temperature (18–25°C).
- (2) Collect blood by venipuncture into a heparinized tube.
- (3) Heparin anticoagulated blood should be separated within two hours of blood sampling.
- (4) Add undiluted blood (8 to 10 mL) to each LeucoPREP tube, then centrifuge for 15 min at 400–600 *g* 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 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 100 *g*.
- (7) Repeat step 5 once again.

##### B. Ficoll-Hypaque gradient system

Ficoll-Hypaque 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 ethylenediaminetetracetic acid (EDTA) in every 100 mL.

- (1) Collect blood by venipuncture into heparinized tube.
- (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 400 g at 8–10°C.
- (5) Collect lymphocytes (middle layer) and wash three times (centrifuge at 100 g for 10 min) with 5 mL F-10 culture medium plus 5% foetal calf serum.

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

### **III-1.2. Freezing the isolated lymphocytes**

After the second wash with F-10 and centrifugation, resuspend the cell pellet by gently vortexing and make a cell suspension in 1:1, F-10 + 40% foetal calf serum (FCS): F-10 + 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°C or in liquid nitrogen.

### **III-1.3. Thawing the isolated lymphocytes**

Take the lymphocyte ampoules out of the freezer and put them directly into a water bath (37°C). When they are slightly melted, transfer the whole suspension into a centrifuge tube (10 mL). Add 10 mL cold (4°C) RPMI + 40% FCS onto the lymphocyte suspensions, slowly drop by drop (in about 30 min), then centrifuge for 10 min at 100 g. Resuspend the cell pellet in 5 mL RPMI + 5% FCS. These mononuclear lymphocytes can be used for PCC experiments.

### **III-1.4. 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 (F-10 + 15% new-born calf serum and antibiotics (penicillin 100 IU/mL and streptomycin 100 µg/mL)). Colcemid (0.1 µg/mL) is added to the exponentially growing cells, and mitotic cells are harvested by a standard selective detachment (shake-off) procedure 4 to 5 hours later. CHO cells can also be grown for more than two cell cycles (~32 hours) in complete medium supplemented with BrdU, (final concentration of 5 µM). Mitotic CHO cells obtained will all be differentially stained and look pale in colour following FPG staining. Therefore, lymphocyte PCC will be better differentiated among CHO mitotic cells.

#### **(1) 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°C.

#### **(2) Thawing mitotic CHO cells**

Take the ampoules of CHO mitotic cells out of the freezer and put them into a water bath at 37°C, then transfer the cell suspension into a centrifuge tube and add 10 mL medium. Centrifuge for 10 min at 100 g. Discard the supernatant, add medium (5 mL) and keep them on ice until use.

### **III-1.5. Preparation of polyethylene glycol (PEG) solution**

Put 400 mg of PEG (M.W. 1450, Sigma, 40% w/v) into a small (10 mL) round bottom centrifuge tube and add 600 µL Hank's balanced salt solution (HBSS) or phosphate buffered saline (PBS) or F-10 medium, and leave the tubes in the water bath at 37°C for 15 min. PEG can also be melted first in an oven and then mixed with HBSS or PBS or F-10 medium.

### III-1.6. Cell fusion

- (1) Interphase lymphocytes and mitotic CHO cells are washed once with HBSS or F-10 (5 mL) separately. Centrifuge for 5 min at 100 g then discard supernatant. In a round bottom culture tube, mix interphase cells with mitotic CHO cells (5:1) in 10 mL F-10 medium and centrifuge for 5 min at 100 g (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 0.15 mL PEG and put it directly into the cell pellet, place in a 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) Add 1.5–2 mL F-10 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 100 g.
- (7) Pour off supernatant completely and add 0.5 mL of culture medium (F-10 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 the test tube at 37°C for 1 hour. By this time cell fusion and induction of PCC are completed.

### III-1.7. Fixation protocol

- (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 100 g.
- (3) Discard supernatant until 0.5 mL above pellet. Cells are fixed in 5 mL methanol: acetic acid (3:1).
- (4) Centrifuge the tube for 5 min at 100 g.
- (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-1.8. Slide preparation

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

### III-1.9. Staining protocols

When mitotic CHO cells are not prelabelled with BrdU, slides can be stained with a 3% aqueous Giemsa solution (Gurr Improved R66) for 5 min.

When mitotic CHO cells are prelabelled with BrdU, slides can be stained according to the FPG technique. (Section 9.3.) Finally, rinse slides in distilled water, allow them to dry and then

mount under a 24 x 60 mm coverslip. However, note the caveat in Section 11.2.1.6 that often this is not the preferred staining method and simple Giemsa staining should be sufficient.

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 x 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 under a coverslip. Note that this is slightly different from the method in section 9.3.3, but both methods work.

For the detection of translocations, whole chromosome specific probes together with a pan-centromeric probe can be used following the same protocol as for metaphases (see Annex II and Fig. 37). Then it is possible to detect dicentrics and translocations simultaneously.

### III-2. PCC BY CHEMICAL INDUCTION

#### III-2.1. Using isolated lymphocytes

- (1) Place 3 mL of heparinized whole blood in a LeukoPREP or Ficol-Hypaque tube.
- (2) Centrifuge at 700 g for 15 min at room temperature.
- (3) Transfer isolated lymphocytes into a 15 mL test tube containing 5ml medium supplemented with 20% fetal calf serum for washing.
- (4) Centrifuge at 200–400 g for 10 min at 4°C.
- (5) Resuspend the lymphocytes in 6 mL of culture medium supplemented with 20% fetal calf serum and PHA.
- (6) Incubate at 37°C for 47 hours (an optional step is to add Colcemid, 40 ng/mL, at 24 hours into the culture time).
- (7) Add calyculin A at a final concentration of 50nM into the culture and incubate at 37°C for 1 hour.
- (8) Prepare a warm (37°C) hypotonic solution, 0.075M KCl.
- (9) Centrifuge the cells at 200–400g for 5–10 min and remove supernatant.
- (10) Add 2 mL of 0.075M KCl to the cell pellet and incubate at 37°C for 20 min.
- (11) Add 30 µL of methanol /acetic acid (3:1) and tap the tube.
- (12) Centrifuge at 200–400 g for 5–10 min at room temperature.
- (13) Add 1.8 mL of methanol:acetic acid after removing the supernatant and transfer into a 2 mL tube.
- (14) Store the tube at -20°C until slide preparation.

#### III-2.2. Using whole blood

- (1) Place 0.75 mL of heparinized whole blood in a 15 mL test tube.
- (2) Bring to a total volume of 10 mL by adding culture medium supplemented with 20% fetal calf serum and PHA.
- (3) Incubate at 37°C for 47 hours. (an optional step is to add Colcemid, 40 ng/mL, at 24h into the culture time).

- (4) Add calyculin A at a final concentration of 30nM into the culture and incubate at 37°C for 1 hour.
- (5) Centrifuge at 200–400 *g* for 5–10 min at room temperature.
- (6) Add 5 mL of 0.075M KCl after removing the supernatant and incubate at 37°C for 25 min.
- (7) Add 30 µL of methanol:acetic acid and tap the tube.
- (8) Centrifuge at 200–400 *g* for 5–10 min at room temperature.
- (9) Add 2 mL of 3:1 methanol:acetic acid.
- (10) Repeat steps 8 and 9 until the cell pellet is clear.
- (11) Transfer the cell suspension into a 2 mL tube.
- (12) Store the tube at -20°C until slide preparation.



## Annex IV

### CYTOKINESIS-BLOCK 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 [1] but for routine biological dosimetry purposes whole blood cultures are adequate.

#### IV-1. STANDARD CYTOKINESIS-BLOCK MICRONUCLEUS PROTOCOL

- (1) The blood sample is collected using lithium heparin anticoagulant.
- (2) Typically 0.5 mL of whole blood is added to 4.5 mL of culture medium (RPMI-1640) supplemented with 10 to 15% heat inactivated fetal calf serum, L-glutamine and antibiotics. 100  $\mu$ L of phytohaemagglutinin (e.g. PHA-M, Sigma, 25 mg/25 mL H<sub>2</sub>O) is added to the culture to give a final concentration of 20  $\mu$ g/mL.
- (3) The blood is cultured in tissue culture flasks at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere.
- (4) 20  $\mu$ L cytochalasin-B (Cyt-B) is added to the culture, at 24 hours post PHA stimulation, to give a final concentration of 6  $\mu$ g/mL. This is the optimum concentration for accumulating BN cells in whole blood cultures. As Cyt-B is difficult to dissolve in aqueous solution a Cyt-B stock solution should be prepared in dimethylsulphoxide (5 mg Cyt-B in 3.3 mL DMSO) and aliquoted and stored until required at -20°C.
- (5) The culture is terminated between 68–72 hours post PHA stimulation. The chosen harvest time should maximize the number of BN cells and minimize the number of mononucleated and multinucleated cells.
- (6) The cells are centrifuged gently at 180 g for 10 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 180 g for 10 min.
- (8) The supernatant is removed and replaced with 5 mL freshly made fixative consisting of methanol: acetic acid (10:1) diluted 1:1 with Ringer's solution (4.5 g NaCl, 0.21g KCl, 0.12 g CaCl<sub>2</sub> in 500 mL H<sub>2</sub>O). The fixative should be added whilst agitating the cells to prevent clumps forming. The cells are then centrifuged again at 180 g for 10 min.
- (9) The cells are washed with two to three further changes of freshly prepared fixative consisting of methanol:acetic acid (10:1), this time without Ringer's solution, until the cell suspension is clear.
- (10) After removing the supernatant to 1 cm or less above the cell pellet (depending on pellet size), the cells are resuspended gently, and the suspension is dropped onto clean glass slides and allowed to air dry.
- (11) For light microscope analysis cells can be stained in 2–6% Giemsa (e.g. Giemsa's Azur-Eosin-Methylene blue solution, Merck) in HEPES buffer (0.03M ; pH 6.5) during 10–20 min in the dark, followed by a quick rinse in distilled H<sub>2</sub>O and air dried. For fluorescence microscopy cells can be stained, alternatively, in acridine orange (10  $\mu$ g/mL in phosphate buffered saline pH 6.9) for 2–3 sec.

#### IV-2. MICRONUCLEUS-CENTROMERE STAINING PROTOCOL

For analysing centromeres in MN a commercial pan-centromeric FISH probe can be used. A pan-centromeric probe can also be made by PCR amplification (forward primer: 5'-GAA GCT TAA CTC ACA GAG TTG AA-3' reverse primer: 5'-GCT GCA GAT CAC AAA GAA GTT TC-3') [2].

Below, the *in situ* hybridisation protocol for the commercial probe is given:

- (1) Slides are prepared according to the standard CBMN protocol given above (up to step 10).
- (2) Dehydrate cells by passing the slides through 70–90–100% ethanol series, 2 min each step and air dry.
- (3) Denature slides:
  - i) denature the chromatin on the slide in 70% formamide in 2xSSC for 2min at 70°C;
  - ii) immerse slides in ice cold 70% ethanol and dehydrate through 70–90–100% ethanol series for 5 min each while shaking.
- (4) Denature probe just before use:
  - i) warm probe to 37°C for 5 min;
  - ii) denature the probe at 85°C for 10 min (10 µL/slide);
  - iii) vortex and spin down quickly;
  - iv) immediately chill on ice and keep in the dark.
- (5) Hybridisation:
  - i) apply 10 µL of probe to the slide, put on a coverslip and seal with rubber cement;
  - ii) hybridize overnight at 37°C in the dark in a humidified chamber.
- (6) Post hybridization wash:
  - i) remove the rubber cement and briefly dip slides into 50% formamide and shake off the coverslip;
  - ii) wash slides in 2X SSC for 5 min at 37°C;
  - iii) wash slides 2 times in 50% formamide at 37°C for 5 min each;
  - iv) wash slides in 2X SSC for 5 min at 37°C;
  - v) wash in Tween washing solution (0.05% in 2xSSC) for 5 min at 37°C;
  - vi) add a drop of DAPI/antifade mountant on a coverslip and put on the slide.
- (7) Slides can be stored at room temperature in the dark or scored immediately under a fluorescent microscope.

#### IV-3 THE ISOLATED LYMPHOCYTE CYTOKINESIS-BLOCK MICRONUCLEUS CYTOME (CBMN Cyt) ASSAY

The detailed protocol for the CBMN Cyt assay has been published recently [1]. For a comprehensive photographic gallery of the various cell types scored in the CBMN Cyt assay see Fenech et al. [3].



#### IV-4 CALCULATING ERROR ON NDI FOR THE CBMN ASSAY

The formulae for calculating the NDI and variance on the NDI (for micronucleus, MN, assay) that are given in Section 12.4.3 is as follows:

$$NDI = \frac{(M_1 + 2M_2 + 3M_3 + 4M_4)}{N} \quad (IV-1)$$

$$\text{var}(NDI) = \sum_{i=1}^4 M_i^2 \text{var}(M_i) + 2 \sum_{i=1}^4 \sum_{j=i+1}^4 M_i M_j \text{cov}(M_i, M_j) \quad (IV-2)$$

In Table IV-1 a worked example for calculating the NDI and variance are presented.

TABLE IV-1. DISTRIBUTION OF MICRONUCLEI

N	Number of cells with 1, 2, 3 or 4 micronuclei				NDI
	1	2	3	4	
500	169 ± 111.878	239 ± 124.758	48 ± 43.392	44 ± 40.128	1.934

In Table IV-1, the numbers of cells with 1, 2, 3 or 4 micronuclei from a total of 500 cells is presented. The NDI is calculated according to Eq. (IV-1) above:

$$NDI = (169 + 2 \times 239 + 3 \times 48 + 4 \times 44) / 500 = 1.934$$

The values of variance on each value are calculated using the binomial equation (Eq. IV-3):

$$\text{var}(M_i) = N(M_i / N)(1 - (M_i / N)) \quad (IV-3)$$

So for  $M_1$ :

$$\text{var}(M_1) = 500 (169 / 500) (1 - (169 / 500)) = 111.878$$

NB: It should be noted that all the figures given here are calculated in Microsoft Excel with each value correct to a large number of decimal places. However the values presented in the text are rounded to the third decimal place for convenience, and thus using a calculator with the presented values will not yield exactly the same results.

To calculate  $\text{var}(NDI)$ , one must first calculate the sum of the square of each value of  $M_i$  times its variance:

$$\sum_{i=1}^4 M_i^2 \text{var}(M_i) \quad (IV-4)$$

However, because the covariance can only correctly be calculated from the total number of cells with  $M = 1$  to 4, one must replace  $M$  in the equation with the following values of  $M'$ :

TABLE IV-2. CALCULATED VALUES OF  $M_i'$  AND  $\text{VAR}(M_i')$

N	Values of $M_i'$ for Eq. (IV-2)			
	1	2	3	4
967	169 ± 139.464	478 ± 241.719	144 ± 122.556	176 ± 143.967

The values of  $M$  have been recalculated so that  $M_1' = 1 \times 169$ ;  $M_2' = 2 \times 239$ ;  $M_3' = 3 \times 48$  and  $M_4' = 4 \times 44$ . The value of  $n$  is the sum of these components, which is calculated as follows:

$$n = (169 + 2 \times 239 + 3 \times 48 + 4 \times 44) = 967$$

The values of variance are recalculated according to Eq. (IV-3), but using the new values of  $M_i$  and  $n$ , for example:

$$\text{var}(M_2) = 967 \times (478 / 967) \times (1 - (478 / 500)) = 241.719$$

The values of  $M_i$  and  $\text{var}(M_i)$  from Table IV-2 can then be used to calculate the first part of the  $\text{var}(\text{NDI})$ , as given in Eq. (IV-4):

$$\begin{aligned} & \sum_{i=1}^4 M_i^2 \text{var}(M_i) \\ &= M_1^2 \times \text{var}(M_1) + M_2^2 \times \text{var}(M_2) + M_3^2 \times \text{var}(M_3) + M_4^2 \times \text{var}(M_4) \\ &= (169^2 \times 139.464) + (478^2 \times 241.719) + (144^2 \times 122.556) + (176^2 \times 143.967) \\ &= 66\,212\,947.630 \end{aligned}$$

Next, according to Eq. (IV-1), one must find the covariance of each data set. This is calculated according to the formula:

$$\text{cov}(M_i, M_j) = -np_i p_j \quad (\text{IV-5})$$

In this equation,  $p_i$  and  $p_j$  are the probability of observing each number of micronuclei in the binucleated cells, so for  $M_1$  to  $M_4$ , the probability is calculated as follows:

$$\begin{aligned} p_1 &= 169 / 967 = 0.175 \\ p_2 &= (2 \times 239) / 967 = 0.494 \\ p_3 &= (3 \times 48) / 967 = 0.149 \\ p_4 &= (4 \times 44) / 967 = 0.182 \end{aligned}$$

So the covariance of  $M_1$  and  $M_2$  is calculated according to Eq. (IV-5):

$$\text{cov}(M_1, M_2) = -967 \times 0.175 \times 0.494 = -83.539$$

The values of covariance must then be similarly calculated for every set of  $M_i, M_j$ :

$$\begin{aligned} \text{cov}(M_1, M_3) &= -967 \times 0.175 \times 0.149 = -25.166 \\ \text{cov}(M_1, M_4) &= -967 \times 0.175 \times 0.182 = -30.759 \\ \text{cov}(M_2, M_3) &= -967 \times 0.494 \times 0.149 = -71.181 \\ \text{cov}(M_2, M_4) &= -967 \times 0.494 \times 0.182 = -86.999 \\ \text{cov}(M_3, M_4) &= -967 \times 0.149 \times 0.182 = -26.209 \end{aligned}$$

Following this, the individual components of the second half of Eq. (IV-2) must then be calculated. For example, for  $i = 1$  and  $j = 2$ :

$$M_1 M_2 \text{cov}(M_1, M_2) = 169 \times 478 \times (-83.539) = -6748429.704$$

Similarly, so that the sums can be made from  $i = 1$  to 4 and  $j = i+1$  to 4:

$$\begin{aligned} M_1 M_3 \text{cov}(M_1, M_3) &= 169 \times 144 \times (-25.166) = -612\,451.806 \\ M_1 M_4 \text{cov}(M_1, M_4) &= 169 \times 176 \times (-30.759) = -914\,897.142 \\ M_2 M_3 \text{cov}(M_2, M_3) &= 478 \times 144 \times (-71.181) = -4\,899\,528.670 \\ M_2 M_4 \text{cov}(M_2, M_4) &= 478 \times 176 \times (-86.999) = -7\,319\,049.001 \\ M_3 M_4 \text{cov}(M_3, M_4) &= 144 \times 176 \times (-26.209) = -664\,238.196 \end{aligned}$$

Once all the individual components have been calculated, these can be summed as per the second half of Eq. (IV-2), to give a total of -21 158 594.519. According to Eq. (IV-2), the variance on the NDI is thus:

$$\text{var}(\text{NDI}) = 66\,212\,947.630 + 2 \times (-21\,158\,594.519) = 23\,895\,758.592$$

To convert this to a normalized value of standard error, for presentation with the value of NDI, the following equation is used:

$$SE(NDI) = \frac{\sqrt{\text{var}(NDI)} / \sqrt{n}}{n} = \frac{\sqrt{\text{var}(NDI)}}{n^{3/2}} \quad (\text{IV-6})$$

Using the values calculated above, this gives a standard error of:

$$SE(NDI) = (23\,895\,758.592)^{(1/2)} / 967^{(3/2)} = 0.163$$

Thus the calculated value of NDI using the data presented in Table IV-1 is  $1.934 \pm 0.163$ .



## Annex V

### CRITERIA FOR DETERMINING MITOTIC INDEX

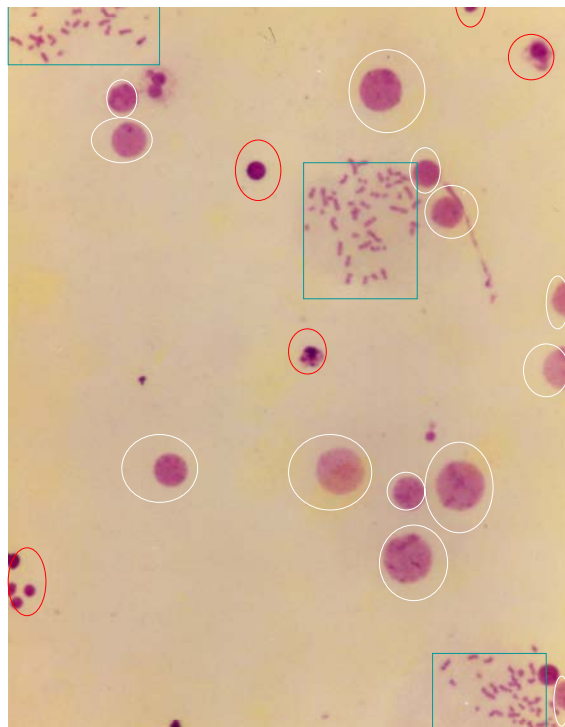
The procedure for determining the mitotic index for the dicentric assay is to:

- Exclude nuclei from polymorphonuclear cells, unstimulated cells (small nuclei), dead or dying cells and micronuclei.
- Count the number of nuclei from mitotic cells and stimulated cells (blast cells with large nuclei) and use Eq. (V-1) to calculate the mitotic index in stimulated cells.

Given the range in sizes of nuclei from stimulated cells, an arbitrary cut-off has to be established between small stimulated nuclei and unstimulated nuclei. 'Metaphase spreads' would include prophases and anaphases.

$$\text{Mitotic Index} = \frac{(\# \text{metaphases}) \times 100}{\# \text{metaphases} + \text{blasts}} \quad (\text{V-1})$$

In Fig. V-1, the mitotic index would be  $(3/(3+12)) \times 100 = 20\%$ , although typically 500 cells are counted for a full mitotic index analysis.



*FIG. V-1. A low magnification view of a typical lymphocyte culture slide. White circles are nuclei counted as blasts, red circles are nuclei which are not counted, boxes are metaphase spreads.*



## Annex VI

### STATISTICAL ANALYSIS

Examples of calculations using statistical procedures for the analysis and interpretation of cytogenetic biological dosimetry data have been given earlier in this publication, notably in Sections 8 and 9. There is a wide selection of statistics text books available, some aimed specifically towards biological and biomedical applications. Therefore this publication does not set out to cover statistics in great detail. However in this Annex a brief introduction is given to the statistical tests and distributions most frequently encountered in the field of cytogenetic biological dosimetry. Part 3 of this Annex provides a software routine for dose response curve fitting.

#### VI-1. BASIC STATISTICAL METHODS FOR CYTOGENETICS

##### VI-1.1. Standard error and standard deviation

The standard deviation (SD) of a set of data is simply a measure of the average dispersion (distance) of the numbers from their mean value. It gives an indication of how widely spread the values in the data set are.

The standard error in the mean (SEM) is a measure of how far the sample mean is likely to deviate from the true population mean. It is equivalent to the estimated standard deviation of the error in the method. The SEM quantifies how accurately the true mean of the population is known. The SEM gets smaller as sample size increases, because the mean of a large sample is much more likely to be closer to the true population mean than the mean of a small sample.

##### VI-1.2. p values

The p value represents the probability of obtaining a result at least as extreme as a given data point, assuming that the data point was the result of chance alone. For example, given a null hypothesis that two population means are identical, a p value of 0.03 would represent a 3% chance of observing a difference as large as the measured difference if the null hypothesis was true. Random sampling from identical populations would lead to a difference smaller than measured in 97% of experiments and larger than measured in 3% of experiments.

For statistical tests, if  $p >$  the significance level (often 0.05), the data do not depart significantly from the expected model and thus the null hypothesis cannot be rejected. It is important to note that, in the above situation, it is impossible to conclude that the null hypothesis is true, just that either 'The null hypothesis can be rejected' ( $p < 0.05$ ) or 'The null hypothesis is not significantly not true' ( $p \geq 0.05$ ).

For multiple comparisons, the p value must be amended as follows: For a number of independent null hypotheses N, the probability of obtaining one or more p values less than the threshold,  $t = 0.05$ , by chance is  $100(1.00 - 0.95^N)$ . The threshold required to ensure that the overall risk of incorrectly rejecting a true null hypothesis is  $\leq 0.05$  is  $1.00 - 0.95^{(1/N)}$ .

##### VI-1.3. The chi-squared test

The chi-squared test (the residual deviance or residual sum of squared deviations (Pearson  $\chi^2$ )) is used to evaluate statistically significant differences between proportions of normally distributed results. The p value for  $\chi^2$  (with the associated number of degrees of freedom) gives the probability that differences between the results are due to chance. It is usual to set the significance level at 95%, which means that for a normally distributed set of data, we would only expect this degree of variation 5% of the time.

The chi-squared test for homogeneity allows comparison of a number of measurements, testing the null hypothesis that the relative frequencies of observed events follow the chi-squared distribution. In cytogenetics, the chi-squared test for homogeneity is used to test for differences between a number of sets of data, for instance observed numbers of dicentrics in scored cells, to determine the number of distinct populations within the data set.

In general, the chi statistic is only reliable for sample sizes greater than  $\sim 5$ . For smaller sample sizes, the Yates correction can be applied in order to reduce the error introduced by approximation of the data to the chi-squared distribution. Effectively, the correction reduces the chi-squared statistic and thus increases the associated p-value. However the applicability of the correction varies and the correction factor may be too great, thus caution is advised in its application.

In the special case of comparison of two samples, the data are expected to be distributed binomially. In this case,  $\chi^2$  is calculated using the normal approximation to the binomial distribution, this is the chi-squared test for one degree of freedom. A binomial version of the chi-squared test can be used to compare a single set of observed and expected counts, for example the number of dicentrics in a control, unexposed, blood sample to the number in an exposed sample.

#### **VI-1.4. The t-test**

The t-test is a statistical hypothesis test for which the null hypothesis is true if the test statistic,  $t$ , is t-distributed. The test is valid for small samples, for which the population cannot be specified as normally distributed because the population standard deviation is uncertain. The t-test takes the effect of chance into account, by incorporating information about the number of samples. In cytogenetics, the t-test is usually used to test for the significance of a difference between the two Poisson counts, comparing the means to determine whether the two sets of data have come from the same population. Again, the p value is used to identify whether differences between samples are significant and it is usual to set the significance level at 95% or 0.05.

There are a number of different forms of the t-test, which are valid in different situations. The paired t-test is used for samples with direct dependence. An example of this would be numbers of dicentrics scored by two different scorers on the same set of slides. For the paired t-test, the size of the two samples, e.g. the numbers of cells scored, must always be equal. The unpaired t-test is for independent sets of data, for example numbers of dicentrics scored by two different scorers on two different sets of slides. In this case the sample sizes may be the same or different. T-tests may be one or two sided. A one sided test is used to determine whether one sample is significantly larger than a second sample. A two sided test is used to determine whether differences between data sets are significant in either direction, i.e. sample one is larger or smaller than sample two.

#### **VI-1.5. The F-test**

The F-distribution is a continuous probability distribution which is equivalent to the ratio of two chi-squared distributions. An F-test, based on this distribution, can thus be used to compare data to see whether they come from the same distribution. The F-test or z-test can be used to test for significance of the coefficients produced by curve fitting by maximum likelihood. In the case where there is evidence of lack of fit (e.g. from the  $\chi^2$  test), the t-test should be used to test for significance of the coefficients. In contrast to the t-test, which is used to compare means, the F-test compares variances of data sets. The most common use of the F-test is in analysis of variance testing.



## **VI-1.6. ANOVA**

Analysis of Variance refers to a collection of several methods which are used to test equality of means. ANOVA uses the F-distribution to test for differences among three or more independent, normally distributed groups, with homogeneity of variances, or between repeated measurements. ANOVA evaluates the importance of one or more factors by comparing the response variable means at the different factor levels.

The p value for each factor describes the probability that the high variance among the groups compared to the variation within groups, is by chance. The p value can be thought of as the probability that random sampling would result in means as far apart (or more so) as observed in the experiment.

In cytogenetics, ANOVA may be used in any circumstance when comparison of three or more groups, or two or more factors, is required. This may be, for instance, to test for the combined effects of radiation dose level and dose fractionation or of radiation and chemical exposure. There are a large number of different forms of the test but most commercially available data manipulation packages have ANOVA capabilities and further guidance can be found in statistical texts.

Although in principal, ANOVA is a parametric method of analysis, which can usually only be applied to Normal data, the type of data most frequently encountered in cytogenetics (i.e. Poisson distributed), approximates the Normal distribution in a manner sufficient to ensure that ANOVA can be applied. Alternatively, a large number of non-parametric analyses are available, as discussed below.

## **VI-1.7. Non-parametric tests**

In cases where the condition of Normality cannot be met, non-parametric tests can be applied. The Wilcoxon test is a non-parametric test that is analogous to the paired t-test. It can be used to compare one or two sets of data. The test is a signed rank test, and as such requires that data are measured at repeated intervals. The test statistic looks for equality of population medians. For independent samples, the Mann Whitney test can be used. This is the non-parametric version of the t-test which can be used to test whether two sets of unpaired data come from the same distribution. For comparisons of multiple data sets, the Kruskal Wallis test is an extension of the Mann Whitney test which is analogous to ANOVA.

## **VI-2. STATISTICAL DISTRIBUTIONS**

There are several forms and classes of distributions that can be used to model the probability of occurrence of events. The type of distribution chosen is very important for accurate data analysis, and several models have been proposed and implemented in assessment of cytogenetic data. A selection of the most commonly used models, and their applicability to radiation cytogenetics, are discussed below.

### **VI-2.1. The Poisson distribution**

The Poisson is a discrete probability distribution which expresses the probability of occurrence of rare random events. The Poisson distribution is by far the most widely recognized and commonly used type of distribution for cytogenetic data analysis. Chromosome aberration data are usually fairly small in number, and Edwards et al. showed that it is much more realistic to assume that chromosome aberrations follow the Poisson distribution than the Normal distribution [4]. Merkle showed that Poisson-based goodness of fit tests, including the  $\chi^2$ , variance and u-test that are all discussed in this publication, were shown to be applicable for cytogenetic data, particularly in the case of large sample sizes [5]. For curve fitting, regression analysis has been shown to be applicable for Poisson data. The resulting forms of maximum

likelihood [6] and/or weighted least squares [7] fitting are now almost universally used for creating dose based calibration curves for chromosome aberrations, such as dicentric or micronuclei.

### **VI-2.2. Binomial distribution.**

The binomial distribution is a discrete probability distribution which describes the probability of the number of successful outcomes from a sequence of independent experiments, each with one of two possible outcomes. In each case, if outcome 1 has an associated level of probability of  $p$ , outcome 2 will have probability  $1 - p$ . In cytogenetics, a good example of a set of data that can be modelled with this distribution is counting numbers of damaged cells, where the two 'binomial' outcomes are a cell is either damaged, or intact. Indeed, the binomial distribution is often used to calculate standard errors associated with yields of damaged cells.

### **VI-2.3. The mixed Poisson model**

Sasaki [8] presented a method of analysis for chromosome aberration data, in an attempt to deal with the problems of inappropriate estimation of average dose which result from inhomogeneity. The cell population consists of a mix of sub populations, each exposed to a different dose, causing a different amount of damage. The distribution of chromosome damage in cells can therefore be expressed in terms of a mixed Poisson distribution, and 'unfolding' of this creates a dose distribution profile. The model was demonstrated to provide adequate fits for the linear-quadratic dose response for simulated and real data.

### **VI-2.4. The negative binomial distribution**

Like the Poisson distribution, the negative binomial distribution is a discrete probability distribution, however the negative binomial has an additional parameter which can be used to represent overdispersion. As the overdispersion parameter tends to 0, the negative binomial tends to Poisson [9]. The negative binomial distribution has been used by several authors in place of the Poisson, for example in a 2008 study of frequency of translocations in airline pilots [10].

### **VI-2.5. The Neyman type-A distribution**

The Neyman distribution was first proposed in 1939 by Neyman, who introduced this new class of distribution to be used to test the difference between means of two samples with different variances. This is in contrast to other standard test such as the z-test and t-test, for example, which are based on normally distributed data with known and unknown population standard deviations respectively, and for which variance must be similar if not identical. The Neyman type-A distribution tends towards the generalized Poisson distribution with increasing sample size [11]. In 2008, Morand et al. published a technical note describing the NETA computer program, which can be used to calculate the 95% confidence limits of Neyman type A distributed events [12]. Morand and colleagues found that the confidence limits calculated using the Neyman distribution were smaller than those calculated using the traditional Poisson-based method for low sample sizes (numbers of cells) [12].

### **VI-2.6. Other distributions**

The Beta distribution defines a family of continuous probability distributions, which are defined on the interval 0–1 by two shape parameters, usually referred to as  $\alpha$  and  $\beta$ . The Dirichlet distributions are an extension of the Beta distribution for multiple ( $>2$  parameters). Stiratelli et al. [13] compared the Poisson and Binomial distributions for chemically induced chromosome damage with the beta-binomial, negative-binomial and correlated-binomial distributions. In contrast to the Poisson and simple Binomial distributions, these models do not rely on independence of cellular response. The authors found that all the Beta distribution based

models showed improved fits with respect to the Poisson and Binomial models (as tested by the  $\chi^2$  test). The Beta-binomial model provided the best fit with respect to the author's data set [13].

The log-normal distribution was formally described by Aitchison and Shen in 1980. Logistic transformation of a d-dimensional normal distribution produces a log-normal distribution over the d-dimensional simplex. This distribution can be applied in statistical diagnosis where classification of the basic cases is subject to uncertainty, such as chromosomal aberration data. The authors give examples of usage, for instance in the direct statistical description and analysis of compositional and probabilistic data and also as a substitute for the Dirichlet conjugate prior class in the analysis of multinomial and contingency table data [14].

### VI-3. A ROUTINE FOR FITTING DOSE RESPONSE CURVES

Curve fitting software has been described in Section 8.3. In this Annex a worked example is presented using one of the software options, the R-based tool, applied to the  $^{60}\text{Co}$  data shown in Table 4. Whereas CABAS and Dose Estimate are available as ready-to-use packages, the R procedure needs a routine to be written by a mathematician. The required routine has been composed (by H. Braselmann) and is presented here in Box 1 in full because it has not been published elsewhere.

The routine has four parts. The first is used to input the observed data i.e., doses, numbers of aberrations, number of cells scored and the distribution index (disp). For this index there are two options; either to use a constant value for every dose point or to ascribe a separate value to each dose. In the worked example a constant value of 1.0 is used. The alternative, which is also shown, would be to use the individual  $\sigma^2/y$  values shown in Table 4. (Note that all lines in the routine starting with a '#' symbol are for information only and will not run). The next part is for entering optimal settings; i) the sigma correlation coefficient, for which it is recommended to use the value 1 or else to estimate this coefficient; ii) the required weight and; iii) the function that one wishes to fit. For this, enter either '1' for a linear fit or 'lq' for linear quadratic. The remaining two parts of the routine should only be modified by developers of the script. If one wishes to fit data to the linear dose response function, the data that would be entered using the  $^3\text{He}$  data in Table 4 are shown beneath Box 1. Thereafter the routine is identical to that shown in Box 1.

To run the routine one should download the R programme from the internet site (see Section 8.3). Using a PDF version of this publication, copy and paste directly into a word processor software the routine shown in Box 1. Replace the  $^{60}\text{Co}$  worked example input data with your own data and select your desired options such a '1' or 'lq'. With the R programme on your screen, paste in the routine after the symbol >.

The output is shown in Box 2 where x0, x1 and x2 are, respectively, the C,  $\alpha$  and  $\beta$  coefficients as shown in Eq. (2) together with their standard errors. The z value is a test of the significance of each coefficient with its probability (Pr). Also shown are the variance and covariance values for each coefficient. It may be noted that the values of the coefficients are identical to those shown in Table 5 and the variance / covariance values with those shown in Section 9.7.3. The R output also presents the data points and the fitted curve as a graph (Fig. VI-1).

## BOX 1. THE CURVE FITTING ROUTINE APPLIED AS A WORKED EXAMPLE TO <sup>60</sup>CO DATA

```

## latest changes: H. Braselmann, 2010, April 9th
##           Helmholtz Zentrum München, Department of Radiation Cytogenetics, Germany
## contact details: braselm@helmholtz-muenchen.de
## user part: data
# cobalt-60 gamma (86)
dose<-c(0,0.1,0.25,0.5,0.75,1,1.5,2,3,4,5)
ab<-c(8,14,22,55,100,109,100,103,108,103,107)
cells<-c(5000,5002,2008,2002,1832,1168,562,332,193,103,59)
disp<- 1.0
#disp<- c(1.0,1.0,1.08,0.97,1.03,1.0,1.06,1.14,0.83,0.88,1.15)
## user part: option settings
sigma<- 1 # regression sigma 1 or
#sigma<- NULL # NULL (regression sigma estimated)
wt<- 1/disp # weight setting, required!
model<- "lq"
#model<- "l"
# "l" for linear or "lq" for linear quadratic
# a background value (c) is fitted in both options
#####
## execution part: changes recommended only for developers of the script ##
#####
if (length(disp)==1) disp<- rep(disp,length(dose))
kurvendaten<-data.frame(dose,ab,cells,disp)
print(kurvendaten)
x0<-cells
x1<-cells*dose
x2<-cells*dose*dose
modelldaten<-list(x0,x1,x2,ab)
if (length(wt)==1) wt<- rep(wt,length(dose))
if (model=="lq" & sigma==1)
  result<-glm(ab ~ -1 + x0+x1+x2,family=poisson(link = "identity"), weights=wt, data=modelldaten)
if (model=="lq" & is.null(sigma))
  result<-glm(ab ~ -1 + x0+x1+x2,family=quasipoisson(link = "identity"), weights=wt, data=modelldaten)
if (model=="l" & sigma==1)
  result<-glm(ab ~ -1 + x0+x1,family=poisson(link = "identity"), weights=wt, data=modelldaten)
if (model=="l" & is.null(sigma))
  result<-glm(ab ~ -1 + x0+x1,family=quasipoisson(link = "identity"), weights=wt, data=modelldaten)
smry<-summary(result,correlation=TRUE)
#smry$coefficients
#smry$correlation
corma<-smry$correlation
bstat<-smry$coefficients
seb<-bstat[,2]
vakoma<-corma*outer(seb,seb)
vakoma<-vcov(result)
#####
## output of results ##
#####
cat("\n")
cat("Result of curve fit 'result'\n")
cat("-----\n")
print(result)
cat("\n")
cat("assumed sigma\n")
print(sigma)
cat("\n")
cat("Coefficients 'bstat'\n")
print(bstat)
cat("\n")
cat("variance-covariance matrix 'vakoma'\n")
print(vakoma)
cat("\n")
cat("correlation matrix 'corma'\n")
print(corma)

```

```

par(lwd=2)
plot(dose, ab/cells)
if (model=="lq") curve(bstat[1,1]+bstat[2,1]*x+bstat[3,1]*x*x,0,max(dose), add=TRUE)
if (model=="l") curve(bstat[1,1]+bstat[2,1]*x,0,max(dose), add=TRUE)

```

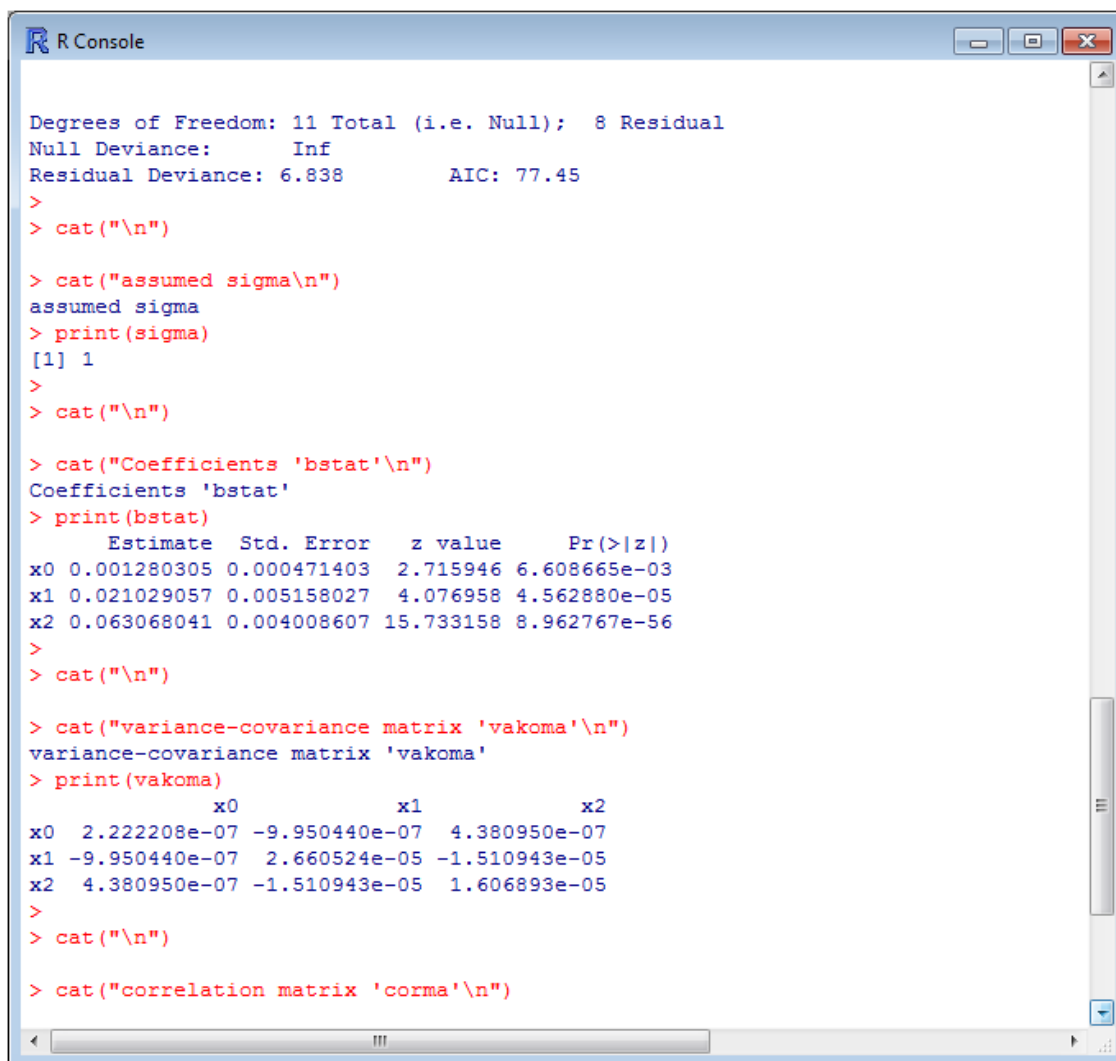
The input data for fitting  $^3\text{He}$  data to the linear model.

```

# 20 MeV helium  $\alpha$ - particles (87)
dose<-c(0,0.051,0.104,0.511,1.01,1.536,2.05,2.526,3.029)
ab<-c(3,19,27,199,108,96,120,148,108)
cells<-c(2000,900,1029,1136,304,142,137,144,98)
disp<- 1.19
sigma<- NULL
wt<- 1/disp
model<- "l"

```

## BOX 2. THE OUTPUT FOR THE FIT TO THE $^{60}\text{Co}$ DATA



```

R Console
Degrees of Freedom: 11 Total (i.e. Null); 8 Residual
Null Deviance:      Inf
Residual Deviance: 6.838      AIC: 77.45
>
> cat("\n")
> cat("assumed sigma\n")
assumed sigma
> print(sigma)
[1] 1
>
> cat("\n")
> cat("Coefficients 'bstat'\n")
Coefficients 'bstat'
> print(bstat)
      Estimate Std. Error  z value    Pr(>|z|)
x0 0.001280305 0.000471403  2.715946 6.608665e-03
x1 0.021029057 0.005158027  4.076958 4.562880e-05
x2 0.063068041 0.004008607 15.733158 8.962767e-56
>
> cat("\n")
> cat("variance-covariance matrix 'vakoma'\n")
variance-covariance matrix 'vakoma'
> print(vakoma)
           x0           x1           x2
x0 2.222208e-07 -9.950440e-07  4.380950e-07
x1 -9.950440e-07  2.660524e-05 -1.510943e-05
x2  4.380950e-07 -1.510943e-05  1.606893e-05
>
> cat("\n")
> cat("correlation matrix 'corma'\n")

```

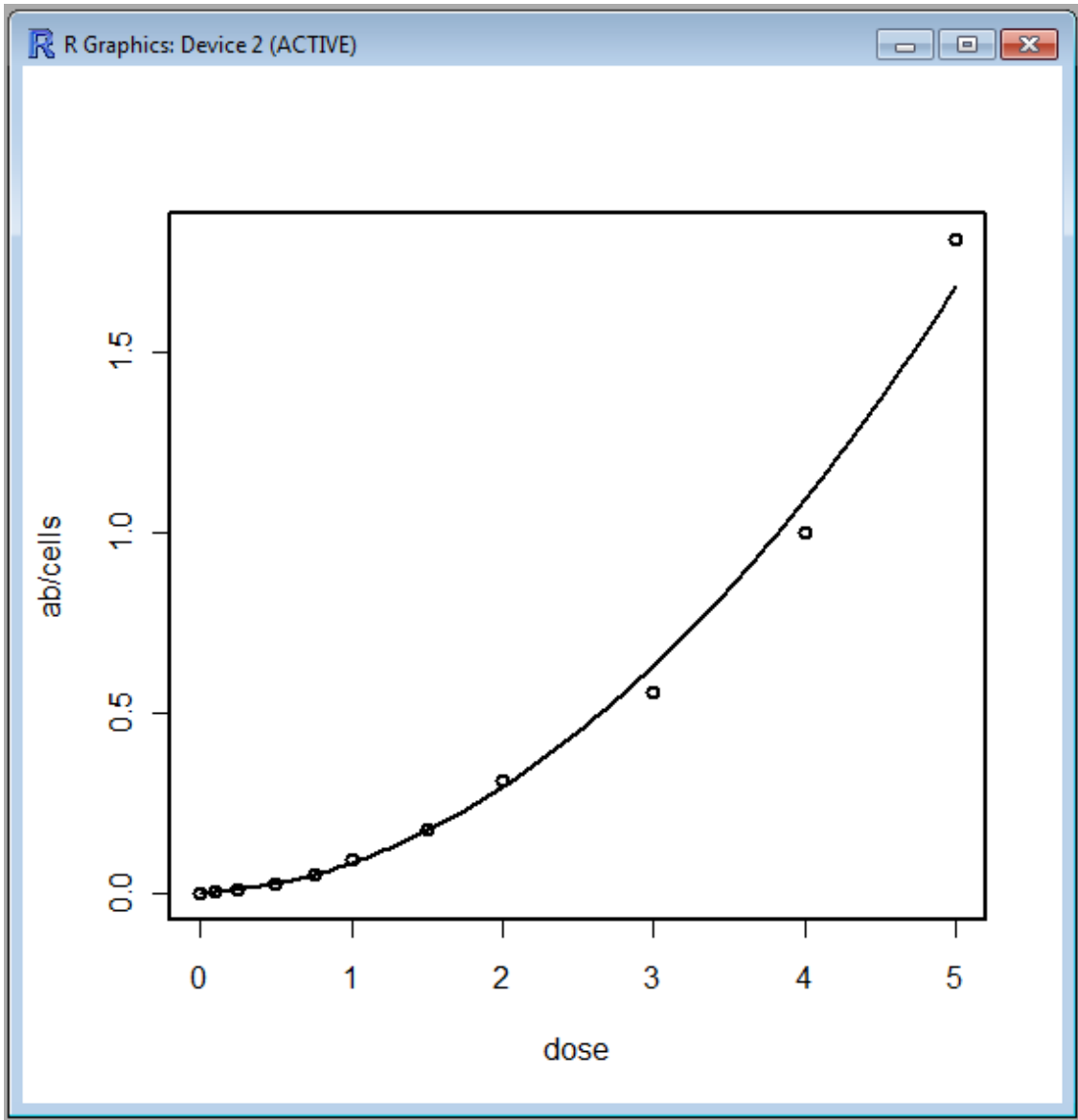


FIG. VI-1. The output for the  $^{60}\text{Co}$  data presented as a graph showing the observed data points and the fitted linear quadratic curve.

## Annex VII

### AN EXAMPLE OF AN INTERLABORATORY COMPARISON EXERCISE FOR QUALITY ASSURANCE

This Annex provides an example of an international interlaboratory comparison performed among 14 biological dosimetry laboratories. The exercise comprised the analysis of slides of metaphase preparations from blood that had been irradiated *in vitro* to 0.75 and 2.5 Gy with  $^{60}\text{Co}$   $\gamma$ -rays. Participating laboratories were required to report the frequency of dicentric chromosomes that they obtained and the estimated dose after the analysis of 50, 100 cells (triage mode) and after conventional scoring of 500 cells or stopping sooner if 100 dicentric chromosomes was reached. For this laboratory interlaboratory comparison, the performance of each laboratory and reproducibility of the exercise was evaluated using robust methods (algorithms A and S) described in ISO standards ISO 5725-5 and ISO 13528:2005 [15, 16].

This annex shows as a worked example just a subset of the interlaboratory comparison results, those obtained after analysing 500 cells at 0.75 Gy. Full details are described in [17].

To determine the laboratory performance the z-test was used:

$$z = \frac{(x_i - x_{ref})}{\sqrt{(s^*)^2 + u_x^2}} \quad (\text{VII-1})$$

For each laboratory the z-test considers its reported values for the frequency of dicentric chromosomes observed or the estimated dose derived by referring the dicentric frequency to its own pre-existing dose response curve ( $x_i$ ). For the frequency analysis,  $x_{ref}$  was a consensus value (robust average,  $x^*$ , obtained by the algorithm A), and for the dose estimation analysis it was the physical dose administered. The z-test also takes into account the robust standard deviation ( $s^*$ ) obtained by the algorithm A and the standard uncertainty of the consensus or reference value ( $u_x$ ). When frequencies were evaluated  $u_x$  was calculated as follows:

$$u_{ref} = 1.25s^* / \sqrt{p} \quad (\text{VII-2})$$

where  $p$  is the number of participating laboratories.

Regarding dose estimation, the uncertainty, designated  $u_x$ , on the physical measurements of the actual doses delivered to the blood samples was considered to be negligible according to the criteria shown in Eq. (VII-3). Regarding dose estimation  $u_x$  was the uncertainty on the physical dose delivered. For each analysis,  $u_x$  was considered negligible according to the following criteria:

$$0.96 \leq \frac{s^*}{\sqrt{(s^*)^2 + u_x^2}} \leq 1 \quad (\text{VII-3})$$

To evaluate the laboratory performance, the following criteria were applied:

$ z  \leq 2$	satisfactory
$2 <  z  < 3$	questionable
$ z  \geq 3$	unsatisfactory

For the dicentric frequency, the z-score obtained for each laboratory is shown in FIG. VII-1. In this case the robust average used as  $x_{ref}$  was 0.05 and  $s^*$  was 0.01. All z values fell between -1.66 and 1.21 and, according to the acceptance criteria, were all considered as ‘satisfactory’.

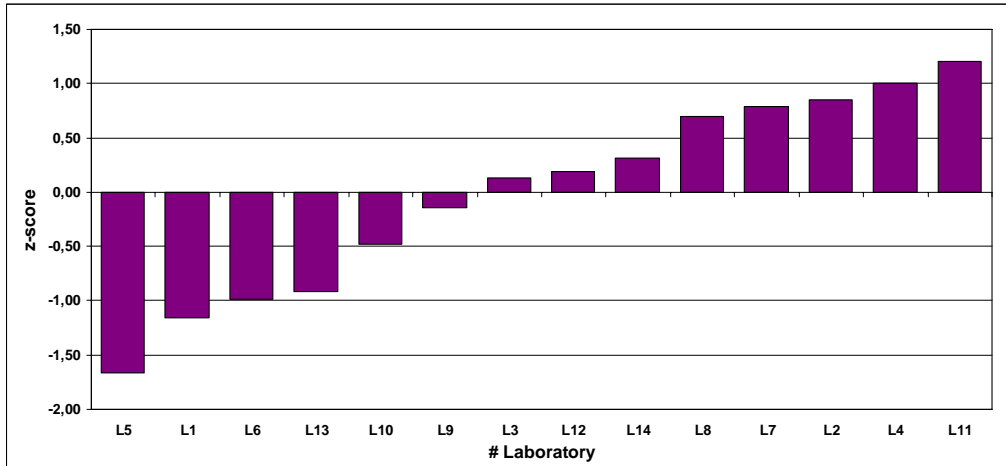


FIG. VII-1. For dicentric frequency, z-score obtained for each participating laboratory.

FIG. VII-2 shows for each laboratory the reported dose estimation with its 95% confidence interval. The solid horizontal line represents the physical dose delivered, the dotted lines are the interval  $1.96s^*$ . In this case only L11, which reported an estimated dose of 0.98 Gy, was considered as ‘questionable’ by the z-score (2.12).

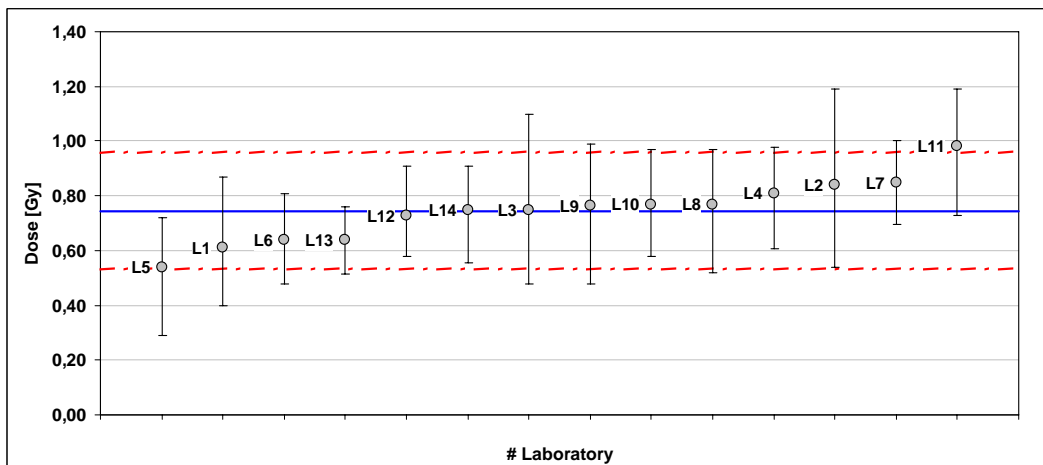


FIG. VII-2. Doses estimated for each participating laboratory.

The reproducibility of the exercise was evaluated by the reproducibility standard deviation ( $S_R$ ):

$$S_R = \sqrt{(s^*)^2 + S_r^2 \left(1 - \frac{1}{n}\right)} \quad (\text{VII-4})$$

This formula considers the variability between laboratories by the robust standard deviation ( $s^*$ ) obtained using algorithm A and the intralaboratory variability by the repeatability standard deviation ( $S_r$ ) obtained using the algorithm S. In Eq. (VII-4),  $n$  represents the replicate measure



for each participating laboratory that in the present exercise was 2. After the analysis of 500 cells at 0.75 Gy the  $S_R$  values were 0.013 for the frequency and 0.116 for the dose. To compare the reproducibility of both measurements, frequency and dose, the coefficient of variation (CV) was defined.

The CV indicates the global dispersion of the results and it is calculated as the ratio  $S_R/x^*$ . In the present example the obtained coefficients were 24.4% and 15.6% for frequency and dose respectively. These results indicated a better reproducibility when estimated doses were considered rather than the dicentric frequencies.

Future interlaboratory comparisons among the same laboratories will determine if the reproducibility can be improved. If it cannot be improved, then the obtained value will be accepted as the variability associated with the random errors of the method.



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## LIST OF ABBREVIATIONS

ace	acentric fragment
AFRRI	Armed Forces Radiobiology Research Institute (USA)
ANOVA	analysis of variance
ARS	acute radiation syndrome
AS	abasic sites
ATP	adenosine triphosphate
BD	base damage
BER	base excision repair
BN	binucleated
BrdU	bromodeoxyuridine
BSS	Basic Safety Standards
CABAS	chromosomal aberration calculation software
CBMN	cytokinesis-block micronucleus assay
CBMN Cyt	cytokinesis-block micronucleus cytome assay
CCD	charge-coupled device
CHO	Chinese hamster ovary
CP	centromeric probe
CRP	Co-ordinated Research Programme
Cyt-B	cytochalasin-B
DAPI	4',6'-diamidino-2-phenylindole
DCA	dicentric chromosome assay
df	degrees of freedom
dic	dicentric chromosome
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DPC	DNA-protein cross-links

DSB	double strand break
EDTA	ethylene diamine tetra acetic acid
ESR	electron spin resonance
FISH	fluorescence <i>in situ</i> hybridization
FPG	fluorescence plus Giemsa
HBSS	Hank's balanced salt solution
HIV	human immunodeficiency virus
HPBL	human peripheral blood lymphocytes
HRR	homologous recombination repair
HUMN	human micronucleus
IAEA	International Atomic Energy Agency
ICRP	International Commission on Radiological Protection
ICRU	International Commission on Radiation Units and Measurements
IND	improvised nuclear devices
IRSN	Institut de Radioprotection et de Sûreté Nucléaire (France)
ISO	International Organization for Standardisation
IU	international unit
LCL	lower confidence limit
LET	linear energy transfer
LIMS	laboratory information management system
M1, M2, ...	first, second, ... <i>in vitro</i> division metaphase
MDS	multiple damage sites
MEM	minimum essential medium
mFISH	multicolour fluorescence <i>in situ</i> hybridization
MN	micronucleus (micronuclei)
MNCM <sup>-ve/+ve</sup>	micronucleus centromere negative/positive cell

NBUD	nuclear bud
NDI	nuclear division index
NHEJ	non-homologous end-joining
NER	nucleotide excision repair
NIRS	National Institute of Radiological Sciences (Japan)
NPB	nucleoplasmic bridge
NPP	nuclear power plant
OA	okadaic acid
PAINT	Protocol for Aberration Identification and Nomenclature Terminology
PBS	phosphate buffered saline
PCC	premature chromosome condensation
PCR	polymerase chain reaction
PEG	polyethylene glycol
PHA	phytohaemagglutinin
QA	quality assurance
QC	quality control
RBE	relative biological effectiveness
RDD	radiological dispersal device
REAC/TS	Radiation Emergency Assistance Center/Training Site (USA)
RED	radiological exposure device
RICA	rapid interphase chromosome assay
RNase	ribonuclease
SD	standard deviation
SE	standard error
SEM	standard error of the mean
SI	International System of Units

SSB	single strand break
SSBR	single strand break repair
SSC	saline sodium citrate
TLD	thermoluminescence dosimeter
UCL	upper confidence limit
UN	United Nations
UV	Ultraviolet
WHO	World Health Organization



## DEFINITIONS<sup>3</sup>

**absorbed dose (D)\*.** The fundamental dosimetric quantity  $D$ , defined as:

$$D = \frac{d\bar{\varepsilon}}{dm}$$

where:

$d\bar{\varepsilon}$  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 energy can be averaged over any defined volume, the average dose being equal to the total energy imparted in the volume divided by the mass in the volume.
- Absorbed dose is defined at a point; for the average dose in a tissue or organ, see organ dose.
- Unit: gray (Gy), equal to 1 J/kg (formerly, the rad was used).

**accident\*.** Any unintended event, including operating errors, equipment failures and other mishaps, the consequences or potential consequences of which are not negligible from the point of view of protection or safety.

**criticality accident.** An accident involving criticality.

- Typically, in a facility in which fissile material is used.

**acentric (ace).** Terminal or interstitial chromosome fragment of varying size lacking a centromere. An acentric formed independently from a dicentric, tricentric, or centric ring aberration is usually referred to as an excess acentric.

**alpha radiation.** Particle radiation emitted in the nuclear disintegration of certain radionuclides. Alpha particles consist of two neutrons and two protons and are identical with the nucleus of the helium atom. They are readily absorbed by a few centimetres of air and therefore the main hazards come from internally incorporated alpha emitting nuclides.

**aneugen.** An indirect mutagen able to affect cell division and the mitotic spindle apparatus resulting in the loss or gain of whole chromosomes, thus inducing aneuploidy.

**ankylosing spondylitis.** Chronic, inflammatory arthritis which affects the spine and the sacroiliac in the pelvis. Many decades ago, large field external beam radiation was used to treat the inflammation of the spines in these patients.

**anticoagulant.** A drug which prevents the clotting (coagulation) of blood.

**background frequency/level/value.** Incidence (or number) of chromosome aberrations or micronuclei recorded in the general population.

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<sup>3</sup> Definitions apply for the purposes of the present publication. Definitions marked with an asterisk are taken from Ref. [357].

**becquerel (Bq)\*.** The SI unit of activity, equal to one transformation per second.

- Supersedes the non-SI unit curie (Ci). 1 Bq = 27 pCi ( $2.7 \times 10^{-11}$  Ci) approximately. 1 Ci =  $3.7 \times 10^{10}$  Bq.

**beta radiation.** Particle radiation comprising electrons with positive or negative charge emitted in the nuclear disintegration of certain radionuclides. The penetration of beta particles is a few centimetres to metres in air and a few millimetres to centimetres in soft tissue or plastic.

**bias.** Deviation of results or inferences from the truth or processes leading to such deviation.

**binucleated.** Having two nuclei. Binucleated cells occur at the end of the nuclear division cycle and can be accumulated using a cytokinesis-block inhibitor such as cytochalasin-B. Binucleated cells are scored for the presence of micronuclei and nucleoplasmic bridges in the cytokinesis-block micronucleus assay.

**biological dosimetry/biodosimetry.** The use of biomarkers to verify exposure to radiation and to estimate absorbed dose.

**biological effects.** Range of possible consequences on living material, organisms, tissues, or cells, depending on type and degree of cellular damage that may result from exposure to an external agent, such as ionizing radiation.

**biomarker.** An indicator of normal biological or pathogenic processes. Within the scope of biological dosimetry, they are used to distinguish radiation-induced biological damage from that produced by other agents.

**Bragg-Gray cavity theory.** Relates the ionization produced within a gas-filled cavity inside a medium to the energy absorbed in that surrounding medium. In the context of chromosome aberration formation, applying the theory means that the size of the cell nucleus is so small that the total energy absorbed is only due to electrons passing through the nucleus. Therefore secondary particles can be ignored.

**5-Bromodeoxyuridine (BrdU).** An analogue of thymidine in which the methyl group at the 6' position in thymine is replaced by bromine. BrdU is used in biological dosimetry for differentially labelling newly synthesized DNA to identify cells having passed through mitosis more than once.

**buffy coat.** The layer of an anticoagulated blood sample after centrifugation that contains most of the white blood cells.

**calibration curve.** In biological dosimetry, a graphical or mathematical description of the dose effect relation derived by the *in vitro* irradiation of blood samples to known doses. The curve is used to determine, by interpolation, the absorbed radiation dose to a potentially exposed individual.

**C-banding.** See 'chromosome banding'.

**centromere.** Primary constriction region of a chromosome that is visualized during mitosis and joins together the chromatid pair.

**chain-of-custody.** The complete record of a blood sample by tracking its handling and storage from point of specimen collection/receipt to final disposition of the specimen.

**charged particle equilibrium.** Occurs when the number of each type of charged particles leaving a given volume is equal to those entering it.

**chromosome banding.** A technique for the differential staining of chromosomes, most commonly using Giemsa stain. Depending on the method, a selective staining of certain chromosomal regions such as centromeres (C-banding) or characteristic patterns along the arms (G-banding) are visualized. The specific pattern of dark and light stripes (bands), unique to each chromosome pair, is used to identify them and evaluate their structure.

**clastogen.** A physical or chemical agent that breaks DNA in chromosomes, leading to rearrangements such as the aberrations observed in metaphase.

**clustered DNA lesions.** More than two sites of DNA damage generated by ionizing radiation within 20 bps of the same molecule.

**colchicine/Colcemid.** Alkaloid compounds that inhibit spindle formation during cell division. They are used to collect a large number of metaphase cells by preventing them from progressing to anaphase. Colcemid is a synthetic analogue of the natural, plant-derived, colchicine.

**complex rearrangement.** An aberration involving three or more breaks in two or more chromosomes and is characteristically induced after exposure to densely-ionizing radiation or high doses of sparsely ionizing radiation.

**confidence interval.** An interval estimate for a variable of interest, e.g. a rate, constructed according to a chosen distribution (e.g. the Poisson) so that this range has a specified probability of including the true value of the variable. Thus, a confidence interval (described by the upper and lower confidence limit) is used to indicate the reliability of an estimate. How likely the interval is to contain the parameter is determined by the confidence level or confidence coefficient. Increasing the desired confidence level will widen the confidence interval.

**confound.** To 'disturb' the correlation between an influencing variable (e.g. exposure to ionizing radiation) and effect (e.g. induced aberrations) investigated in a study by another variable (confounder, e.g. age, smoking). If confounders are not taken into consideration a correlation that does not exist in reality can be pretended or a real correlation can be blurred.

**contaminated poisson method.** A mathematical analysis of centric ring and dicentric chromosome frequencies which permits dose assessment in cases of suspected partial body exposures. The method permits dose assessment by considering the distribution of dicentrics among all the scored cells and gives additional information about the irradiated volume of the body. See also Qdr method.

**contamination\*.** Radioactive substances on surfaces, or within solids, liquids or gases (including the human body), where their presence is unintended or undesirable, or the process giving rise to their presence in such places.

**control group.** A group of cells, animals, or test persons being exposed to the best possible identical conditions as the exposed individuals, with the exception that the effect to be investigated is not administered.

**covariance.** A measure of the correlation of the variance between two (or more) dependent sets of data, in other words, how the data vary together. It can be positive or negative, indicating a positive or negative linear relationship between the data sets. If the data are independent, the covariance is zero.

**covariance of curve parameters.** The parameters (C, alpha, beta) do not deviate independently from its ideally true values (see standard deviation, variance), but do so together up to a certain amount of correlation because they are simultaneously calculated from the same data set. Thus it reduces the error term in combined calculations like Eq. (7) as when calculated with variances alone. Corresponding correlations could also be calculated from the variances and covariances (covariance divided by square root of the product of the two variances, i.e. covariance divided by the product of the standard deviations).

**curve fitting.** Identifying an equation which describes the best fit to a series of data points, possibly with a number of other constraints, including weighting the fit by the reliability of each data point (assessed by the standard error on the point) and/or constraining the fit to a measured baseline value.

**cytochalasin B.** A natural compound, of fungal origin, with the unique property of inhibiting cytokinesis in mammalian and human cells used in the cytokinesis-block micronucleus assay.

**cytogenetics.** A branch of genetics that deals with the study of chromosomes

**cytokinesis-block micronucleus cytome assay (CBMN Cyt).** The CBMN Cyt assay is a more advanced version of the CBMN assay in which a wider range of biomarkers of chromosome damage (micronuclei, nucleoplasmic bridges, nuclear buds in binucleated cells) as well as cell death (necrotic and apoptotic cells) and cytostasis (nuclear division index based on ratios of mononucleated, binucleated and multinucleated cells) are measured. The micronucleus and nucleoplasmic bridge biomarkers are the biomarkers in this system that are best validated for biological dosimetry of ionizing radiation exposure.

**densely-ionizing/high-LET radiation.** Radiation which deposits its energy in closely spaced interactions along its track (e.g. alpha particles, neutrons). This spatial distribution is reflected in the relative biological effectiveness. See also 'linear energy transfer, LET'.

**detection limit.** The dose represented by the lowest frequency of a given biodosimetric marker that can be discriminated above the background frequency with a certain level of confidence, normally 95%.

**deterministic effect.** A health effect of radiation for which generally a threshold level of dose exists above which the severity of the effect is greater for a higher dose. Such an effect is described as a 'severe deterministic effect' if it is fatal or life threatening or results in a permanent injury that reduces quality of life.

**dicentric (dic).** Aberrant chromosome bearing two centromeres derived from the misrepair of two broken chromosomes.

**diploid.** The species specific number of chromosomes in a somatic cell; in humans: 46 (22 pairs of autosomes and two sex chromosomes).

**deoxyribonucleic acid (DNA).** The molecule contained within chromosomes which encodes the genes responsible for the structure and function of an organism and allows for transmission of genetic information from one generation to the next.

**dose rate.** A measure of the rate at which energy is transferred from radiation to a target; the dose delivered per unit time. Although dose rate could, in principle, be defined over any unit of time (e.g. an annual dose is technically a dose rate), usually the term dose rate is used in the context of short periods of time, e.g. dose per second or dose per hour.

**dosimeter, radiation.** A physical device that measures exposure to ionizing radiation.

**double strand break (DSB).** Type of DNA damage; the DNA is cut completely due to scission of the phosphodiester backbone in both strands of the double helix at the same locus or nearby loci.

**electron spin resonance (ESR)/electron paramagnetic resonance (EPR).** A technique for studying chemical species that have one or more unpaired electrons, such as organic and inorganic free radicals or inorganic complexes possessing a transition metal ion. It can be used as a radiation dosimeter.

**emergency\*.** A non-routine situation that necessitates prompt action, primarily to mitigate a hazard or adverse consequences for human health and safety, quality of life, property or the environment. This includes nuclear and radiological emergencies and conventional emergencies such as fires, release of hazardous chemicals, storms or earthquakes. It includes situations for which prompt action is warranted to mitigate the effects of a perceived hazard. nuclear or radiological emergency. An emergency in which there is, or is perceived to be, a hazard due to:

(a) The energy resulting from a nuclear chain reaction or from the decay of the products of a chain reaction; or

(b) Radiation exposure.

- Points (a) and (b) approximately represent nuclear and radiological emergencies, respectively. However, this is not an exact distinction.
- Radiation emergency is used in some cases when an explicit distinction in the nature of the hazard is immaterial (e.g. national radiation emergency plan), and it has essentially the same meaning.

**enzyme.** A protein molecule that catalyzes chemical reactions of other substances without itself being destroyed or altered upon completing the reactions.

**epidemiology.** The study of the distribution and determinants of health-related states or events in specified populations, and the application of this study to control of health problems.

**exposure\*.** The act or condition of being subject to irradiation.

Exposure should not be used as a synonym for dose. Dose is a measure of the effects of exposure.

- Exposure can be divided into categories according to its nature and duration (see exposure situations) or according to the source of the exposure, the people exposed and/or the circumstances under which they are exposed (see exposure, types of).

**external exposure.** Exposure to radiation from a source outside the body.

- Contrasted with internal exposure.

**internal exposure.** Exposure to radiation from a source within the body.

- Contrasted with external exposure.

**exposure situations\*.**

**acute exposure.** Exposure received within a short period of time. Normally used to refer to exposure of sufficiently short duration that the resulting doses can be treated as instantaneous (e.g. less than an hour).

**chronic exposure.** Exposure persisting in time. The adjective 'chronic' relates only to the duration of exposure, and does not imply anything about the magnitude of the doses involved. Normally used to refer to exposures persisting for many years as a result of long lived radionuclides in the environment. The International Commission on Radiological Protection uses the term prolonged exposure to describe the same concept as chronic exposure. Both terms are contrasted with acute exposure.

**false negative.** A test result which indicates that an individual is unaffected when he or she is actually affected; i.e. a negative test result in an affected individual.

**false positive.** A test result which indicates that an individual is affected when he or she is actually unaffected; i.e. a positive test result in a truly unaffected individual.

**fast neutrons.** Neutrons, the energy of which exceeds some arbitrary level, usually around 1 MeV.

**first responders\*.** The first members of an emergency service to respond at the scene of an emergency.

**fission neutrons.** Free neutrons, usually produced during nuclear fission, a nuclear reaction in which a heavy nucleus of an atom splits into two approximately equal parts (nuclei of lighter elements), accompanied by the release of a relatively large amount of energy.

**fluorescent in situ hybridization (FISH).** A technique used to identify the presence of specific chromosomes or chromosomal regions through attachment of fluorescently-labelled DNA probes to denatured chromosomal DNA.

**interphase FISH.** Probes are introduced directly to the interphase cell. This permits visualization of changes at the level of chromosomal domains.

**metaphase FISH.** Probes are introduced to the chromosomes in metaphase. This permits visualization of chromosomal aberrations.

**FISH techniques:**

**multicolour FISH (mFISH).** mFISH utilizes ‘whole chromosome paint’ DNA probes which are labelled with a unique combination of fluorescent colours for each of the human chromosomes.

**pq-mFISH.** A variant of mFISH whereby individual chromosome arms are discriminated.

**fluorochrome.** Molecules that are fluorescent when appropriately excited. They are used for FISH cytogenetics to highlight specific chromosomal regions.

**gamma radiation.** Electromagnetic radiation (photons) of high frequency (very short wavelength) emitted by the nucleus or from the particle decays or annihilation events. Gamma rays penetrate tissue farther than do beta particles or alpha particles, but have a lower linear energy transfer. Heavy materials such as lead or concrete must be used for shielding from gamma radiation.

**G-banding.** See ‘chromosome banding’.

**genotoxin.** Any chemical or physical agent that causes damage to or mutation of genetic material.

**G-function.** A time dependent factor used to modify the dose squared coefficient of the linear quadratic dose response relationship to allow for the effects of dose protraction.

**gray (Gy)\*.** The SI unit of kerma and absorbed dose, equal to 1 J/kg. It replaced the rad. 1Gy = 100 rad.

**haematopoiesis.** The formation of blood cellular components.

**half-life ( $T_{1/2}$ )\*.**

(1) For a radionuclide, the time required for the activity to decrease, by a radioactive decay process, by half.

- Where it is necessary to distinguish this from other half-lives (see (2)), the term radioactive half-life should be used.
- The half-life is related to the decay constant,  $\lambda$ , by the expression:

$$T_{1/2} = \frac{\ln 2}{\lambda}$$

(2) The time taken for the quantity of a specified material (e.g. a radionuclide) in a specified place to decrease by half as a result of any specified process or processes that follow similar exponential patterns to radioactive decay.

**biological half-life.** The time taken for the quantity of a material in a specified tissue, organ or region of the body (or any other specified biota) to halve as a result of biological processes.

**effective half-life ( $T_{\text{eff}}$ ).** The time taken for the activity of a radionuclide in a specified place to halve as a result of all relevant processes.

$$\frac{1}{T_{\text{eff}}} = \sum_i \frac{1}{T_i}$$

where:

$T_i$  is the half-life for process  $i$ .

**radioactive half-life.** For a radionuclide, the time required for the activity to decrease, by a radioactive decay process, by half.

- The term ‘physical half-life’ is also used for this concept.

**half-time.** The time required for a parameter to fall 50% of its original value. For biological dosimetry application, this term is used to describe the turnover of peripheral blood lymphocytes.

**harlequin chromosome.** A chromosome which has been differentially labelled and stained after uptake of BrdU during two cycles of replication. After appropriate staining one of the sister chromatids appears dark and the other appears light.

**incorporation.** The intake of a radionuclide into the body by inhalation, ingestion or across intact or damaged skin.

**insertion.** A chromosome abnormality in which an acentric piece of one chromosome has been inserted within an arm of another chromosome.

**inter-comparison exercise.** A procedure, used for quality assurance and control. In biological dosimetry materials are exchanged between laboratories for comparative analysis.

**interphase.** Period of a cell cycle between the mitotic divisions in which the cell spends most of its time and performs the majority of its functions such as preparation for cell division.

**inversion.** A chromosomal rearrangement in which a segment of a chromosome has inverted from end to end, and reinserted into the chromosome at the same breakage site. It can be peri- or para-centric depending on whether the centromere is included or not, respectively.

***in vitro.*** Observed or performed in the test tube.

***in vivo.*** Observed or performed in the living organism.

**ionization.** The physical process whereby one or more orbital electrons are ejected from an atom or molecule, converting them into an electrically charged state (an ion). The process produces a localized release of a large amount of energy, approximately 33eV per ionizing event, which is more than enough to break a strong chemical bond.

**isotope.** Atoms of one and the same chemical elements with the same number of protons (same atomic number but different nuclear number) and electrons but



different number of neutrons. Isotopes are the same chemical but have different nucleonic characteristics.

**karyotype.** Systematic arrangement of the homologous chromosomes pairs of a single cell based on size, centromere position and banding pattern according to a standard classification.

**kerma, air (kinetic energy released per unit mass)\*.** The quantity  $K$ , defined as:

$$K = \frac{dE_{tr}}{dm}$$

where:

$dE_{tr}$  is the sum of the initial kinetic energies of all charged ionizing particles liberated by uncharged ionizing particles in a material of mass  $dm$ .

- Unit: gray (Gy).
- Originally an acronym for kinetic energy released in matter, but now accepted as a word.

**leukocyte.** A generalized term describing all types of white blood cells. (e.g. lymphocytes, neutrophils, eosinophils, basophils, plasma cells and monocytes).

**linear energy transfer (LET) ( $L_{\Delta}$ )\*.** Defined generally as:

$$L_{\Delta} = \left( \frac{dE}{d\ell} \right)_{\Delta}$$

where:

$dE$  is the energy lost in traversing distance  $d\ell$ , and  $\Delta$  is an upper bound on the energy transferred in any single collision.

- A measure of how, as a function of distance, energy is transferred from radiation to the exposed matter. A high value of linear energy transfer indicates that energy is deposited within a small distance.
- $L_{\infty}$  (i.e. with  $\Delta = \infty$ ) is termed the ‘unrestricted linear energy transfer’ in defining the quality factor.
- $L_{\Delta}$  is also known as the ‘restricted linear collision stopping power’.

**linear model.** Common mathematical model used to describe the biological response to radiation, in which the effect  $Y$  is a linear function of dose  $D$ :

$$Y = \alpha D.$$

**linear quadratic model.** Common mathematical model used to describe the biological response to radiation, in which the effect  $Y$  is a linear-quadratic function of dose  $D$ :

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

**lymphocyte.** A type of white blood cell mainly found in the blood, lymph, and lymphatic tissues that forms the body's immunologically competent cells and their precursors.

**meta-analysis.** Studies where the results of similar, independent investigations on a certain topic are pooled, summarized, and evaluated according to certain parameters.

**metaphase.** Stage of mitosis in the eukaryotic cell cycle in which chromosomes are condensed to their minimum length and align in the middle of the cell before being separated into each of the two daughter cells.

**micronucleus (MN).** A micronucleus is an additional small nucleus found in cells with chromosomal damage. A MN typically originates from a lagging whole chromosome or acentric chromosome fragment at anaphase. The diameter of an MN is usually  $1/16^{\text{th}}$  to  $1/3^{\text{rd}}$  of that of the main nuclei in a mononucleated or binucleated cell. MN have the same texture and staining properties of nuclei.

**mitogen.** Reagent inducing cell proliferation.

**mitosis.** The part of the cell division process in which a eukaryotic cell separates its chromosomes into two identical sets.

**mitotic index.** Percentage of cells in mitosis at any given time.

**mutagen.** Any chemical or physical agent with the property of inducing genetic mutation at the base sequence of the DNA molecule or at the chromosomal level of organization.

**mutation.** Any alteration in a gene from its natural state; may be disease-causing or a benign, normal variant.

**neutron radiation.** Neutrons, one of the components of the atomic nucleus, are electrically neutral elementary particles. They are particularly released during nuclear fission and have a high penetration capacity.

**nuclear bud.** Micronucleus-like bodies attached to the nucleus by a thin nucleoplasmic connection; nuclear abnormality.

**nuclear division index (NDI).** A measure of the average speed of cell cycling which is taken from the relative number of cells in a population which has reached each successive stage of division, normally to fourth division.

**nucleoplasmic bridge (NPB).** Occurs when the centromeres of dicentric chromosomes or chromatids are pulled to the opposite poles of the cell at anaphase. In the CBMN assay, binucleated cells with NPB can be observed because cytokinesis is inhibited, preventing breakage of the anaphase bridges from which nucleoplasmic bridges are derived.

**nucleosome.** These structures form the fundamental repeating units in the packaging of eukaryotic chromatin within the chromosome.

**nuclide.** Type of atom characterized by proton number (atomic number) and nuclear number.

**odds ratio.** The ratio of two odds, calculated to show the likelihood of zero dose versus a suspected (e.g. badge) or a physical measurement of dose.

**OSL dosimeter (optically stimulated luminescence).** Type of physical dosimeter which is used to measure exposure to ionizing radiation.

**PAINT (Protocol for Aberration Identification and Nomenclature Terminology).** Terminology used in FISH analysis for describing chromosomal aberrations.

**pan-centromeric probe.** DNA probes labelling the centromeric region of each chromosome.

**phantom.** A device which absorbs and scatters  $\gamma$ - and X-radiation in approximately the same way as the tissues of the body. (e.g. for dosimetry measurements or irradiation of blood samples needed to generate a dose-response calibration curve).

**polymerase chain reaction (PCR).** A procedure that enzymatically amplifies a DNA sequence through repeated replication by DNA polymerase.

**Qdr method.** A mathematical analysis of centric ring and dicentric chromosome frequencies which permits dose assessment in cases of suspected partial body exposures. The method permits dose assessment by considering the distribution of dicentrics and rings among just the damaged cells. See also contaminated Poisson method.

**Qpcc method.** A mathematical analysis identical to Qdr (above) but based instead on the distribution of prematurely condensed chromosome fragments.

**relative biological effectiveness (RBE)\*.** A relative measure of the effectiveness of different radiation types at inducing a specified health effect, expressed as the inverse ratio of the absorbed doses of two different radiation types that would produce the same degree of a defined biological end point.

**ring, centric/acentric.** Aberrant circular chromosome containing/lacking a centromere that has resulted from the joining of two breaks within one chromosome.

**röntgen (R)\*.** Unit of exposure, equal to  $2.58 \times 10^{-4}$  C/kg (exactly).

- Superseded by the SI unit C/kg.

**service laboratory.** The term refers in the context of this publication to a specialized laboratory dedicated to the performance of biological dosimetry measurements.

**sievert (Sv)\*.** The SI unit of equivalent dose and effective dose, equal to 1 J/kg.

**significance, statistical.** One talks about statistical significance if a result of a given experiment or study clearly deviates from the expected result. The statistical significance of a result of a given experiment or study is an estimated measure of the degree to which it is 'true', i.e. caused by something other than just chance. In general, a deviation between observed and expected results is considered to be significant if this error probability is less than 5 %.

**single strand break (SSB).** Type of DNA damage in which the DNA is broken only across one strand of the double helix.

**sparsely-ionizing/low-LET ionizing radiation.** See ‘densely-ionizing/high-LET’ and ‘linear energy transfer’.

**stable aberration.** A non-lethal chromosomal rearrangement or exchange between or within chromosomes without loss of genetic material during mitosis.

**stable cell.** Cell without unstable aberrations. It may be entirely undamaged or it may contain stable type aberrations only.

**standard deviation (SD).** A quantitative measure of the amount of variation in a sample of measurements from a population, giving an indication of how widely spread are the values in a data set. For data that approximate to a Normal distribution, the standard deviation of a curve parameter indicates how far it is likely to deviate from the true value, which can be ideally thought of as given by the mean of a large population of calibration donors (or experiments) based on large numbers of cells for each one. See also covariance.

**standard error of the mean (SEM).** A quantitative measure of the amount of variation in a sample of measurements from an approximately Normal population, giving an indication of how far the sample mean is likely to deviate from the true population mean, thus quantifying how accurately the true mean of the population is known.

**stochastic effect (of radiation).** A radiation induced health effect, the probability of occurrence of which is greater for a higher radiation dose and the severity of which (if it occurs) is independent of dose. Stochastic effects may be somatic effects or hereditary effects, and generally occur without a threshold level of dose. Examples include thyroid cancer and leukaemia.

**synchronization.** When applied to cell culturing, the process refers to manipulating cells into the same stage of the cell cycle.

**telomere.** The end of each chromosome arm which consists of tandem repeat TTAGGG sequences that protect against chromosome fusions and formation of dicentric chromosomes. Telomere sequences may be lost with each DNA replication cycle or microdeletions in the sequence.

**TLD dosimeter (thermoluminescent).** Type of physical dosimeter which is used to measure exposure to ionizing radiation.

**translocation.** A chromosome alteration in which a whole chromosome or segment of a chromosome becomes attached to or interchanged with another whole chromosome or segment, the resulting hybrid segregating together at meiosis.

**reciprocal translocation (2-way or complete translocation).** The exchange of terminal portions of two separate chromosomes.

**non-reciprocal translocation (1-way or incomplete translocation).** Translocation of a single chromosome segment; no reciprocal exchange.

**triage.** A process of prioritizing patients based on the severity of their condition. Cytogenetic triage is the use of biological dosimetry results to support medical decisions.

**tricentric.** Aberrant chromosome bearing three centromeres derived from the joining of parts from three broken chromosomes. For calculation purposes they are

considered as equivalent to two dicentrics and are accompanied by two acentric fragments.

**tritiated water.** A form of water where the usual hydrogen atoms are replaced with tritium, a radioactive isotope of hydrogen.

**unstable aberration.** A chromosomal rearrangement in a cell that can be lethal to itself or its daughter cells because it leads to loss of genetic material or unbalanced transmission of genetic material during mitosis.

**unstable cell.** Cell with an unstable aberration such as a dicentric, ring or acentric fragment.

**variance.** A statistical measure of distance of values from a central value, calculated as the square of the standard deviation.

**X-radiation.** Short-wave electromagnetic radiation (photons) emitted by electrons either in orbitals outside of the nucleus, or while being accelerated to produce Bremsstrahlung-type radiation. They are shorter in wavelength than UV rays and longer than gamma rays. Heavy materials such as lead must be used for shielding from X-rays.



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