1.0. Introduction

1.1. Radiation

Radiation is a form of invisible energy travelling through space. Based on the energy of the individual photons, they are categorised as ionising (carries >10 eV) (X-rays, gamma [γ] rays, alpha [α] particles, beta [β] particles and neutrons) and non-ionising (radio waves, micro waves, light and heat). Ionising radiation (IR) has many potential applications such as power generation, drug discovery, medical diagnosis, cancer treatment, etc. While adopting IR for those beneficial applications, a definite amount of radiation exposure to human is unavoidable. Both natural and translation application resulted man made sources combined exposure to the public is estimated to be 2.8 mSv per year. Figure 1.1 shows the sources and their proportion together contributing to background radiation (1). However, considering the benefits of IR, as well as reported side effects, use of radiation application is firmly regulated and monitored. Thus to decrease the risk of harm associated with radiation, its use is often regulated by the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR), International Atomic Energy Agency (IAEA), and International Commission on Radiological Protection (ICRP) to evaluate radiation risks, provide recommendations, and promote safe use of radiation technologies, which evolve rapidly and gain in complexity.

Figure 1.1: Sources that contribute to background radiation

Source: world-nuclear.org
1.2. Health effects of radiation exposure

A harmful effect of radiation overexposure includes deterministic effects (e.g. radiation sickness, skin radiation burn, cataracts, infertility) and stochastic effects (e.g. cancer). Manifestation of these effects can take from weeks to years. The severity depends upon multiple parameters including total radiation absorbed dose, dose rate, volume of body exposed, tissues involved, radiation source, as well as personal characteristics of overexposed people (e.g. age, health status). Effect of radiation and its symptoms with respect to its dose and organ exposed is given in figure 1.2 (2).

*Figure 1.2: Effect of radiation on various organs, symptoms of radiation exposure and changes of death rate based on exposure level*

Source: Guardian.co.uk: World Nuclear Association

1.3. Biological effects of radiation

While the beneficial effects of radiation are well accepted and being expanded into many areas, the concern related to those application are better understood by knowing the effect of its exposure on the living system. When radiation traverses through a biological system, it deposits energy along its track (tissues, cells). In turn if the energy of the photon is more than that of the binding energy between the atoms in a molecule, it disrupts the target and damages the molecule either by direct deposition of energy or indirectly through free radicals.
Chapter I: Introduction

resulting from the lysis of a biomolecule. Nevertheless, the actions are not uniform for different types of radiation: direct deposition of energy is the dominant process in interaction of high linear energy transfer (LET) particulate radiations. Whereas, low LET radiations such as $\gamma$- and X- radiation lose its energy indirectly; in specific, radiation interacts mainly with water molecules within the cell to produce free radicals which are short-lived yet extremely reactive. Examples are $\text{H}_2\text{O}^+$ (water ion) and $\text{OH}^-$ (hydroxyl radical). These free radicals in turn can cause damage to the target within the cell (3).

The deposited energy alters cellular structure and functions by interfering with the structure of genetic material, the DNA. The changes include single-strand breaks, DNA protein cross-links, double strand breaks (DSBs), etc. Magnitude of all those alterations determines the final outcome of the exposure. Cell damage caused by altered DNA structure provokes cell signalling, transcription genes and recruits its enzymes as a response for cellular stress by means of complex signal transduction cascade pathways. Cell cycle arrest occurs and based on DNA damage it sends cells to apoptosis or DNA repair machinery. Eventually, those changes could result into stochastic effect is also called as mutational effect on single cells at low dose and non-stochastic or cell killing effect (4-6). The mean lethal dose for a cell is 1.5 Gy if the irradiated area includes the nucleus, whereas the viability of the cell remains unaffected even after 250 Gy if only the cytoplasm is irradiated (7). This clearly demonstrates that the biological effects of irradiation are due to its interaction with the nucleus. Figure 1.3 summarises the information on biological effects of IR.

1.4. Biomarker

The term ‘biomarker’ has been adapted from epidemiology by free radical biologists to describe a change in a biological molecule (lipids, nucleic acids, proteins) that has arisen from attack by reactive oxygen/ nitrogen or halide species. The commonly available biomarkers of radiation exposure are classified based on either its adopted methodology or on end products. A summary of various biomarkers are shown in table 1.1.
Figure 1.3: Sequential events on the biological effects of IR exposure

Ionizing radiation

Direct action

Indirect action

Free radical generation

H₂O (Absorption)

H₂O⁺, H₂O*, e⁻ (Excitation & Ionization)

H₂O⁺, OH⁻ H⁻ (Recombination)

H₂O, H₂O₂, OH⁻, H⁻

Intracellular damage

Damage to ECM/Neighbouring cells

Bystander phenomenon

Mediated via release of GFs, cytokines, interleukins, ROS, ect.

DNA
- Base lesions
- Intercalation
- Strand breaks
- MN, CA

Lipid
- Lipid peroxidation
- Changes in membrane viscosity and dynamics

Protein
- Amino acid conversions
- Inter and intra-strand cross linking
- Cleavage
- Oxidation
- Carbonylation

No repair

Apoptosis

Repair

Normal Cells

Cell survival

Mutation / Carcinogenesis
### Table 1.1: Biomarkers of radiation exposure and their classification

<table>
<thead>
<tr>
<th>S.No</th>
<th>Techniques/Biomarkers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><strong>Cell based assays</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Haematological changes</td>
<td>(8, 9)</td>
</tr>
<tr>
<td>2</td>
<td><strong>Chromosome based assays</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dicentric chromosome (DC)</td>
<td>(10)</td>
</tr>
<tr>
<td></td>
<td>Premature chromosome condensation (PCC)</td>
<td>(11)</td>
</tr>
<tr>
<td></td>
<td>Micronucleus (MN)</td>
<td>(12, 13)</td>
</tr>
<tr>
<td></td>
<td>Translocation measurements by fluorescence in situ hybridisation (FISH) and GTG banding</td>
<td>(14, 15)</td>
</tr>
<tr>
<td></td>
<td>Sister chromatid exchange (SCE)</td>
<td>(16)</td>
</tr>
<tr>
<td>3</td>
<td><strong>DNA based assays</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypoxanthine guanine phosphoribosyltransferase (HPRT) mutation</td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td>Fragmented DNA by Comet assay</td>
<td>(18)</td>
</tr>
<tr>
<td></td>
<td>Glycoporphin A (GPA) mutation</td>
<td>(19)</td>
</tr>
<tr>
<td></td>
<td>T-cell antigen receptor (TCR) mutation</td>
<td>(20)</td>
</tr>
<tr>
<td></td>
<td>Adenine phospho-ribosyl-transferase(APRT) mutation</td>
<td>(21)</td>
</tr>
<tr>
<td></td>
<td>Human leukocyte antigen-A (HLA-A) mutation</td>
<td>(22)</td>
</tr>
<tr>
<td></td>
<td>Beta globin (β-globin) mutation</td>
<td>(23)</td>
</tr>
<tr>
<td></td>
<td>Lactate dehydrogenase-X (LDH-X) sperm cell mutation</td>
<td>(24)</td>
</tr>
<tr>
<td>4</td>
<td><strong>Gene expression assays</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transcription genes (example p53, Stat-3)</td>
<td>(25)</td>
</tr>
<tr>
<td>5</td>
<td><strong>Protein biomarkers</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>γH2AX foci</td>
<td>(26)</td>
</tr>
<tr>
<td></td>
<td>C-reactive protein (CRP)</td>
<td>(27)</td>
</tr>
<tr>
<td></td>
<td>Serum amyloid A (SAA) protein</td>
<td>(28)</td>
</tr>
<tr>
<td></td>
<td>fms-like tyrosine kinase receptor-3 ligand</td>
<td>(29)</td>
</tr>
<tr>
<td></td>
<td>Citrulline</td>
<td>(30)</td>
</tr>
<tr>
<td>6</td>
<td><strong>Radiation metabolomics</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Levels of creatine, histamine, taurine, amylase, and prostaglandins in the urine</td>
<td>(31)</td>
</tr>
<tr>
<td>7</td>
<td><strong>Physical techniques</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Electron paramagnetic resonance (EPR) or Electron spin resonance (ESR) spectroscopy</td>
<td>(32-34)</td>
</tr>
<tr>
<td></td>
<td>Luminescence dosimetry</td>
<td>(35)</td>
</tr>
<tr>
<td></td>
<td>Activation techniques (radioactivity measurement in biological and non-biological samples)</td>
<td>(36)</td>
</tr>
<tr>
<td>8</td>
<td><strong>Computational techniques</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Analytical dose reconstruction</td>
<td>(37)</td>
</tr>
<tr>
<td></td>
<td>Numerical approach of dose reconstruction</td>
<td>(38)</td>
</tr>
</tbody>
</table>
Available biomarkers are grouped into three categories based on its stability. (i) **Early markers** - Before these damages get repaired, they are measured by the very sensitive protein based assays such as γH2AX assay, CRP etc. Since these damages return back to normal within a few days of exposure, it has to be measured as soon as possible and the techniques rely on the signal strength. Besides, early cellular effects and tissue responses are measured from the profiles of blood cell counts, gene expression and protein markers present in the serum. These measurements also need to be done within few days after exposure.

(ii) **Unstable rearrangements** - Misrepaired DNA damages are seen as chromosome aberrations such as DC, break, ring and MN. They are called as unstable rearrangements present in non-diving cells; nevertheless, these inheritable damages are removed once the cells enter into cycle.

(iii) **Stable rearrangements and mutations** - Chromosomal translocations are the stable rearrangements that can transmit to daughter cells and cause the serious health effects to the exposed individuals. Translocations are mitotically stable and thus it does not affect the frequency in stable cells (39).

1.5. **Biodosimetry**

Quantification of those changes on DNA can be related to predict the response of the exposed individual and also to estimate the dose. The estimation of absorbed dose using the biomarkers is known as biodosimetry. All those biomarkers have a potential to be a good biodosimeter. Of which, old and gold standard biomarker is DC; that this is being used for several decades for dose estimation in accidental and uncontrolled acute or protracted radiological events (40). Thus it helps the clinicians for providing better medical management for severely to lesser radiation exposed individuals.

1.6. **Need for radiation biodosimetry**

IR is being used in enormous field for improving the human life. The field of nuclear technology is also associated with risk. Through quantification of absorbed dose, those risk can be better manageable. While generally it is well regulated and radiation workers and the workplace are monitored with suitable devices. However, the biodosimetry is needed for the following: (i) verification and evaluation of certain or suspected accidental overexposure in the absence of physical dosimetry data due to non-use of individual dosimeters in controlled zones, (ii) verification and evaluation of certain or suspected accidental
Chapter I: Introduction

overexposure of persons operating in zones where only environmental, not individual, dosimetry is carried out, (iii) checking of uncertain results of physical dosimetry for various reasons, including "spurious" exposure and exposure of the dosimeter to chemical agents or other physical agents, (iv) biological type dosimetric integration for diagnostic, prognostic and therapeutic purposes and (v) evaluation of population involvement after a nuclear emergency. Overall, cytogenetic biodosimetric investigations not only have a reassuring psychological function, but also helps in finding false claims of exposure (41).

1.7. Chromosomal aberration (CA)

The entire process in the formation of CA by radiation is complex and involves chain of events. Radiation interaction on cells produces a random pattern of primary particle tracks in those cells. Later, the charged particles lose their energy by creating δ-rays which are secondary electron tracks starting on the path of the primary particle and thus the randomness of energy deposited is constrained. It is these constraints to randomness which distinguish radiations of different quality. Secondary electron tracks, by direct ionisation and through the agency of chemical radicals cause damage to the chromatin structure. If those changes are not rectified (misrepair) then it will end up in the formation of CAs (42).

In an exposed system, the induced DNA damages resulted in the formation of various form of CAs due to mis-rejoining. Those CAs are very sensitive biological end point, and reflects the effect of radiation induced DNA damage on the whole genome. Chromosome alterations such as DC, MN in binucleated cells, fragments and DC in PCC in peripheral blood lymphocytes (PBL) are being used to estimate the absorbed dose in acute radiation exposure. FISH technique using chromosome specific paint probes, a pan-centromeric and telomeric probe for whole genome, has opened up the possibility of detecting stable CAs in an exposed individuals, and made it possible to perform retrospectively biological dosimetry (43).

Initial event in the DC formation is the induction of two DSB on two different chromosomes; an exchange between the centromeric pieces of the two broken chromosomes which in its complete form is accompanied by an acentric chromosome (AC) fragment composed of the acentric pieces of these chromosomes. Particularly after high doses, multicentric configurations can be
formed. Centric ring (CR) is much rarer than the DC; it is an exchange between two breaks on separate arms of the same chromosome and is also accompanied by an AC fragment. These are considered as radiation specific aberration (44, 45). Formations of DC and CR are given as simple illustration (figure 1.4 and 1.5).

**Figure 1.4: Formation of DC**

<table>
<thead>
<tr>
<th>Exposure of radiation on two different chromosomes</th>
<th>Double strand break occurs on chromosomes</th>
<th>Formation of dicentric chromosome(DC) &amp; acentric fragment (AC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome I</td>
<td></td>
<td><img src="image1.png" alt="Diagram of DC formation" /></td>
</tr>
<tr>
<td>Chromosome II</td>
<td></td>
<td><img src="image2.png" alt="Diagram of DC formation" /></td>
</tr>
</tbody>
</table>

**Figure 1.5: Formation of CR**

<table>
<thead>
<tr>
<th>Exposure of radiation on chromosome</th>
<th>Double strand break occurs on chromosome</th>
<th>Formation of centric ring(CR) &amp; acentric fragment (AC)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image3.png" alt="Diagram of CR formation" /></td>
<td><img src="image4.png" alt="Diagram of CR formation" /></td>
<td><img src="image5.png" alt="Diagram of CR formation" /></td>
</tr>
</tbody>
</table>
1.8. Conventional methods on the analysis of DC and dose estimation

DC assay is the conventional biodosimetric assay. The blood sample from the exposed individuals is the preferred choice because of its invasive nature of sampling. Furthermore, as analyses of chromosomes with traditional cytogenetic techniques necessitate cells that are dynamically dividing, they are stimulated to enter into cycle under *in vitro* condition to get sufficient metaphase chromosomes requisite for analysis (46). Development of defined growth media, commercial availability of many purified reagents used in cell cultures resulted into accurate identification of various chromosomes and its abnormality. For which the cells are stimulated with phytohemagglutinin and arrested at metaphase by using colcemide or colchicine. Then cells were harvested, casted and fixed on a glass slides. The metaphase chromosomes were giemsa stained and analysed under light microscope at high resolution. To calculate the frequency of DC, around 1000 metaphases are scored and then related it to the dose by substituting the same to reference calibration curve coefficients (47).

One has to remember that scoring huge number of metaphases in uniformly stained preparation requires lot of time. Moreover, interpretation of abnormality on poor metaphase preparation is cumbersome. To overcome these limitation, the assay has undergone technical and technological developments to meet the growing demand. Even the developments are not devoid of limitations; which also summarised (table 1.2) and detailed in the forthcoming paragraphs.
### Table 1.2: Developments on DC assay

<table>
<thead>
<tr>
<th>Type of assay</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C-banding</strong></td>
<td>▪ Confirmation of DC from that of twist in the chromosomes</td>
<td>▪ Chemical used in this procedure alters chromosome morphology</td>
<td>(48-50)</td>
</tr>
<tr>
<td>• Centeromeric FISH</td>
<td></td>
<td>▪ More expensive</td>
<td></td>
</tr>
<tr>
<td>• mBAND (multicolor band)</td>
<td></td>
<td>▪ Highly expertise dependent</td>
<td>(5, 39, 51-59)</td>
</tr>
<tr>
<td>• Spectral karyotyping (SKY)</td>
<td></td>
<td>▪ Need quality fluorescent microscope with different band pass filters</td>
<td></td>
</tr>
<tr>
<td><strong>Triage mode scoring (~50 metaphases)</strong></td>
<td>▪ Reduces scoring burden and use less time</td>
<td>▪ Decreases assay sensitivity</td>
<td>(60-63)</td>
</tr>
<tr>
<td><strong>Automated analyser</strong></td>
<td>▪ Scoring is automated</td>
<td>▪ Difficult to distinguish first and second mitotic cell</td>
<td>(64, 65)</td>
</tr>
<tr>
<td>▪ No scorer variation</td>
<td>▪ Captures all possible metaphase in the slide</td>
<td>▪ Chances of considering adjacent spread DC for scoring</td>
<td></td>
</tr>
<tr>
<td><strong>High throughput system</strong></td>
<td>▪ Completely automated and hence eliminates manual processing</td>
<td>▪ Eliminates DC present in twist or overlap</td>
<td>(66, 67)</td>
</tr>
<tr>
<td>▪ No man power required</td>
<td>▪ Able to process more number of samples at a time</td>
<td>▪ High cost</td>
<td></td>
</tr>
<tr>
<td>▪ RABiT</td>
<td></td>
<td>▪ Affordability</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>▪ Care should be taken to incorporate sample collection, transport and processing into the local emergency response plan in an intelligent way, in order to fully benefit from these systems</td>
<td></td>
</tr>
<tr>
<td>Laboratory networking</td>
<td>Helps to ensure the robustness, accuracy and reproducibility of its procedures</td>
<td>Sample transport</td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Harmonize protocols and to share the workload during radiation triage</td>
<td>Involves experimental variables</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Needs regular networking exercises to meet consistency</td>
<td></td>
</tr>
<tr>
<td>Quick scan</td>
<td>Quickly prioritize samples for full DC analysis</td>
<td>Individual centromeres were not counted</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Provides highly accurate dose estimates to those individuals who received clinically significant radiological doses</td>
<td>Only detects obvious damages</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Feasible to produce initial dose estimates for up to 360 individuals per day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Web-based scoring</td>
<td>Supports the feasibility of networking</td>
<td>Very good resolution in capturing and uploading images is required</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A fast manual rejection of metaphases not representing first mitoses and non scorable metaphases was necessary</td>
<td></td>
</tr>
<tr>
<td>Imaging cytometry</td>
<td>Combine the speed and increased sample size of a flow cytometer and the imaging capabilities of a microscope assay to verify DC by visualization</td>
<td>Efficiency and accuracy of the protocols remains limited</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC Score™</td>
<td>Significantly reduces the scoring time and the observation fatigue</td>
<td>Requires manual pre-processing and post-processing review of DCs, especially at low radiation doses</td>
<td></td>
</tr>
<tr>
<td>Automatic dicentric chromosome identifier (ADCI)</td>
<td>Useful for the identification of radiation exposed individuals with occupational, military or clinical overexposures</td>
<td>Accuracy is highly dependent on the quality of images in the samples</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Relevant for assessing long term effects of radiation therapy in cancer patients</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Image repository | ■ Advantageous in the case of small radiation accidents, as new galleries with 150 images could be generated and uploaded within half an hour. Automatically captured images from one laboratory could then be analysed by scorers in other labs  
■ Immediately increases the scoring capacity by assessing external scorers who could be located anywhere in the world  
■ Reduces turnaround time for dose estimates and allow the opportunity to quickly get a second opinion in difficult cases | ■ Does not have neither operational arrangements for its use in a real emergency response, not enough flexibility nor capacity to be an appropriate instrument for a large-scale accident where several thousand or more persons are potentially exposed | (75) |
1.9. Inter-laboratory comparison (ILC) and networks for DC assay

As per the regulatory requirements, many nations have established reference cytogenetic biological dosimetry laboratories. Many biodosimetry laboratories (Argentina, Brazil, China, Chile, Cuba, Finland, France, Germany, Greece, Israel, Italy, Japan, Hungary, Netherlands, Philippines, Republic of Korea, Russian Federation, Spain, Turkey, UK and USA) are in the IAEA coordinated research programme on cytogenetic biodosimetry. 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 intercomparison studies to ensure suitable performance of individual laboratories. These developments are to use cytogenetic networks to enhance the capabilities at the time of triage and dose assessment for mass casualty radiation events (76, 77). When a network is established, it is imperative to perform regular intercomparison between the laboratories of the network to maintain and assess confidence, capabilities, capacities, accuracy and throughput of the participating laboratories. It also helps to bring up well defined procedures and protocols for certain issues like packaging, labelling and shipping biological samples (78). Laboratories that have established national and/or regional networks to enhance their capabilities are listed in table 1.3.
Table 1.3: Established networks and participant biodosimetry laboratories

<table>
<thead>
<tr>
<th>Network</th>
<th>Location of network and Name of the network</th>
<th>Participating laboratories</th>
<th>References</th>
</tr>
</thead>
</table>
| National                     | Cytogenetic Emergency Network (CEN) Canada  | • Consumer and clinical radiation protection bureau, Health Canada (Ottawa),  
  • Defence research and development Canada (DRDC, Ottawa),  
  • Atomic energy of Canada limited (AECL, Chalk River) and the McMaster institute of applied radiation sciences (McIARS, Hamilton),  
  • >18 clinical laboratories those are located within a 400 km radius of these four main laboratories. | (79)       |
| National                     | Reseau de dosimetry biologique France     | • Institut de radioprotection et de surete nucleaire (IRSN),  
  • Two laboratories from French alternative energies and atomic energy commission (CEA),  
  • National museum of natural history (MNHN) | (77)       |
| National                     | The Chromosome Network Japan              | National institute of radiological sciences (NIRS) with seven surrounding laboratories                                                                                                     | (80)       |
| Regional                     | Korean Radiation Biodosimetry Network South Korea | Korea institute of radiological & medical sciences with six surrounding laboratories                                                                                                                                                                                                           | (77)       |
| Regional                     | North America                             | • Health Canada (HC),  
  • Defence research and development Canada-Ottawa research centre (DRDC),  
  • Canadian nuclear laboratories limited (CNL),  
  • McMaster university,  
  • Oak ridge institute for science education (ORISE),  
  • Armed forces radiobiology research institute (AFRRI). | (78)       |
<table>
<thead>
<tr>
<th>Region</th>
<th>Network</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latin America</td>
<td>Latin American Biological Dosimetry Network (LBD Net)</td>
<td>Laboratories from Argentina, Brazil, Chile, Cuba, Mexico, Peru, Uruguay, France, Spain, Finland, Germany, Turkey, Europe and United Kingdom</td>
</tr>
<tr>
<td>Asia-Pacific</td>
<td>Asia-Pacific Network</td>
<td>Argentina, Australia, Austria, Canada, Germany, India, Indonesia, Japan, Malaysia, Philippines, Republic of Korea, Singapore, Thailand, USA, Vietnam</td>
</tr>
<tr>
<td>Europe</td>
<td>Tri-Partite</td>
<td>France, Germany and the UK</td>
</tr>
<tr>
<td>Europe</td>
<td>MULTIBIODOSE</td>
<td>Stockholm university (SU), Sweden, Bundesamt fur strahlenschutz (BfS), Germany, Universiteit gent (UGent), Belgium, Health protection agency (HPA), United Kingdom, Institut de radioprotection et de surete nucleaire (IRSN), France, Instituto superiore di sanità (ISS), Italy, Norwegian radiation protection authority (NRPA), Norway, Radiation and nuclear safety protection (STUK), Finland, Universitat autonoma de barcelona (UAB), Spain, Institute of nuclear chemistry and technology (INCT), Poland, Helmholtz zentrum munchen (HMGU), Germany, Bundeswehr institut fur radiologie in verbindung mit der universitat ulm (BIR), Germany, Gray institute for radiation oncology and biology, University of Oxford (UOXF), United Kingdom, European radiation dosimetry group (EURADOS), European network registered in Germany</td>
</tr>
<tr>
<td>Region</td>
<td>Network</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Europe</td>
<td>Realising the European Network of Biodosimetry (RENEB)</td>
<td>Twenty three organisations from sixteen European countries</td>
</tr>
<tr>
<td>Global</td>
<td>World health organisation (WHO’s) BioDoseNet</td>
<td>Sixty three labs from Belgium, Bulgaria, Finland, France, Germany, Greece, Italy, Poland, Portugal, Romania, Spain and UK</td>
</tr>
<tr>
<td></td>
<td>IAEA’s Response and Assistance Network (RANET)</td>
<td>Continues to change</td>
</tr>
</tbody>
</table>
1.10. Scoring less number of cells towards triage (triage scoring method)

IAEA has recommended to score 1000 metaphases at lower doses or to score either 1000 metaphases or 100 DC at high doses for reliable dose estimation. Efforts are being made to reduce the cells need to be scored to get a meaningful dose estimation to meet emergency; in particular, nuclear disaster, triage is a priority besides dose estimate. It has been suggested that where medical treatment would be administered only to those receiving more than 2.0 Gy. In these situations, the sensitivity of the assay can be reduced by decreasing the number of metaphase cells scored which subsequently greatly reduces the time required for analysis. Standard triage DC analysis now consists of analyzing only 50 metaphase spreads, providing a threshold of detection of 1–2 Gy; still adequate to guide treatment of acute radiation syndrome (ARS). The time efficiency of triage based scoring has been further improved dramatically, without losing accuracy in the dose estimate, by introducing a scoring technique termed ‘DCA Quick Scan’. The basis for this method is that individual centromeres are not counted and metaphase spreads are only rapidly examined for obvious damage, thereby eliminating the counting of individual chromosomes to ensure the completeness of the analysed cell, as done in the conventional DC scoring. This method has been demonstrated to be as accurate as conventional triage scoring, while reducing the time for scoring by a factor of about 6. For a mass casualty situation, scoring 20 cells using Quick Scan would provide excellent triage dose estimation (78, 86).

1.11. International organization for standardisation (ISO) guidelines for DC as a quality measure

Despite the dose estimation with DC method which is well established, a stringent quality measures are required to maintain its accuracy. ISO described two quality policy for the above purpose: ISO 19238 (Radiation protection - Performance criteria for service laboratories performing biological dosimetry by cytogenetics) and ISO 21243 (Radiation protection - Performance criteria for laboratories performing cytogenetic triage for assessment of mass casualties in radiological or nuclear emergencies - General principles and application to dicentric assay) (87). Principal parameters of ISO regulations are, (i) incorporation of good laboratory practices, (ii) appropriate documentation of protocols, (iii) development of an own dose response curve at each laboratory, excluding inter-laboratorial differences, (iv) set-up of DC scoring only by trained technicians, (v)
demonstration of scoring experience by intra- and inter-laboratory comparisons, using standard scoring criteria (88, 89).

1.12. Why DC remains as gold standard?

Since mid-1960s analysis of solid stained DC has been used as a primary biodosimetry tool. From that time onwards DC assay have seen great improvements in bringing the technique to a point where DC analysis has become a routine component of the radiation protection programmes of many countries. Experience with DC analysis in the evaluation of hundreds of cases of suspected or verified radiation overexposures throughout the world has demonstrated the usefulness and limitations of this technique for the purpose of providing personal absorbed dose estimates in the absence of physical dosimetry. For many years the DC assay using blood lymphocytes was the only method of biological dosimetry available, and still today it is the technique most frequently used. It represents the most robust cytogenetic bioassay for early response dose assessment (77) due to the following reasons:

- High specificity to IR (10)
- Low background frequency in non-exposed populations (about 1–2 in 1,000 cells) (90)
- High occurrence among unstable aberrations (in the order of 60%) (91)
- Considerable range of dose detection. Lower detection limit is about 0.05 and 0.01 Gy for low- and high-LET radiation, respectively (76, 92)
- It can provide information on whole-body (homogeneous) vs. partial-body (heterogeneous) exposures (47)
- Low cost method for chromosome staining (conventional staining with giemsa)
- No special microscope is needed for analysis
- Easy to analyse large numbers of cells through semi-automation (64)

1.13: Need for establishing individual dose response curve for a laboratory

Even though, procedure to estimate dose with DC is well established, IAEA recommend that each service providers to establish their own dose response curve due to many variables known to influences the dose estimation when using DC assay. Some of the known variables which are listed over a period of research are as follows.
 **Temperature:** DC yields have been measured after applying various temperatures between 4 to 37 °C before, during and after X-irradiations and in the radiation free intervals. It was found that chromatin lesion repair was completely suppressed at ≤21 °C, whereas lesion formation is reduced only <17 °C. The interaction between repairable lesions which leads to DC is also suppressed by low temperatures. Hypothermic suppression of chromatin lesion repair and interaction is fully reversible at least up to 12 hour of maintenance of the stored state of these lesions. Thus temperature has an effect on DC formation (93).

 **Culture time:** The effect of culture time on DC yield was studied by Hone et al., (2005) and found that DC yields at 3.0 Gy for 30 minutes exposure to γ- radiation and harvested at 48th hour were approximately 10% lower than 3 minutes exposure; however, the yield was not differ each other. Whereas, DC yields from the 3 minutes exposed cells cultured over different durations (45-72 hours) remained constant up to 51 hours and then rose to a different value beyond 60 hours. The increase at 60-70 hours compared with the yield at 48 hour and the rise was about 50% (94).

 **Culture medium:** The choice of culture medium on the rate of cell proliferation was studied by Purrot et al., (1981). They used four different culture mediums namely Ham's F10 or RPMI 1640 or Eagle's MEM or TC 199. Among these Ham’s F10 or RPMI 1640 media yielded significant numbers of good mitotic cells than others (95).

 **Colcemid exposure:** DC frequencies and resultant dose estimations were compared from 48 and 72 hour cultures with colcemid added at the beginning, after 24 hour or for the final 3 hours. The frequencies of DC in first division cells increased with the cell culture time, providing better dose estimations. It was suggested that the combination of a lower than usual concentration of colcemid combined with its earlier addition and longer culture time provide metaphases better suited to estimate the dose (50).

 **Scoring:** Knowledge on scoring criteria, skill of the scorer and number of metaphases scored plays an important role for determining the assay sensitivity and importantly dose estimation (63). The scorer should have the efficiency of fast scoring and to identify DC present in acrocentric
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Standardization and validation of dicentric chromosome and gamma-H2AX assays for radiation triage and to quantify radiation absorbed dose. (Ph.D. (RR)/243/FT/VII/2011)

chromosome or in twisted chromosome with its associated fragments. Scoring less number of cells (20-50 metaphases) decreases the assay sensitivity to 1.0 Gy which is sufficient for preliminary medical triage. However, additional scoring should be performed in order to get a higher certainty of dose estimation and potentially to re-categorize the exposed populations.

- **Importance of background aberration:** The background frequency of aberration has been reported as an important variable that can influence the coefficient of reference calibration curve and the dose estimation. Generally the DC frequency is reported to varies between 1-2/1000 metaphases (96). Inter-laboratory trials using experimental studies and photomicrograph data from metaphase analyses reported considerable variance in results due to individual scorer selection of metaphases and scoring of aberrations with a low frequency (97).

- **Effect of Age, gender and smoking:** There is evidence for an age related increase in CAs (excluding gaps) (90). There was good evidence from studies using FISH, that stable translocations also increased with age. The evidence regarding unstable chromosomal changes such as DC was unclear, with both positive and negative findings reported, which may have been affected by the method used to score DC. There is no evidence for any gender related cytogenetic alterations. It was also noted that smoking may be a risk factor for DC formation. The increase in unstable aberrations (e.g. DC) was evident in heavy smokers (>20 cigarettes/day) (98).

- **Effect of drinking alcoholic beverages:** An elevated frequency of CAs was documented in PBLs from alcoholics. No information was retrieved on the short term effects of alcohol drinking on DNA damage in PBLs (99).

- **Effect of diet:** Kazimirova et al., (2004) studied the effect of vegetarian and non-vegetarian diet on DC frequency and did not found any significant difference. Hence, diet does not play a role in the formation of DC (100).

- **Effect of micronutrients:** There was no evidence from the available limited trials that vitamin supplementation independently gives rise to
cytogenetic damage in PBLs. There was some limited evidence that vitamin supplementation may affect sensitivity of PBLs to chemically induced cytogenetic damage, but the data are inadequate to draw any firm conclusions particularly with regard to specific vitamins that might be relevant with regard to reduction of chemically induced cytogenetic damage (101).

- **Effect of Genotype:** A relatively small association has been reported between slow N-acetyltransferase (NAT2 acetylator) genotype and cytogenetic damage assessed by metaphase analysis and FISH analysis (using chromosomes 1,2,4) in PBLs, although this finding was particularly evident in smokers. There was evidence for an increase in baseline frequency among GSTM1-positive subjects, CYP1A1, MSPI heterozygotes (in new borns), CYP2E1 wt/*5B heterozygotes and EPHX ‘low activity’ genotype. However, the authors were cautioned that these data were derived from investigations of relatively few individuals and need to be examined in further studies (102).

1.14. Technical limitation of DC assay

Even today DC assay stands as a “gold standard” method for estimation of dose for accidental radiation exposure (103). Like any methodology, the dose estimation with DC is not devoid of limitations. (i) DC have a limited applicability in procedures that involve chronic and retrospective dose estimation (104), (ii) necessity of highly skilled personnel with good experience for accurate scoring (105), (iii) requirement of 52-55 hours time from sample receipt to culture the blood sample, followed by scoring of CA to derive dose (106), it became unsuited for large scale emergency scenarios (107) and (iv) even difficulty in automated analysis system with compromised sensitivity.

1.15. Alternate for DC assay

Owing to those above mentioned shortfalls, newer, simple, rapid and reproducible tool in quantifying radiation absorbed dose are being developed.

1.15.1. PCC

PCC is one of the alternate for DC assay which does not require culturing of cells i.e. it condense chromatins when it is not in mitosis (108). This was achieved by fusing interphase cells to mitotic Chinese hamster ovary (CHO) or
HeLa cells using fusing agent like polyethylene glycol (PEG). The advantage of this method is that damage can be observed shortly after blood sampling. FISH (109) or C-banding (110) is also possible with this for accurate identification of specific aberrations. The major drawback of PCC is that it needs a pre-prepared CHO mitotic cell which is not possible all time. And hence radiation accidents are rare unexpected event one cannot spend too much of resources for maintaining the CHO mitotic cells in expecting for exposed sample. To overcome these cells maintaining issues PCC were also induced using chemicals namely calyculin-A (111) or okadaic acid (112). This assay is not suitable for low dose range (< 1 Gy), cell analysis is more complicated than conventional lymphocyte cell culture, requiring the use of carcinogenic chemicals and also needs good technical person to perform the assay. Thus PCC is not suited technique for emergency situations. Hence the search is continued to identify the better alternative biodosimetric technique. One such is the newly emerged biodosimetric technique is γH2AX assay which has the potential to overcome those difficulties.

1.15.2. γH2AX and its formation

Variant of histone H2A is H2AX which constitutes 2–25% of mammalian histone depending on the organism and cell type. H2AX is composed of a central globular domain, flanked by N-terminal and C-terminal tails which possess sites for a variety of post-translational modifications such as acetylation, biotinylation, phosphorylation, methylation, and ubiquitination. H2AX is structurally similar to other H2A species except for the presence of a unique COOH terminal tail, containing a serine four residues from the C terminus (omega-4). Upon induction of a DSB, the H2AX omega-4 serine residue becomes rapidly phosphorylated to form γH2AX. This modified γH2AX is easily visualized in the nucleus after immunofluorescence staining with specific fluorescence antibodies and serves as a sensitive indicator of DNA DSB formation (26, 113). H2AX is rapidly phosphorylated at Ser-139 (termed γH2AX) by members of the phosphatidylinositol 3-kinase (PI(3)K) family and upon DSBs induction, form foci at these sites and promote recruitment of other DNA damage response proteins (114). This change in the level of phosphorylation can be quantified using fluorescence microscopy (115), flow cytometry(116), western blotting (117) and image cytometry (118) (figure 1.6).
Figure 1.6: Phosphorylation of H2AX molecule and its measurements

Ionising radiation produces DSBs on DNA

DSBs triggers the activation of multiple factors involved in DNA repair and chromatin remodelling and initial phosphorylation of the histone H2AX molecules

Subsequent phosphorylation of the histone H2AX molecules

Immunofluorescence staining

Quantification of DNA damage by microscopy/flow cytometry/western blotting
1.15.2.1. γH2AX and its response to radiation

It is well accepted fact that a living system exposed to IR induces DSB. γH2AX foci formation was observed within seconds of induction of DNA breaks by IR and the level of phosphorylation increases linearly with the amount of damage (119). The linear relationship is maintained from 30 minutes to 16 hours in human PBL after irradiation with γ- radiation for doses in the range 0.05 to 2 Gy (120). Several reports have described large residual γH2AX foci that are formed several hours after irradiation (121-124) and these foci can persist for months (125). For an X- radiation dosage of 1 Gy, there is always around 1-2% of H2AX that becomes γH2AX (26). Doses as low as 0.05 Gy can be detected with this marker after in vivo exposure (126). Using rapid flow cytometry method, the dose response of γH2AX induction in lymphocytes and lymphocyte subsets showed a linear dose response up to 40 Gy with a gradual saturation above this dose (26, 127-132).

This assay has also been used as a marker of in vivo radiation exposures; γH2AX foci analysis could be used as a tool for estimating of DNA damages after cardiac catherization (133) computed tomography (134). γH2AX foci analysis has also been used for estimation of individual radiation doses by comparisons of in vivo and in vitro exposed lymphocytes to fractionated irradiation or angiography (135). In addition, several reports showed that γH2AX foci analysis can be used to measure DNA damage induced by local radiotherapy; analysis of γH2AX foci in prostate cancer biopsies after in vivo radiation showed reproducible quantifications of foci numbers in the prostate specimens in the dose region 0-1 Gy (136, 137). These findings support the γH2AX assay as a potential biodosimetric tool in estimating a radiation absorbed dose.

1.15.2.2. Recent developments on γH2AX

While majority of studies employed fluorescence microscopy and flow cytometry for quantitative and qualitative measurement of γH2AX, a dual method has been developed to determine fluorescence yield using high-speed microscope imaging analysis. This workstation has been designed to fully automate the γH2AX immunocytochemical protocol, from the isolation of human blood lymphocytes to the image acquisition step (138). Histolab™ is considered as semi-automatic since it still requires that the images be loaded separately by the user during analysis (120). The major advantage of this software is that user
interface is accessible, rendering parameters setting for foci detection very easy. Furthermore, in the current version of HistoLab™, the operator has the possibility of removing manually the cells with aberrant staining or morphology. Foci counter (139) is another semi-automated system. It was developed specifically to score foci, with a simple interface and few parameters to change (faster to set up). These programs are ideal for simple scoring of a reduced number of cells/conditions, where the quality of the images/slides frequently requires operator intervention. Since these programs require user intervention to select nuclei, the time of the analysis is their main disadvantage.

Cell profiler, a freeware that allows fully automated analyses were operator intervention is not possible. Cell type selection has to be done graphically using measurements of nucleus staining and morphology. For the nucleus analysis, an adaptive algorithm is applied on the counterstained nucleus pictures to detect nuclei with a range of diameters. Inside these nuclei, another module can use a various choice of algorithms on the γH2AX pictures (pre-subjected or not to a treatment) to detect foci with possibly minimum and maximum diameters. The last modules measure the area and intensity of both nuclei and foci. Cell profiler is a program that offers a great number of measurements and detection options. The main drawback of this freeware is a less user-friendly graphical interface that may render the vast number of detection algorithms and measurements overwhelming for a beginner. Cell profiler is also able to measure the mean intensity of the associated colour channel inside each cell (140). It can also perform with ELISA-based assays (141) and high content screening assays (142).

A rapid automated biodosimetry tool (RABiT) system is designed to be completely automated, from the input of the capillary blood sample into the machine, to the output of a dose estimate. It will serve both to reduce panic by reassuring those who were not significantly exposed, as well as triaging those in need of medical attention (118).

Owing to the advantages and potential for the radiation triage, γH2AX assay also validated by ILC. Rothkamm et al., (2013) have done manual versus automated γH2AX foci analysis across five European laboratories and concluded that the γH2AX assay may be useful for rapid triage following a recent acute radiation exposure with regular re-calibration or inclusion of reference samples to ensure its consistent results between laboratories or over long time periods (143).
Followed this, Barnard et al., (2015) have conducted RENEB with eight European laboratories, which consisted of a telescoring comparison of 200 circulated foci images taken from eight samples, and a comparison of ten fresh blood lymphocyte samples that were shipped overnight to participating labs after 4 or 24 hours post-exposure. Despite large variations between laboratories in the dose response relationship for foci induction, the obtained results indicated that the network should be able to use the γH2AX assay for rapidly identifying the most severely exposed individuals within a cohort who could then be prioritised for accurate chromosome dosimetry (115).

1.15.2.3. Issues associated with γH2AX assay

The half-time of dephosphorylation was estimated from the time response curves and found to be 3-12 hours (144). The level of γH2AX returning to background levels after 24 hours, it would be difficult to obtain blood samples quickly enough after exposure to be able to measure a signal. Based upon the rapidly changing kinetics of γH2AX and the large inter-individual variability in response, it would only be possible to use this assay for determining the dose received by an individual under precisely controlled exposure conditions (145).

Consistent with CA, many variables are known to influence the dose estimate with the γH2AX assay. Those variables are age (146), ethnicity, race, alcohol consumption (147), smoking (148), inflammation (149), stress (150), temperature (151), heat (152), genetic factors (153), inter-individual variables (154), difference in foci size and its signal intensity, shipment, antibody, staining quality (115).

1.16. Need for standardization and validation

Quantification of radiation absorbed dose, using DC assay, requires very good standardization. To quantify radiation absorbed dose, the DC frequency obtained from exposed individuals are inferred from the standard in vitro reference dose response curve of biodosimetry laboratory validated by the IAEA or the authorized regulatory agency of respective country. The most important feature is that a dose response curve established in one laboratory cannot be readily used by others due to influence of many factors like variation on baseline frequency of DC, dose and dose rate employed for in vitro irradiation, culture variables, scoring criteria adopted, inter-individual variations in scoring and error/uncertainties in calibration and curve fitting (155). The interpretation of dose
using a calibration curve produced elsewhere may introduce substantial extra uncertainty, and therefore, it must be recommended that any laboratory intending to carry out biological dosimetry should establish its own dose response data. The DC is considered to represent the gold standard for diagnostic biosimetry, which should be used as a reference method to validate new tools such as γH2AX for rapid dose estimation or triage. This would support clinical decision making in the management of exposed individuals.

1.17. Aim

Validation of DC and γH2AX fluorescence intensity measurements in the blood lymphocytes exposed in vitro and in vivo to ionizing radiation to quantify radiation absorbed dose.

1.18. Objectives

✓ To construct a reference dose response curve for DC assay from human PBL samples exposed in vitro to γ- and X- radiation and stained with giemsa.
✓ Authenticate the scoring of DC in giemsa stained metaphases with centromere specific FISH and inter-laboratory comparison.
✓ To establish a reference dose response curve for γH2AX assay (flow cytometry) from human PBL samples exposed in vitro to γ- and X- radiation.
✓ Dose reconstruction using DC and γH2AX assay from the blood lymphocytes of cancer patients underwent radiotherapy.
1.19. References


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