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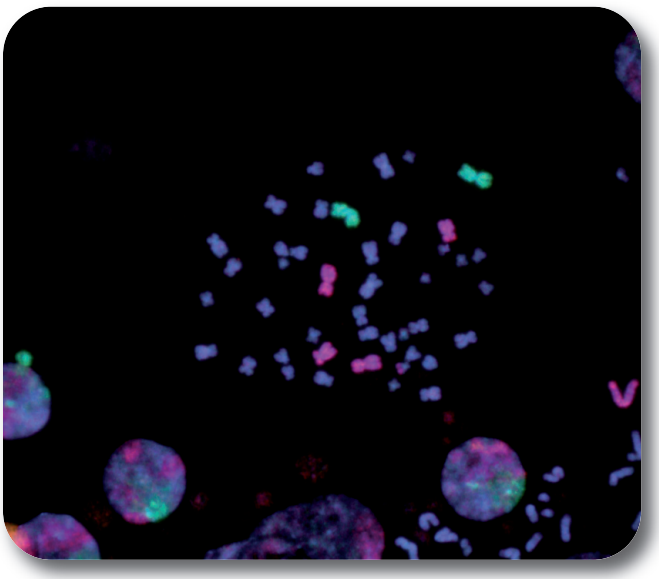
BIOLOGICAL DOSIMETRY

HOW TO MEASURE THE ABSORBED DOSE IN DIFFERENT SCENARIOS

ANNA GIOVANETTI, ANTONELLA SGURA, GIORGIA AVERSA



Biological dosimetry

How to measure the absorbed dose in different scenarios

Anna Giovanetti, Antonella Sgura, Giorgia Aversa

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1. THE USE OF BIOLOGICAL DOSIMETRY

Ionizing radiations are a ubiquitous component of our life and major concerns have been raised on their carcinogenic potential. We are all exposed as population, but there are specific modalities of exposure for workers and for patients submitted either to diagnostic procedures or to therapeutic radio-treatment. Recently it has been raised concern on the possibility for the population to be exposed to radiation due to a nuclear incident or a terrorist attack. The radiation protection International and National Agencies have established specific criteria and procedures for decreasing radiation exposure and for minimizing unwanted effects in all these different scenarios. Prerequisite for planning any intervention is the knowledge of the absorbed dose.

Workplaces and exposed workers are equipped with physical dosimeters allowing the qualified experts to assess the dose to each worker and to apply the envisaged measures. No information can be achieved on the effects, on previous exposures or on differences in individual radiosensitivity.

The use of ionizing radiation for medical procedures, in particular for diagnostic purposes, has recently dramatically increased. In US in 2006, around 377 million diagnostic and interventional radiological examinations and 18.6 million nuclear medicine examinations were performed resulting in a very significant growth in cumulative exposure in the low doses range. Although the general radiobiologic principles underlying external beam and radionuclide therapy are the same, there are significant differences in the biophysical and radiobiologic effects (Kassis, 2008). In radiology dose is delivered at constant dose-rate while in nuclear medicine dose-rate is declining, function of the initial uptake, distribution, biological and physical half-life (Chianelli et al., 2011).

Moreover, in nuclear medicine, protection from radiation involves not only patients but also their relatives and medical staff (Salvatori and Lucignani, 2010). ICRP has edited a reference manual on Radiological Protection in Medicine (ICRP, 2007) with regard to the medical exposure of patients, including their comforters and carers, and volunteers in biomedical research. It addresses the proper application of the fundamental principles (justification, optimisation of protection, and application of dose limits) of the Commission's 2007 Recommendations to these individuals. In diagnostic and interventional procedures, justification of procedures and management of the patient dose are the appropriate mechanisms to avoid unnecessary or unproductive radiation exposure. With regard to comforters and carers, and volunteers in biomedical research, dose constraints are appropriate. For therapeutic applications, it is not considered appropriate to apply dose limits or dose constraints, because such limits would often do more harm than good.

Often, there are concurrent chronic, severe, or even life-threatening medical conditions that are more critical than the radiation exposure. Nevertheless medical procedures need to be justified and radiological protection has to be optimized or for and also for the medical applications it is not planned entering data on individual cumulative radiation exposures. The dose absorbed due the medical treatment can be, not always, be calculated basing on the delivered dose.

No information is available on cumulative exposure, radiation effects or radio-sensitivity, that are key information for planning further radio-treatment or countermeasures for minimizing side effects. In case of nuclear or radiological emergencies physical dosimeters are not utilizable and also type of exposure, route of entry in the organism and other crucial information may be not available in the first hours.

However, triage decisions have to be undertaken very soon in order to split the exposed subjects: no- or very low exposure (people are not requiring medical assistance and can go home), exposure to discrete amount of radiation (immediate hospitalization, application of medical countermeasures, isolation) or subjects exposed to lethal doses (hospitalization, application, if feasible of bone marrow transplant o other relevant interventions). The knowledge of the range of the absorbed dose will permit to focus medical staff and instrumental resources only on subjects needing medical assistance. Otherwise the medical structures can be overwhelmed and unable to properly intervene (Etherington et al., 2011). Thus there is the urgency to have early biomarkers for knowing, within the first hour after the emergency, the individual absorbed dose.

In conclusion, biological dosimetry is needed when physical dosimetry can not be used or does not provide sufficient information.

2. BIODOSIMETERS

Biological dosimetry does not measure the exposure in real time, but, as previously stated, the biological changes induced by radiation. There are both indicators of exposure or effects. Often the two aspects overlap as in the case of deterministic effects induced by high-doses, as for the acute radiation syndrome clinic (ARS) that is characterized by damages in skin and in haematopoietic, gastrointestinal, and cerebrovascular systems; the severity of the lesions depends on the amount of the absorbed dose. In the case of stochastic effects, induced by low doses, the biomarkers used to measure the absorbed dose, not always imply a clear detriment of health. It was, however, often demonstrated that an increase in the frequency of these indicators is associated with an increased risk of radiation-induced cancer and may be indicative of radio-sensitivity.

To be used effectively a biodosimeter must:

- to be measured on tissues or fluids easily obtainable.
- the effect must be specific of radiation.
- the response should vary directly depending on the dose.
- it has to measure also chronic or repeated exposure.
- it must be possible to measure retrospectively exposure also after years.
- the measure must be simple, fast or automated.

In this report we first briefly introduced the main methods currently used to measure radiation exposure paying particular attention to the dicentric assay, considered to be the gold standard, the FISH technique and the Comet Assay.

2.1 Blood count

The lympho-hematopoietic elements are among the most highly replicated tissues in mammals and as such are among the most radiosensitive and the peripheral blood count may well serve as a biological indicator of damage. The “normal” range for absolute lymphocyte count can vary, even in a healthy adult population. Samples collected from small number of “healthy” workers at AFRRI (Armed Forces Radiobiology Research Institute, US) suggest a “normal” range from about 1.35-3.5 x 109 cells/liter. The kinetics of the lymphocyte response to radiation is likely to be at least as important as the absolute lymphocyte count.

Lymphocyte counts can be depressed (or in some cases, increased) by drugs, infection, and many clinical disorders unrelated to radiation. The hematopoietic syndrome develops in the dose range between 2–3 Gy and 8 Gy, while mitotically active hematopoietic progenitors are unable to divide after a whole-body exposure > 2–3 Gy, which results in a hematologic crisis in the ensuing weeks. AFRRI has developed a lymphocyte depletion kinetics algorithm based on the AFRRI BAT program. (Waller et al., 2009)

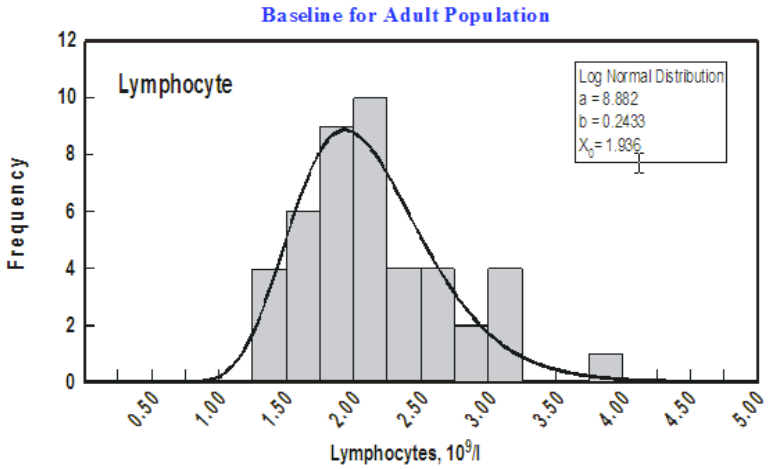


Fig 1 *Source: Biodosimetry Assessment Tool (BAT), Armed Forces Radiobiology Research Institute*

At lower doses (< 2 Gy) radiation induces mild cytopenias without significant bone marrow damage (Anno et al., 1989). Peripheral blood lymphopenia may develop within the first 6-24 hours after a moderate - to high-dose exposure. In addition to inducing apoptosis whose effect is not seen before the first cell cycle, radiation alters recirculation properties of lymphocytes (Fliedner et al., 1996).

Data extrapolated from mass casualty event at Chenobyl Nuclear Reactor accident (Vorobiev 1997) demonstrated that after significant (> 1 Gy) radiation dose all blood elements can be affected adversely. Increasing dose will have increasing effect, and effects will be seen earlier. As showed in *Fig 2* and *Fig 3*, colored by Dr. William Dickerson (AFRRI), based on Figure 6 from Vorobiev (1997).

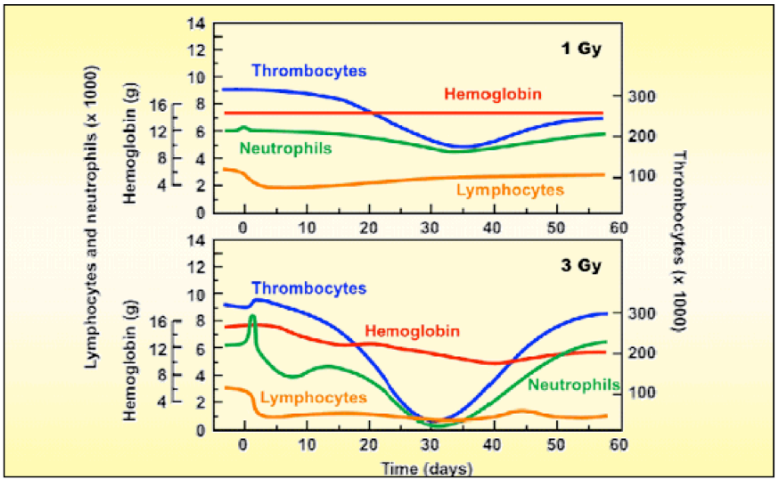


Fig 2 *Radiation Effects on Blood Counts - 1 Gy vs 3 Gy*

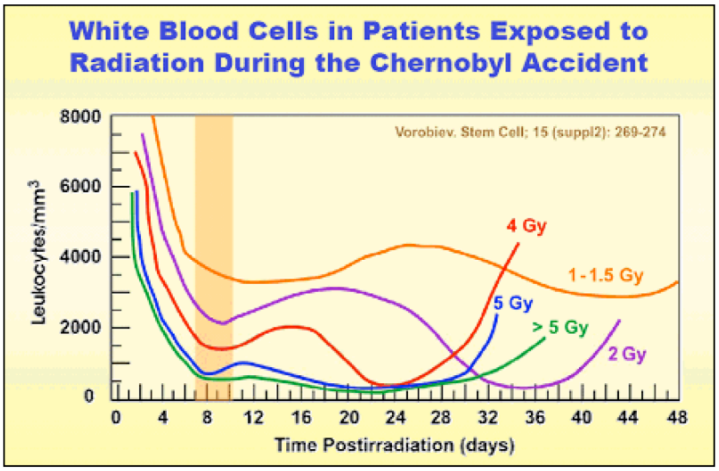


Fig 3 *White blood cell count over time after exposure to different doses of ionizing radiation*

2.2 Cytogenetic biomarkers

As proposed by Hutchinson in 1966 the main target for mutagens, in particular ionizing radiation is DNA (Hutchinson, 1966). DNA, along with proteins, constitutes the chromosomes, localized in the nucleus of every eukaryotic cell. During mitosis or meiosis, the chromosomes condense and become identifiable. In human have been identified as 23 pairs chromosomes. Initially, the pairs of chromosomes were classified according to size and shape, following the use of banding techniques and probes the ability to more accurately identify individual chromosomes and chromosomal regions has been greatly improved.

2.2.1 Dicentrics

Of the biological methods adopted for dosimetry purposes, cytogenetic analysis has been the most popular one. The occurrence of chromosome aberrations in peripheral blood lymphocytes has been used for over 20 years as the most sensitive biological indicator of external irradiation (Bauchinger et al., 1984).

Dicentric chromosomes are an abnormal chromosome with two centromeres (*Fig 4*) rather than the normal one. A dicentric chromosome is doubly tethered (by its two centromeres) and is pulled to the opposite poles of the spindle when the cell divides, causing the chromosome to break. The broken ends of the chromosome fuse with each other in the daughter cell and form a new dicentric chromosome. Dicentric chromosomes therefore lead to chromosome instability.

Fig 4 *Example of dicentric chromosome in human metaphase*

In some cases, the biological dosimetry is the only possibility to get information about the assumed radiation exposure. In this context, it was demonstrated that the dicentric assay is able to assess health risks and guide medical treatment decisions in large scale radiation accidents like Chernobyl (Piatkin EK, et al., 1989) or Goiânia (Ramalho AT and Nascimento AC, 1991). When an acute exposure occurred and a blood sample for chromosome analysis should be obtained as soon as possible, the dicentric assay represents the method of choice.

In 1962 it was suggested by Bender and Gooch that dicentric chromosomes in peripheral lymphocytes could well be used for the detection and dose assessment of human radiation exposures and these authors have used this method for the first time in the sense of biological dosimetry at the occasion of the so-called Recuplex criticality accident at Hanford, USA.

Calibration dose-response curves using a reference radiation are essential for estimating the absorbed dose in a biodosimetric test. For dicentric scoring in blood lymphocytes, calibration curves have been established for many years and are very reliable and universally used (Bender et al., 1988).

It has been shown that there is no significant difference in the aberration frequencies after whole body irradiation of patients and their blood in vitro with X-rays (Leonard A. et al., 1995) demonstrating that calibration curves can be used for the conversion of an observed frequency of exchange aberrations in an exposed individual into a dose.

Protocol

Blood/cells culture

To collect chromosomes two different techniques usually are used: conventional colcemid-block and chemical-induced premature chromosome condensation (PCC). The latter seems to be a powerful method for biological dosimetry, as it allows some problems exhibited by the conventional colcemid block method to be overcome. In particular, when peripheral blood lymphocytes are cultured according to standard

protocol, the mitotic index is extremely low in some individuals, such as in the elderly, after accidental exposure to high radiation doses, as a consequence of immunological diseases. The use of calyculin A, an inhibitor of type 1 and 2A protein phosphatases, has been demonstrated to be a simple method for measuring chromosome damage both in G1 and G2/M chromosomes (Gotoh et al., 1999).The population observed is not biased by cell-cycle delay. For the very high dose experiments, the mitotic index is too low and PCC must be used to obtain data on chromosomal damage (Kanda et al., 1999).
A volume of 0,5 ml of blood is added to 4.5 ml of complete medium in a culture flask, and flasks incubated a 37° C in a humid atmosphere with 5% CO_2. Growth media is RPMI 1640 supplemented with 10% foetal calf serum, 1%L-glutamine, 1.5 % penicillin/streptomycin 5000UI.ml-1. T-lymphocytes are stimulated in vitro to proliferate by 2% mitogen phytohaemagglutinin (PHA). Following 47 h incubation, calyculin A (50nM) is added to the medium. Flasks is kept in calyculin A 1 h for the analysis of G1, G2 and M-phases.

Harvest
Following incubation and PCC, cells are transferred in centrifuge tubes and centrifugated for 5 min at 1000 rpm. Pellet is carefully resuspended in KCl 75mM and incubated at 37 °C. After 20 min, freshly prepared Carnoy's fixative (methanol:acetic acid= 3:1) is slowly mixed into the solution. Tube centrifuged and pellet are resuspended in pure Carnoy's fixative. Tubes are kept 20 min on ice and pellets are washed again three times in the fixative. Tubes filled with fixative could be stored at -20 in the dark.

Slide preparation
Cells in fixative are dropped on pre-cleaned warm and humid slides. The metaphase preparations can be further processed for conventional Giemsa staining (3% of Giemsa in water for 10 min).

Scoring criteria
Scoring of chromosomal aberrations is performed analysing 500 cells, generally considered as sufficient to obtain a reliable result. In some cases it might be helpful to increase the number of cells.

Background frequency of dicentric chromosomes
An important prerequisite for dose reconstruction of exposed persons is the knowledge of the background levels of the corresponding chromosome aberration types, determined in blood samples of healthy unexposed persons.

A careful analysis of the baseline frequency of chromosome aberrations is important because this information has a direct bearing on the precision of dose estimate, especially when individuals are suspected to have been exposed to a low dose of radiation. Numerous studies have been performed to evaluate the spontaneous background levels of dicentric chromosomes. The frequencies vary extensively among different studies (Bauchinger M., 1995) and can be attributed, at least partly, to the scoring criteria of the observer. In consequence, for purposes of biological dosimetry, each laboratory should have its own control data and its own dose-response curves.

2.2.2 *Micronuclei*

Because the needs of trained personnel and the time factor of chromosomal aberration assay, attention has been turned to the micronucleus assay because of the close relationship between the micronucleus formation and the presence of chromosomal aberrations. Since micronuclei are derived from chromosomal aberrations, they can serve as an indirect measure of chromosomal breakage. In vitro studies on radiation-induced micronuclei revealed dose-response relationship, which indicates that micronuclei have a potential to serve as a biological indicator of radiation exposures. Compared to the traditional chromosomal analysis, the micronucleus technique does not require highly trained personnel and is much faster (Heddle, 1973). This permits an easy analysis of many thousands of cells in a very short time.

Micronuclei are small round bodies found in cytoplasm outside the main nucleus which they resemble in shape, structure, and staining properties. Micronuclei arise from acentric fragments that fail (because of the lack of a centromere) to incorporate into the daughter nuclei during cell division (Heddle and Carrano, 1977). Micronuclei can also be formed by entire chromosomes that lag behind during mitosis due to a failure of mitotic spindle, or by complex chromosomal configurations that pose problems during anaphase. Thus, formation of micronuclei can be induced by both clastogenic agents and mitotic inhibitors. The problem over the fate of micronuclei in cells that have divided more than once, was solved by Fenech and Morley (1985) with an elegant method in which cytokinesis is blocked after 44 h of culturing by adding cytochalasin B, which results in the formation of binucleated cells, i.e. cells that divided only once. Micronuclei are then scored only in these cells (*Fig 5*).

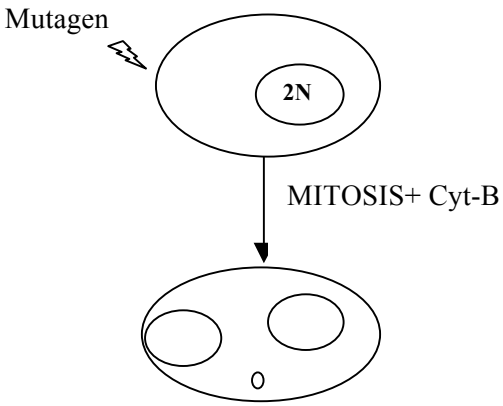


Fig 5 *Scheme of MN formation in binucleated cells*

Spontaneous frequencies of micronuclei in binucleated cells of eight donors was 4.4 micronuclei per 500 cytokinesis-blocked cells, and a linear dose-response was obtained after in vitro irradiation of lymphocytes of the eight donors with l-4 Gy.
Due to its simplicity and the short time needed for analysis, most attractive use of the micronucleus method will be in:

1. Screening large populations for hypersensitivities to clastogenic agents prior to start of occupational exposures (e.g. pre-employment screening of nuclear plant workers);
2. rapid screening of individuals for hypersensitivities to radiation or specific drugs prior to the start of radiotherapy or chemotherapy;
3. Rapid estimation of acute radiation overexposures when serious health effects are expected; and
4. Rapid screening for the most exposed individuals in the cases of large-scale radiation accidents.

Protocol

Blood/cells culture (see as for dicentric)
Harvest
To obtain binucleated cells, cytochalasin B (Sigma), previously dissolved in DMSO, was added at a final concentration of 6 mg/ml to cell cultures 24 hr before fixation. Cells were pelleted by centrifugation (10 min at 1,000 rpm), resuspended in 0.075 M KCl, incubated for 2 min at room temperature, and gently fixed three times with methanol:acetic acid (5:1), stored at -20 °C for staining procedures (Giemsa or DAPI).

Scoring criteria
For each experimental point, a minimum of 1000 binucleated cells are analysed on coded slides for MN induction. Only MN not exceeding 1/3 of the main nucleus diameter, not overlapping the main nucleus and with distinct borders are included in the scoring.

2.2.3 Fluorescence in situ hybridization for the detection of chromosome aberrations

FISH is a method for localizing and detecting specific nucleotide sequences in morphologically preserved tissue sections or cell preparations by hybridizing the complementary strand of a nucleotide sequence (the probe) with the DNA of interest (also called target). There are essentially five types of probe that can be used in performing *in situ* hybridization (Read and Strachan, 2003):

1. Oligonucleotide probes
2. Single-stranded DNA probes
3. Double-stranded DNA probes
4. RNA probes
5. PNA probes.

The probe could be directly or undirectly labeled. If the probe has been labeled indirectly, an extra step is required for visualization of the non-fluorescent haptene that uses an enzymatic or immunological detection system.
Whereas FISH is faster with directly labeled probes, indirect labeling offers the advantage of signal amplification by using several layers of antibodies, and might

therefore produce a signal that is brighter compared with background levels. A large variety of commercial probes are now available, so as to allow hybridization even in less optimal conditions.
Scoring of FISH-painted chromosomes is relatively straightforward and fast, whereas dicentric scoring in Giemsa-stained cells must be performed by expert cytogenetics and is very time-consuming.

Chromosome Painting
FISH using whole human chromosome-specific DNA probes has opened new possibilities for detecting stable aberrations, and nowadays it is widely used for biological dosimetry of ionizing radiation (Tucker J. D., 2001). In fact, the analysis of dicentrics (unstable aberrations) in solid-stained chromosome preparations is very reliable to estimate recent and acute radiation exposures, but not for chronic or past exposures because the yield of dicentric chromosomes decreases with time after irradiation (Bauchinger M., 1995). For this reason, chromosome painting (*Fig 6*) was mainly developed to allow detection of stable exchange-type aberrations.
For a correct analysis of aberrations in painted (as well as in solid-stained metaphases), the detection of centromeres may be critical, especially in the case of mouse acrocentric chromosomes: it is obtained either by DAPI counterstainingthat, by itself, after alkaline/heat denaturation, gives a bright signal to centromeric heterochromatin or by centromeric FISH staining. In solid-stained metaphases, the conventional nomenclature classifies each simple interchange as a single event (e.g., one dicentric plus an acentric fragment), and distinguishes between complete (if no unrejoined breaks are found) and incomplete exchanges (e.g., a dicentric chromosome without any acentric fragment).

The use of painting techniques has led to the development of new nomenclature systems. These are mainly based on the recognition of color switches between the fluor-conjugated probe and the counterstaining dye. According to the Protocol for Aberration Identification and Nomenclature Terminology (PAINT) (Tucker et al., 1995) each abnormal painted chromosome or fragment is described individually using the letters “A” and “a” to indicate counterstained chromosomal material, the letters “B” and “b” to indicate painted material, capital letters to designate centromere containing regions, lower case letters to indicate acentric regions.

Thus, typical PAINT-classified aberrations are t(Ab), dic(AB) or ace(ab), where t stands for translocation, dic for dicentric, and ace for acentric fragment. Another independently developed nomenclature system is that of Savage and Simpson (S&S) (Savage J. R. and Simpson P. J., 1994) which is the method of choice for an interpretation of the mechanistic aspects of aberration formation.

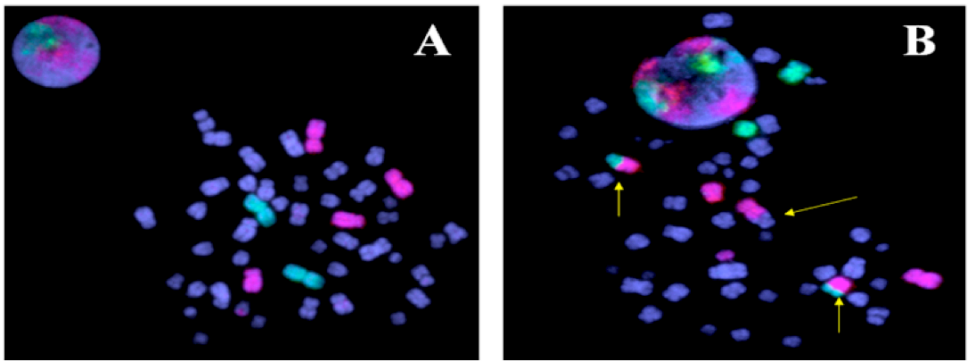


Fig 6 *Example of metaphases with 1, 4 (red staining) and 2 (green staining) painted chromosomes. Metaphases with normal painted chromosomes (A) and chromosomal aberrations in the form of translocations (B) are reported as representative. DNA was counterstained with DAPI*

When FISH painting is applied to the analysis of aberrations induced by chemical mutagens (Sgura A. et al., 2005) a further type of aberration, not encountered in radiation studies, must be considered for the correct assessment of induced effects: it is the presence of one or more supernumerary fully painted chromosomes in a metaphase with the correct chromosome number for the specific species. These supernumerary chromosomes derive from chromatid-type exchanges in the pericentromeric region, which are induced by radiation only when G2 cells are targeted.

Unless all chromosomes are hybridized with their specific painting probes conjugated with different fluorochromes and a computerized image analysis system is used to assign a different color to each of them, only a portion of the whole karyotype is usually painted (*Fig 6*). Aberrations involving painted chromosomes thus represent only a subset of all induced aberrations. It may be of importance to estimate the total number of aberrations induced to predict the fate of exposed cells or individuals. Assuming that aberrations are randomly distributed over different chromosomes, to convert the frequency of observed aberrations into an estimate of the frequency of total aberrations/genome, one has to know the proportion of the genome covered by each color probe (or probe cocktail).

Then, if p represents the proportion of genome painted in a given color, q the proportion of genome painted in a second color and r the unpainted fraction of the genome, the fraction of detectable exchanges is calculated as $S = 2pq + 2pr + 2qr$. Multiplying the number of scored metaphases by S, one obtains the number of cell (genome) equivalents. The ratio between the number of observed aberrations and the number of cell equivalents gives the estimated frequency of aberrations/genome. Also, assuming that n is the recommended number of metaphases to be scored when the whole genome can be scanned for aberrations, n/S gives the number of painted metaphases to score to obtain the amount of information equivalent to that provided by n fully analyzed metaphases (Tucker J. D., et al., 1997).

Protocol

There are almost as many methods for carrying out *in situ* hybridization. The basic principles for *in situ* hybridization are the same. Here we report a brief outline of the common procedural cytogenetic steps.

Slide Preparation and Fixation

Proper technique for preparing slides is considered essential for bothclassical cytogenetics (banding) and molecular cytogenetic procedures (FISH). Different protocols were specifically designed for use with particular types of biological material or cells or for particular purposes such as detection of structural aberrations, numerical aberrations, or sequence amplifications. To preserve morphology, the biological material must be fixed. For metaphase chromosome spreads, methanol–acetic acid fixation is usually sufficient. In the basic protocol, cells are resuspended in 3:1 methanol–acetic acid fixative(s) and dropped or smeared on commercially precleaned slides, which are dried at various temperatures. For paraffin-embedded tissue sections, use formalin fixation.

Cryostat-sliced sections fixed for 30 min with 4% formaldehyde have been used successfully. Unfortunately, a fixation protocol that can be used for all substrates has not yet been described. The fixation must be optimized for different applications

Aging

Slides are subjected to dry heat and/or ethanol, to denature the proteins, to remove water and fixative from the preparations, and to enhance the adherence of the material to the glass.

Chromosome painting: chromosomes dropped onto clean microscope slides are left to age for 24hrs at room temperature.

RNase Treatment

RNase treatment serves to remove endogenous RNA and may improve the signal-to-noise ratio in DNA–DNA hybridization.

Chromosome painting: for chromosome painting there is no treatment with RNase

Permeabilization and Pretreatment

Pretreatment serves to increase target accessibility by digesting the protein that surrounds the target nucleic acid. The most common enzyme used is pepsin because it has the advantage that it can be easily inactivated by pH changes, and the reaction is easier to control

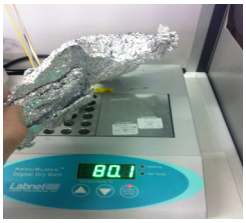
Chromosome painting: for chromosome painting there is no need of permeabilization and pretreatment

Denaturation

Denaturation of DNA, which separates the two strands, is obtained by heat or alkaline treatment. Heat treatment can be performed on hot plate.

Chromosome painting: The slides are incubated in denaturing solution (70% formamide/2X SSC) for 2 min at 65 °C. Denatured slides are dehydrated through a series of 3-min ethanol washes (70, 85, 100%) and air dried.

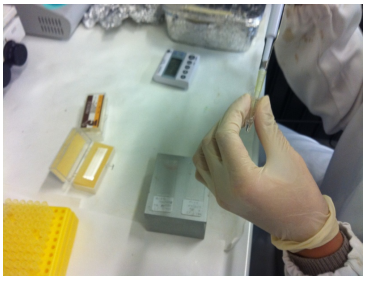
The DNA probes are denatured 10 min in a 72 °C water bath.

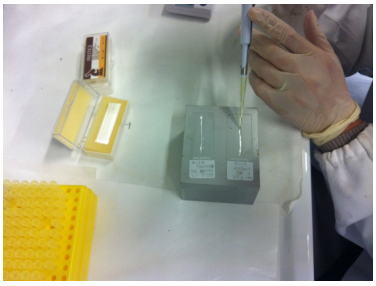


Hybridization

The hybridization step consists in simply mixing the single-strand probes with the denatured target DNA.

Chromosome painting: the probe is added to the slides on a slide warmer at 45 °C. The slides are covered with coverslips





Provided that different probes are conjugated with fluorochromes with nonoverlapping emission wavelengths, multiple targets can be simultaneously hybridized in the same cells.

Sealed with rubber cement (glass cover slip sealant) and incubated overnight at 37 °C in a moist chamber.

Humidified chamber can be prepared using a clean plastic box with a lid.

-Soak several paper towels in water and place them at the bottom of the tray.

-Put the lid on top and place chamber in the 37 °C incubator.

It would be better to darken the box with aluminum foil (to protect the labeled probe from the light).

Detection and Amplification of FISH Signal

The detection of the probes is the final step of FISH. Is the probe is labeled by a fluor-dUTP the probe-target hybrids can be visualized under a microscope immediately after the hybridization reaction. When a DNA probe is not labeled with fluorochrome-dUTP, the fluorescent signal is usually provided by a fluor-labeled molecule (primary detection reagent) that binds the dUTP with high affinity.

To amplify the signal, another fluorescent-labeled molecule (secondary detection reagent) that recognizes the primary reagent is used. (For details: Pacchierotti F, Sgura A. Fluorescence in situ hybridization for the detection of chromosome aberrations and aneuploidy induced by environmental toxicants. Methods Mol Biol., 2008;410:217-39)

Chromosome painting: usually for chromosome painting are available direct labeled probe so, in this case, we do not need to detect or amplify the signal.

Posthybridization Washes

Labeled probe can hybridize nonspecifically to sequences that are partially but not entirely homologous to the probe sequence. They can be dissociated by performing washes of various stringencies. The stringency of the washes can be manipulated by varying the formamide concentration, salt concentration, and temperature.

Chromosome painting: the slides are washed 5 min in 0.5X SSC at 72 °C and 2 min in 2X SSC/0.005% Tween 20 at room temperature in a choplin jar.

Counterstaining

Fluorescent DNA counterstaining is usually performed with red fluorescent propidium iodide (PI) or with blue fluorescent DAPI.

Chromosome painting: unlabelled chromosomes are counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

An antifade agent should be mixed with the counterstaining dye solution to retard fading. Chromosome aberrations are viewed with a fluorescence microscope equipped with specific filters and a CCD camera.

Microscopy

Finally the signals are evaluated by fluorescence microscopy. A fluorescent microscope contains a lamp for excitation of the fluorescent dye and a special filter that transmits a high percentage of light emitted by the fluorescent dye. There has been considerable improvement in both the hardware and software that are used for the analysis of FISH

images. Cooled charge-coupled-device (CCD) cameras and fluorescence filter sets for microscopy that are more specific and efficient have improved the sensitivity and resolution of imaging, and sophisticated software facilitates the acquisition and processing of images.

2.2.4 Comet Assay

The Comet Assay, also known as the Single Cell Gel Electrophoresis Assay, is a rapid, sensitive and relatively simple method that permits quantitative assessment of the effects of DNA damaging or apoptosis inducing agents and mechanisms in a wide variety of important target cell types. The assay also has great utility in studies of DNA repair. It combines the simplicity of biochemical techniques for detecting DNA single and double strand breaks (strand breaks and incomplete excision repair sites), alkali-labile sites, and cross-linking, with the single cell approach typical of cytogenetic assays.

This was first introduced by Ostling and Johanson in 1984 as neutral assay in which the lysis and electrophoresis were done under neutral conditions. Electrophoresis causes the migration of unwound or fragmented DNA out of the nucleus of the cells, resulting in a characteristic appearance that can be visualized microscopically, with fluorescent staining of the DNA. The image obtained looked like a "comet" with a distinct head, comprising of intact DNA and a tail, consisting of damaged or broken pieces of DNA hence the name "Comet" Assay. The approach of Ostling and Johanson was based on previous work published by Cook et al., (1976), who developed a method for investigating nuclear structure based on the high salt lysis of cells in the presence of non-ionic detergents. The more versatile alkaline method of the comet assay was developed by Singh and co workers in 1988.

The unwinding and electrophoresis processes at pH 13 facilitates the detection of double strand breaks, expresses alkali labile sites (ALS) in addition to all types of lesions listed above (Miyamae et al., 1997). This method was developed to measure low levels of strand breaks with high sensitivity.

The main advantages of the Comet Assay include the collection of data at the level of the individual cell, allowing more robust statistical analyses; the need for a small number of cells per sample (<10,000); sensitivity for detecting DNA damage and use of any eukaryote single cell population both in vitro and in vivo, including cells obtained from exposed human populations and aquatic organisms for eco-genotoxicological studies and environmental monitoring (Collins et al., 1997; Dixon et al., 2002; Lee and Steinert, 2003; Jha, 2004; Giovanetti et al., 2008). In combination with certain bacterial enzymes (e.g. formamidopyrimidine glycosylase Fpg, endonuclease III, uracil-DNA glycosylases etc.), which recognise oxidised purines and pyrimidine bases, this assay has been used to determine oxidative DNA damage which has been implicated in several health conditions (Collins et al., 1993; Collins et al., 1997a; Collins et al., 2001; Kruman et al., 2002).

In combination with the fluorescence in situ hybridisation (FISH) technique (Comet-FISH), the application of this assay has also been extended to determine sequence or gene specific damage and repair (Santos et al., 1997; McKenna et al., 2003) as well as of possible diagnostic use (Kumaravel and Bristow, 2005).

In addition, the assay is being used in translational research to assess whether tumour radio-sensitivity (Fisher et al., 2007) and chemo-sensitivity (Smith et al., 2007) can be determined.

The Comet Assay is based on the ability of negatively charged loops/fragments of DNA to be drawn through an agarose gel in response to an electric field. The extent of DNA migration depends directly on the DNA damage present in the cells. It should be noted that DNA lesions consisting of strand breaks after treatment with alkali either alone or in combination with certain enzymes (e.g. endonucleases) increases DNA migration, whereas DNA-DNA and DNA-protein cross-links result in retarded DNA migration compared to those in concurrent controls (Tice et al., 2000).

This technique provides 9 main points.

Protocol (*Alkaline Comet Assay)*

1. Preparation of slides
Soak the microscope slides in a solution of 1% normal melting agarose (1% NMA diluted in 0.01M of PBS) for almost 30 seconds.
Afterwards dry the slides and lay them in a stove at 50 °C for 1 hour.
Later, lay the slides in their own boxes at room temperature until their use.
The gel on the slides must be uniform to guarantee the visualization of comets with low background.

2. Deposition of cells
The day of the experiment 20 µl of cells are mixed with 180 µl of 0.5% low melting agarose (diluted in 0.01M of PBS).
This mix must be positioned in the center of the slides prepared before and covered with coverslip.
Then the slides are let on ice for 10 minutes to solidify the gel.
During the procedure put some tinfoil sheets between the slides and the ice to avoid the wet of slides.

3. Lysis
Take off very gently the coverslip from the slides and position them in coplins containing 50 ml of lysis buffer, for 1 hour at 4 °C in the dark.
The lysis buffer (pH 10) contains:

in 200 ml H_2O d

29,22 g NaCl (2,5 M)

7,44 g Na_2EDTA (100 mM)

0,24g Tris-HCl (100 mM)

1,6 g NaOH

pH needs to be adjusted using HCl and NaOH 1 M.

Buffer can be stored at room temperature.

Shortly before use add 1% (volume) Triton X-100 and 10% DMSO.

4. Rinsing

For the rising process put the slides in coplins containing 50 ml of electrophoresis buffer for 10 minutes at 4 °C in the dark.

The electrophoresis buffer (pH 13) contains:

in 4 lt H_2O d

1,48 g Na_2EDTA (1 mM)

48 g NaOH

After having magnetic stirrer adjust pH using HCl and NaOH 1 M. Buffer has to be stored at 4 °C.

During the rising process residues and alkaline salts are removed.

5. Unwinding

For this process position the slides inside the electrophoretic chamber and add a volume of electrophoretic buffer able to cover the slides with a layer of 0.5 cm (measure the volume of buffer utilized and use always the same amount) and let slides for 20 min at 4 °C in the dark.

6. Electrophoresis

Then slides, let in the same buffer, are submitted to electrophoresis, 30 min, 25 V, 400 mA.

DNA migrates from anode to cathode and if fragmentation of DNA has occurred the fragments will migrate faster creating the comet tail.

7. Neutralization

After the electrophoresis for neutralizing alkali, slides are washed in coplin jairs with neutralization buffer contains Tris 0.4 M (pH 7.5).

8. Dehydratation

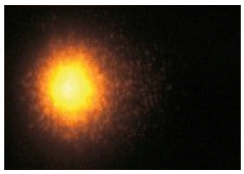
Later, slides are dehydratated with cold methanol, for 1 min, or with different ethanol passages 70%, 80%, 100% (10 min each).

9. Staining of DNA and visualization of comets

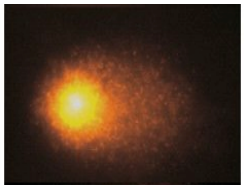
The DNA of damaged cells appears like a comet with a head (nuclear region) and a tail. The tail is determined by fragments of DNA who migrate faster trough the cathode while the undamaged DNA remains confined in the nucleus.

The different types of migration have been classified in 5 categories that correspond to 5 levels of genetic damages (*Fig 7*):

Type 1: Comet without tail where the genetic material remains inside the nucleus.: no genetic damage

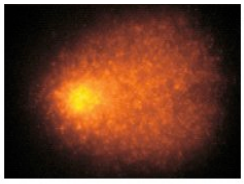


Type 2: Cell with a small tail, little migration of fragments of DNA: light genetic damage



Type 3: Cell with tail with evident migration due to a greater damage

Type 4: Cell with definite tail with a consistent amount of fragments



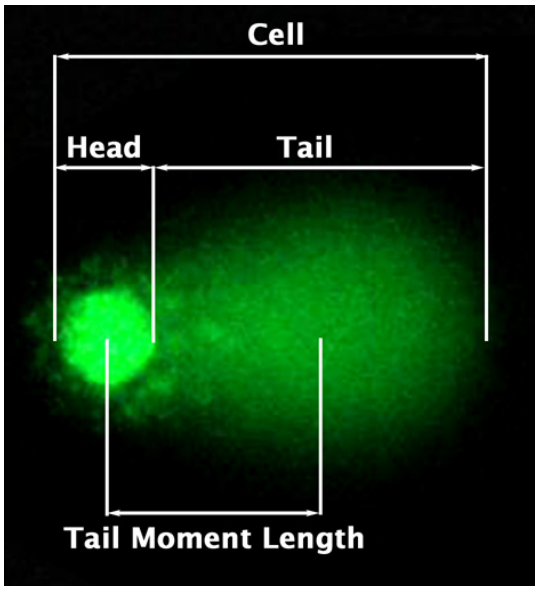
Type 5: Almost all the DNA is present in the tail: severe genetic damage

Fig 7 *The different categories of migration for visual scoring analysis*

The damages can be calculate with different methods:

Visual scoring: for this technique analyze about 200 cells for each slide and classify in according to the 5 types of damage. Then calculate the index of damage: sum the product of the percent of the cells appertaining of each class (type of comet) for the correspondent number of class. ID= 0x(number cells of type 1) + 1x(number cells of type2) + 2x(number cells of type3) + 3x(number cells of type 4) + 4x (number cells of type 5).

Automated analysis: Comets are observed and recorded by a fluorescent microscope provided with a camera associated to computer. The measurement of DNA migration can be performed using different image analysis softwares (please see the Comet Assay interest group *http://cometassay.com/*) The main parameters measured by the softwares are tail area, % DNA of tail, Tail length, or a combination of % of DNA in the tail and Tail length= Tail Moment.



Tail Length = Tail Length is the distance of DNA migration from the center of the nuclear core.

Tail % DNA = the % of DNA present in the Tail.

Tail Moment**:** Tail moment is defined as the product of the tail length and % of DNA present in the Tail.

Because the length of the tail saturates with increasing DNA damage it is suggested to utilize only the % of DNA in the Tail (Collins et al., 2001).

2.2.5 Comet Assay - Fpg

The protocol of the Comet Assay remains unchanged. The additional step consists of an incubation with the enzyme FPG (formamidopyrimidine glycosylase) after the lysis step, for 30-45 min at 37 °C. For each experimental point it is requested to process also control slides not treated with the enzyme. Here below is described the technique using the TREVIGEN, Fpg FLARE^{TM} Assay Kit , but also other companies can provide suitable kits.

Immediately after lysis slides are rinsed for 10 min in coplins containing 1X FLARE buffer in order to rebalance the pH to 8 (pH at which the enzyme works FPG). This buffer can be used for three times. The 1X FLARE buffer prepared by mixing:

25X FLARE buffer	10 ml
Deionized water (milliQ)	240 ml

Meanwhile prepare the humidified chamber where locate slides for the enzymatic reaction (eg racks boxes with absorbent paper soaked in distilled water). Drop on slides the FPG working solution prepared by mixing:

FPG FLARE REACTION buffer	98 µl
Diluted FPG enzyme	2 µl

FPG FLARE REACTION buffer was previously prepared by mixing:

25X FLARE buffer	40 µl
100X BSA	10 µl
Deionized water (milliQ)	up to1 ml

FPG dilutions:

Depending on the type of cells and their sensitivity to the enzyme the dilution can vary:
1:100 (2 µl of Fpg enzyme in 198 µl di REC dilution buffer).
1:50 (4 µl of Fpg enzyme in 196 µl di REC dilution buffer).
With HaCaT dilution to be used is 1: 50.

In control slides drop the FPG FLARE REACTION buffer only. Then put the humidified chambers containing the slides in the oven for 30-45 min at 37 °C.
After incubation with the enzyme the further steps are the same as in the case of the previously described Comet protocol.

2.3 H2AX

Thanks to the advances in molecular biology, new methods to measure exposure to radiation have been recently developed not requiring cell culturing and stimulation, that can be applied to all cell types at any stage of the cycle as the count of phosphorilated histones foci (γH2AX).

One of the earliest steps in the cellular response to DSBs is the phosphorylation of serine 139 of H2AX, a subclass of eukaryotic histone proteins that are part of the nucleoprotein structure called chromatin. Discrete nuclear foci, either induced by exogenous agents such as IR or generated endogenously during programmed DNA rearrangements, can be visualized at sites of DSBs by using a fluorescent antibody specific for the phosphorylated form of H2AX (γ-H2AX).

Briefly cells are grown and irradiated on coverslips, then fixed in 2% paraformaldehyde for 15 min, washed in PBS for 3 × 10 min, permeabilized for 5 min on ice in 0.2% Triton X-100, and blocked in PBS with 1% BSA for 3 × 10 min at room temperature. The coverslips were incubated with anti-γ-H2AX antibody for 1 h, washed in PBS, 1% BSA for 3 × 10 min, and incubated with conjugated goat anti-rabbit secondary antibody for 1 h at room temperature.

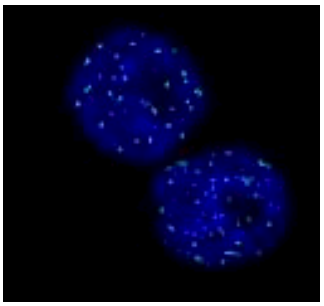


Fig 8 *The foci of γH2AX: nuclei stained with the blue fluorescent which are highlighted in green*

Cells were then washed in PBS for 4 × 10 min and mounted by mounting medium with 4,6 diamidino-2-phenylindole (Vector Laboratories). Fluorescence images were captured by using an epifluorescent microscope (Paul and Amundson, 2008).

The count of H2AX foci is a very sensitive biodosimeter, in fact the number of H2AX foci increased already after 1-3 min from exposure and from exposure to 1 mGy of X rays, however it is not stable during the time because their number rapidly decreased.

2.3.1 Microsatellites

Chromosomal aberrations and breaks do not remain long, they can be repaired or lead to cell death. Non-coding repetitive DNA sequences are sensitive to radiation-induced mutations; these mutations are not harmful to a cell and may accumulate and provide a stable molecular record of genetic damage that can be used to determine cumulative radiation exposure and health risk (Mairs et al., 2007). New mouse and human microsatellite markers have been identified and mouse and human multiplexes assay for small-pool PCR (SP-PCR) have been constructed. Human research protocols have been set up utilizing cultured primary buccal cells and human blood cells. Microsatellite mutations have been found in the offspring of irradiated parents 19 years after the Cesium-137 accident in Goiânia, Brazil, in 1987. Genetic variation of 12 microsatellite loci was surveyed in 10 families of exposed individuals and their offspring and also in non-exposed families. The mutation rate was found to be higher in the exposed families compared to the control group. Details of the experimental protocols are reported in the article of da Cruz et al. (2008).

2.3.2 Gene expression profiles: DNA microarray

All body cells contain the same genetic material, however, not all genes are active in the same cells. Analyze which genes are active and which inactive in different cell types helps to understand both how cells operate normally, and the effects of a treatment.

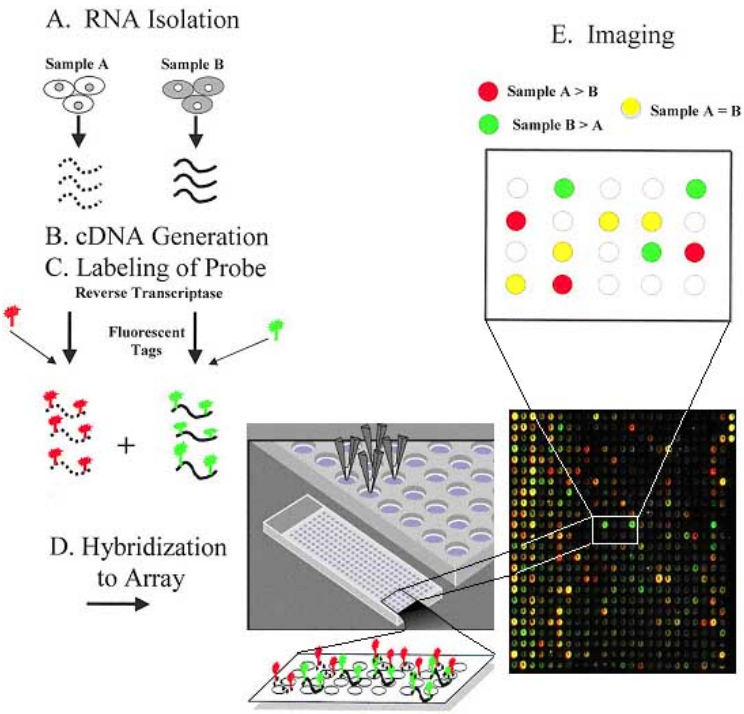


Fig 9 *Scheme of the production of microarrays: from cells treated or untreated mRNA is extracted, converted into cDNA labeled with different fluorescent drop on a slide and scanned with a laser beam, first at one wavelength to collect fluorescence data representing one probe, then is scanned at a second wavelength to collect data representing the second probe. A computer compares the amount of fluorescence at each spot on the microarray for each probe. Through the use of computer software, the ratio of fluorescence is obtained and correlated with the clone address so the investigator knows which gene (spot on the slide) was expressed more in treated tissue as compared to control tissue* (figure taken from http://www.fastol.com)

Until a decade ago it was possible to study only a few genes at a time while the development of DNA microarrays allows the simultaneous analysis of the expression of thousands of genes (Paul and Amundson, 2008). The chips are made up of many molecules of DNA (probes) deposited in a known position on a support to form a microgrid (hence the name microarrays).

The support is usually a microscope slide. Each probe consists of a single-stranded segment of DNA of a gene. The chips exploit the complementarity of the bases that constitutes DNA. The mRNA extracted from cells is converted into cDNA, bound to a fluorescent, and placed on the chip where it pairs with the corresponding complementary nucleotide base emitting fluorescence, red if a gene is only expressed in normal tissue, green if a gene is expressed only in the irradiated tissue and various shades of yellow (red + green) when a gene is expressed in both tissues.

Using image analyzer produces a profile of expression that allows you to compare the paintings of gene expression. Microarray hybridization analysis may provide rapid, automation-capable, non invasive tests for measuring radiation exposure; a set of biomarker genes was developed to build a sensitive quantitative real-time PCR (QRT-PCR) multiplex platform.

2.3.3 Protein biomarkers

Organisms respond to irradiation by altering the expression and/or the post-translational modifications of some proteins in cells, tissues and/or organic fluids, as serum or urine and protein expression profiling can be used to measure radiation exposure or to differentiate between detrimental and harmless upcoming injuries (Guipaud and Benderitter, 2009). Proteins are easily obtained using non-invasive (urine) or semi-invasive (blood) collection methods. Their quantifications using immunodetection techniques in biological fluids (urine, serum), cells (circulating lymphocytes) or tissue (biopsies) are fast and reliable. Two proteins have been proposed as bioindicators for radiation exposure effects: the amylase, an indicator of radiation damage to the parotid gland, and the Flt3-ligand (Flt3-L), an indicator of bone marrow damage, while the amino acid citrulline has been used as a physiologic marker for epithelial radiation induced small bowel damage.

The rise of serum amylase that results from the irradiation of salivary tissue provides a biochemical measure of an early radiation effect (Barrett et al., 1982) It reflects the interphase cell death of serous salivary cells and has also been proposed as biochemical indicators of salivary gland injury during iodine-131 therapy of patients with thyroid carcinoma (Becciolini et al., 1994) and as biochemical dosimeter for exposure to cosmic radiation during prolonged space travel. Serum amylase activity is measured using a clinical blood chemistry analyzer. Elevations in serum amylase activity must be measured early, *i.e.* 0.5 to 2 days, after suspected radiation exposure to serve as a biochemical indicator triage tool for identifying individuals with potentially severe radiation injury.

Flt3-ligand is a hematopoietic cytokine structurally homologous to the stem cell factor (SCF) and the colony stimulating factor 1 (CSF-1). In synergy with other growth factors, Flt3-L stimulates the proliferation and differentiation of various blood cell progenitors. The Flt3-L concentration is increased in the blood of patients with aplastic anaemia and plasma Flt3-L concentration during the first 5 days after radiation therapy directly correlated with the radiation dose in a non-human primate model (Bertho et al., 2001). The variations in plasma Flt3-L concentration has been shown to directly reflect the radiation-induced bone marrow damage during fractionated local radiation therapy, suggesting a possible use for Flt3-L monitoring as a means to predict the occurrence of grade 3-4 leukopenia or thrombocytopenia during the course of radiotherapy (Huchet et al., 2003).

Plasma Flt3-L is measured by a quantitative sandwich enzyme immunoassay using commercial ELISA kits, the assay kits are sensitive (less than 7 pg/ml), specific and show no significant cross-reactivity with other human cytokines.

Citrulline is an amino acid specifically produced by enterocytes and its concentration is correlated with the enterocyte mass in some pathological situations such as small bowel disease (Crenn et al., 2003) and after irradiation (Lutgens et al., 2003). Recently, the citrulline concentration was assessed in patients accidentally irradiated (Bertho et al., 2008). Citrulline can be repeatedly measured enabling monitoring of treatment effects and the assay is simple to apply and relatively cheap by chromatographic methods in plasma, prepared from blood sample taken into heparinized tubes (Lutgens et al., 2007).

2.3.4 Physico-chemical methods: EPR and GPA

The electron paramagnetic resonance (EPR) tooth is used to measure the amount of CO_2-radicals following acute and chronic exposure to ionizing radiation. To measure the CO_2-radicals the enamel is separated by chemical treatment, pulverized and introduced into a quartz tube. It's a long lasting and quite sensitive methods, in fact the amount of CO_2-radicals, with stability> 100 years, is measured by spectrometry and depends linearly on the absorbed dose starting from <100 mGy to 300 Gy. The limiting factor is the need to extract the tooth to be examined. It was recently developed an in vivo method which is not equally sensitive (Swartz et al., 2006).

Mutation test of glycophorin. (GPA). Glycophorin A is a glycoprotein expressed on the erythrocyte membrane, may present the allelic form M or N. The frequency of the variance, as determined by flow cytometry after staining with fluorescent, was found to be linearly correlated with the dose absorbed in the bomb survivors in Japan and as a result of serious accidents. The variance does not increase in case of exposure to medium-low doses even if accumulated over a long period. Another limitation is the fact that only 50% of the population is heterozygous for allele M / N The test is therefore not considered GPA by the International Commission on Radiation Units and Measurements (ICRU) suitable for personal dosimetry, but may be used to estimate the dose to the population for exposures greater than 1 Gy (Lindholm et al., 2004).

2.3.5 The problem of controls

One of the main difficulties when carrying out biodosimetric analysis, is the choice of the unexposed control to compare with the exposed subject. It is particularly critical because lifestyle such as age and smoking increases the frequency of mutations decreasing the signal / background ratio. Three different approaches are used. The first is to use the subject as its own control, while in case of mass casualty samples collected prior to exposure are not available, in the medical field the level of genetic damage before the treatment is needed for evaluating radio-sensitivity and effects of the administered radiations. The second is to select a control population to be processed together with the exposed one, there may be errors in this case the choice of control for confounding. The third approach is moving us towards which is the acquisition of samples to hundreds or thousands of subjects not exposed including different classes of age, diet, lifestyle etc ... with which to compare the subjects in question.

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