

DNA Repair Measured by the Comet Assay

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1. Introduction

The stability of the genome is of crucial importance, and yet the DNA molecule is prone to spontaneous loss of bases, and damage from exogenous and endogenous sources – with potentially mutagenic consequences. Damage can take the form of small alterations to bases (alkylation or oxidation); breaks in the sugar-phosphate backbone involving one or both strands (single or double strand breaks – SSBs or DSBs); bulky adducts combined with bases; and covalent bonds between adjacent bases (intra-strand cross-links), across the double helix (inter-strand cross-links), or between DNA and protein. These lesions can disrupt replication, or cause incorporation of the wrong base.

Cells possess repair enzymes that correct almost all the damage before it can result in permanent change to the genome. Different pathways deal with the various kinds of damage. Repair of SSBs is in most cells a rapid process, consisting of little more than ligation. DSBs are more complicated (and potentially more serious) since the continuity of the double helix is disrupted. Homologous recombination ensures restoration of the correct DNA sequence by using the DNA of the sister chromatid or homologous chromosome as a template, while non-homologous end-rejoining is less precise and can entail loss of sequence. Base excision repair (BER) is concerned with small base alterations and starts with removal of the damaged base by a more or less specific glycosylase, leaving a base-less sugar or AP-site (apurinic/apyrimidinic site). An AP endonuclease or lyase cleaves the DNA at this site, and – after trimming of the broken ends of DNA – the one-nucleotide gap is filled by DNA polymerase β . Ligation is the final stage. Nucleotide excision repair (NER) is a more complex affair, involving recognition of a bulky adduct or helix distortion (such as is caused by the dimerisation of adjacent pyrimidines by UV(C) radiation), endonucleolytic incision on each side of the lesion, and removal of an oligonucleotide containing the damage. This is then filled in by DNA polymerase δ , κ or ϵ and the new patch of nucleotides is ligated into the DNA, completing the repair. NER enzymes are also involved in repair of inter-strand cross-links, removing the linking molecule from one strand, leaving it attached to the other strand as a mono-adduct to be removed in a second NER reaction (according to the simplest, and possibly simplistic, model).

Individual DNA repair capacity is regarded as a biomarker of susceptibility to mutation and cancer. A person with high repair rate is assumed to be at lower risk than one with low repair rate. DNA repair is partially determined genetically, and polymorphisms in repair

genes will affect overall repair activity. However, this variation cannot account for the wide range of individual repair rates as measured in human populations. The intrinsic repair rate is likely to be affected by environmental conditions such as the presence of DNA-damaging agents that induce repair activity, and there is accumulating evidence that nutritional and lifestyle factors – for instance, micronutrients – can also modulate DNA repair.

Levels of mRNA corresponding to DNA repair pathways are frequently assessed by DNA microarray techniques, or by RT-PCR for selected genes. However, gene expression does not necessarily correlate with enzyme activity, and there is no substitute for measurement of repair capacity, i.e. phenotype. This is where the comet assay can be most usefully applied.

The comet assay, with modifications, can measure various kinds of damage, and the corresponding repair pathways. The basic comet assay detects strand breaks (see section 2.1. “The comet assay”), and so is readily applied to SSB repair by monitoring the rejoining of breaks. With a modification to detect particular classes of damage by incorporating a digestion with lesion-specific endonuclease, repair of oxidised and alkylated bases, as well as dimerised pyrimidines, can be followed. There are other specialised modifications of the assay to study cross-link repair. In addition to these assays based on following the removal of damage, there is a method for measuring NER in cells in culture by blocking repair synthesis and accumulating incision events as DNA breaks. Another approach to measuring BER or NER involves an ‘*in vitro*’ assay in which a cell extract is incubated with a DNA substrate containing specific lesions, and again the occurrence of breaks is monitored.

A quite distinct application of the comet assay is to the study of repair rates in different genes, taking advantage of the ability to identify – by the use of specific hybridisation probes – particular regions of the genome.

Here we will describe the different methods, and give examples of their application to cell culture, animal and human studies, where appropriate, without providing an exhaustive review of the literature.

2. Methods

2.1 The comet assay

The comet assay (single cell gel electrophoresis) is a simple, sensitive, economical method for measuring DNA SBs. Cells are embedded in agarose on a microscope slide, lysed, and electrophoresed. Broken DNA is drawn towards the anode, forming a ‘comet tail’; it is stained with a DNA-binding dye and observed with fluorescence microscopy (Figure 1a). The assay depends on the fact that DNA in the mammalian nucleus is organised as a series of DNA loops, attached to the nuclear framework, or matrix, at intervals. The DNA is (negatively) supercoiled, by virtue of its arrangement as nucleosomes, and each supercoiled loop should be regarded as a structural unit. Lysis of cells with detergent and high salt (removing membranes, soluble cell components and most histones), leaves the DNA still attached to the matrix, and known as a nucleoid; the supercoiling is still present, and when this supercoiling is relaxed by a DNA SB, only the loop containing the break is affected. The assay can be carried out at ‘neutral’ pH (around 10 - not high enough to denature DNA [Ostling & Johanson, 1984] or at high pH above pH 13 [Singh et al., 1988]). Both neutral and alkaline versions detect SSBs, since a single SB is sufficient to relax supercoiling. The assay does not depend on alkaline denaturation to reveal SSBs (unlike other assays such as neutral/alkaline elution, and alkaline unwinding), but the apparent analogy has led to much confusion, and it is often stated that the neutral assay only detects DSBs. The neutral

and alkaline comet assays do, however, differ in one important respect; at a high pH, AP-sites are converted to breaks.

The more breaks are present, the more loops are relaxed, and the more intense is the fluorescence of the comet tail relative to the nucleoid core when the nucleoids are stained with an appropriate DNA-binding dye (Figure 2). Comets (normally 30 to 100 per gel) are scored, most commonly, by computer-based image analysis, with '% tail DNA' as the preferred parameter, although an alternative 'visual scoring' technique is still widely used (Collins, 2004). For statistical analysis, the unit of analysis is the mean or median % tail DNA from the comets representing one independent sample of cells. % Tail DNA can be converted to 'real' units such as breaks per 10^9 Da by use of a calibration curve, based on γ - or X-irradiation of cells, since the breakage rate per Gy is known.

The comet assay can be applied to virtually any eukaryotic cell type that can be obtained as a single cell or nuclear suspension. Cell cultures and white blood cells are widely used, but also methods have been developed for disaggregating many kinds of tissue without causing damage to the cells' DNA. Sperm, with highly compacted DNA, can be subjected to comet analysis after treating with protease or dithiothreitol. The most commonly adopted strategy with plant cells is to release the nuclei by simply chopping the plant tissue with a sharp blade. The presence of chloroplasts in leaf tissue can lead to release of free radicals and oxidative damage to DNA unless the isolation is carried out under safelight conditions.

The basic comet assay is limited in its usefulness because only strand breaks (and alkali-labile sites) are detected. An additional step - digestion of the nucleoid DNA, after lysis, with a lesion-specific enzyme - converts various other kinds of DNA damage to DNA breaks (Figure 1b). Thus formamidopyrimidine DNA glycosylase (FPG) recognises oxidised purines, principally 8-oxoguanine (8-oxoG), but also ring-opened purines or formamidopyrimidines (and in addition some alkylated bases). Endonuclease III (EndoIII) converts oxidised pyrimidines to breaks, while 3-methyladenine DNA glycosylase II (AlkA) acts on alkylated bases (principally 3-methyladenine). UV-induced cyclobutane pyrimidine dimers are detected by the UV endonuclease, T4 endonuclease V (T4endoV).

2.2 Measuring DNA repair with the "challenge assay"

The simplest assay for DNA repair is the so-called 'challenge assay' (Au et al., 2010), whereby cells are treated with a damaging agent and the removal of the damage is monitored over time to study the kinetics of repair. Different assays can be used to assess the level of damage remaining at different time points; the comet assay is one of them. It is commonly used to monitor rejoining of SBs by cells, but by incorporating the digestion of DNA (nucleoids) with a lesion-specific endonuclease the removal of different DNA lesions can also be assessed. With this aim FPG is used to convert oxidised purines into SBs, Alk A to convert the alkylated bases and T4endoV to convert the cyclobutane pyrimidine dimers induced by UV. Using all the possibilities, this assay allows us to measure SSB rejoining, BER (removal of oxidised and alkylated bases) and NER (removal of UV-induced cyclobutane dimers).

Different agents are used to induce the desired type of lesion in the DNA depending on the repair pathway to be studied. SSBs are easily induced by a brief treatment with H_2O_2 or by irradiation with X- or γ -rays. Oxidized purines, mainly 8-oxoG, are induced by treating the cells with the photosensitiser Ro 19-8022 plus visible light. Methyl methanesulfonate (MMS) can be used to produce alkylated bases and UV(C) radiation induces cyclobutane dimers.

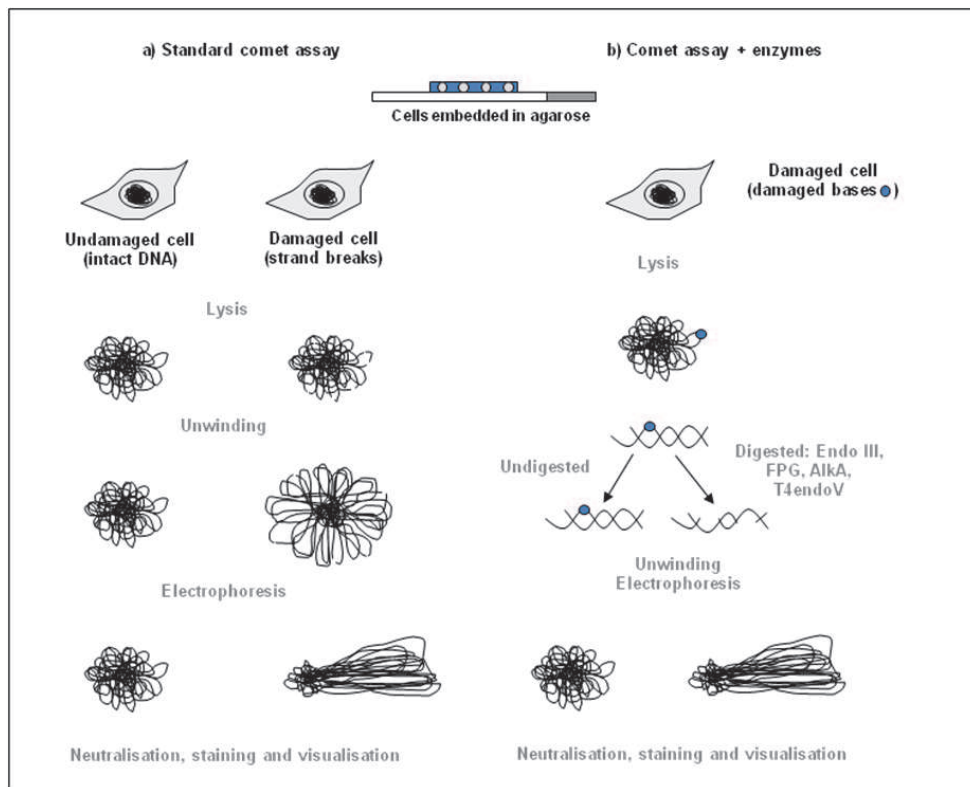


Fig. 1. Scheme of the standard comet assay (a), and the modified assay including digestion with lesion-specific enzymes (b).

The conditions of the treatment can vary depending on the cell type, and it is recommended first to establish optimal conditions; a high level of induced lesions, but not enough to saturate the assay or the capacity of the cells to repair the damage without entering apoptosis.

After the treatment cells are incubated in the appropriate cell culture medium and conditions (normally in an incubator at 37°C with 5% of CO₂) for different times. Just after the treatment (time 0) an aliquot of the cells is taken to check the level of induced damage. Further aliquots are taken at different times of incubation, including times soon after the start of incubation in order to estimate the initial rate of repair accurately.

In the case of adherent cells it is necessary to set up as many cell cultures as there are time-points (in multi-well plates or petri dishes) because at each time-point cells should be trypsinized. If cells are growing in suspension, an aliquot can be removed from the whole cell culture at each time-point. Setting the right times is a very important issue, influenced by the cell type and the repair pathway to be studied and so a prior investigation should be done on this topic also.

To avoid continuing repair of DNA damage while processing the cells after sampling at the different time-points, cells should be kept on ice during their manipulation. This is particularly important when very short intervals of time are tested.

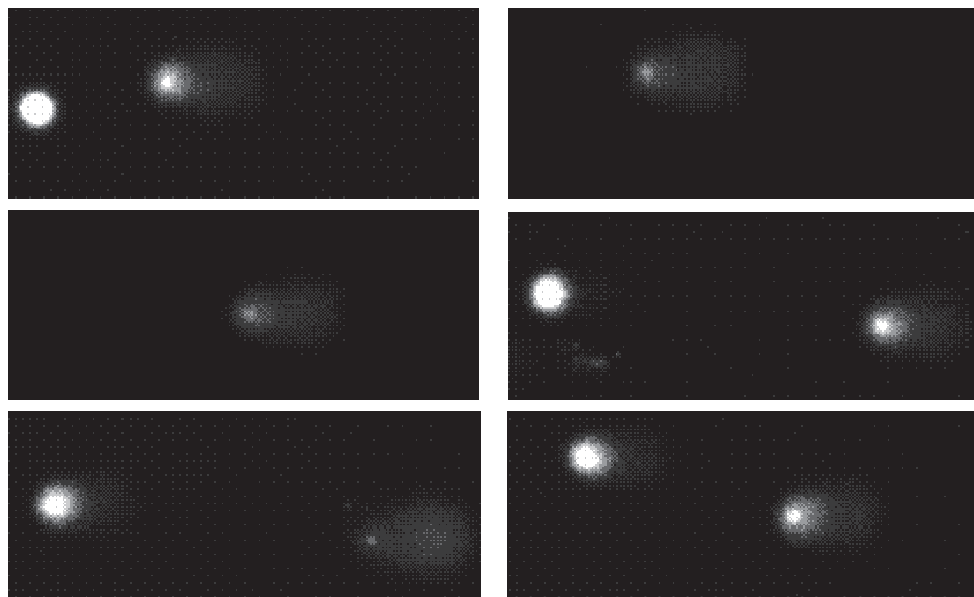


Fig. 2. Comet images with different levels of DNA damage.

The comet assay is done as described above; either the basic version (to assess SSB rejoining) or with an enzyme digestion (to assess BER or NER). The lysis step of the comet assay can last between 1 h and 24 h (or even longer) so gel-embedded cells/nucleoids can be kept in the lysis solution until all of the samples have been processed. Then samples from all time-points can be run in the same experiment, which as well as being practically convenient, avoids experimental variability.

To be able to compare different kinetics of repair, the half time of damage removal ($t_{1/2}$) should be calculated. To obtain an accurate estimation of this parameter the choice of the different time points is crucial. Generally the repair of SSBs is rapid, with a $t_{1/2}$ of 10 minutes or so while the repair of oxidized and alkylated bases and UV-induced cyclobutane dimers takes a few hours (Lorenzo et al., 2009). Another useful parameter is the initial repair rate, but this is difficult to estimate accurately if repair is rapid.

As in all of the assays, proper controls should be included to interpret the results correctly. A non-damaged cell culture should be included at all time points (including time 0) to check for any variation in or problem with experimental conditions.

2.2.1 Applications of the challenge assay

The challenge assay is used in cell culture experiments to check the influence of different compounds on the cellular repair rate. It is also used in animal studies and in human biomonitoring, normally studying lymphocytes.

The residual damage should always be measured at several time points after the incubation, so that the kinetics of the repair can be quantified and compared between different cell types or experimental conditions. Ideally, residual damage should be measured at shorter intervals immediately after treatment, since the initial rate of removal of damage is considered the defining step of the process. Another option, as explained before, is to

calculate the $t_{1/2}$ for lesion removal. Measuring residual damage at a unique late point when most of the damage has been repaired, as is often reported, gives limited and ambiguous information.

For a valid comparison of different cell types or lymphocyte samples, the level of induced damage to be removed should ideally be the same in all cells/samples in the study, a state that in many cases is not easily achieved. It is a good assay to use with cell lines for examining the effect of an agent on repair when the compound to be tested does not affect the level of induced DNA damage. But sometimes cell cultures can be protected from DNA damage by the compound being studied; thus, for example, when an antioxidant micronutrient is tested for an effect on repair, it will obviously decrease the level of induced oxidative damage.

Compared with cell lines, animals and humans have more variability that can affect the level of damage achieved with the challenge compound. In biomonitoring, one subject group can have a higher antioxidant status that protects them against the damaging agent. This problem may well arise and is very difficult to solve. One possibility is to arrange for different doses of damage to each group to ensure the same initial level of lesions but this is in general impracticable.

Another disadvantage of this assay is that it involves a lot of cell culturing, specially when adherent cells are used and trypsinization is needed at all time points; the scheduled times to carry out the assay of residual damage can be inconvenient, and overall the experiment is complicated to perform. This is especially the case in biomonitoring studies, since the large number of samples to be tested precludes such complicated procedures – and there is inevitably day-to-day variation in culture conditions and results. On the other hand its endpoint is the removal of lesions and restoration of normal DNA structure, i.e. overall repair, whereas other methods tend to look only at one step in the repair process.

2.2.2 The challenge assay in cell culture studies

The “challenge assay” is the most suitable comet assay-based approach to measure DNA repair in cell culture and it has been used with different purposes. In 2003, Blasiak et al. demonstrated the temperature-dependence of the DNA repair process with the aim of using hyperthermia in the modulation of cancer therapy. They treated human peripheral lymphocytes and two variants of a human myelogenous leukemia cell line (K562 and its doxorubicin-resistant variant) with doxorubicin and studied the removal of the damage at 37°C and 41°C. They found an increase in the repair rate of the cells incubated at 41°C compared with 37°C. Tsai-Hsiu et al. (2003) studied the effect of S-adenosylhomocysteine (SAH), an inhibitor of most methyltransferases, on the repair rate of a mouse endothelial cell line and a human intestinal cell line. Cells were treated with H₂O₂ before incubating them with different concentrations of SAH or homocysteine as control and the removal of the damage was monitored. They showed that SAH decreased the DNA repair rate in a dose-dependent manner.

Ramos et al. (2008) studied the chemoprotective effects of the flavonoids quercetin and rutin, and the phytochemical ursolic acid, on the DNA damage induced by tert-butyl hydroperoxide (t-BHP) in a human hepatoma cell line. They checked the removal of the DNA damage induced by t-BHP after incubating the cells with different concentrations of quercetin, rutin or ursolic acid for 24h. There was an increase in the DNA repair rate of cells incubated with quercetin and ursolic acid, when the remaining lesions were measured 2 h

after the treatment. The same group showed an enhancement in the repair rate when the human colon carcinoma cell line Caco-2 was preincubated with *Salvia* extracts or luteonil-7-glucoside before H₂O₂ treatment; but there was no effect when the compounds were just present during the repair time (Ramos et al., 2010a).

The repair of both SBs and oxidized bases (induced by treatment with H₂O₂ or with a photosensitiser plus visible light, respectively) were assessed in the human cervical cancer cell line HeLa and in Caco-2 cells incubated with different concentrations of the carotenoid β -cryptoxanthin (Lorenzo et al., 2009). This carotenoid induced a faster removal of both kinds of lesions in both cell lines at very low concentrations. The effect of β -cryptoxanthin on the removal of the 8-oxoG in Caco-2 cells is shown in Figure 3a.

Rejoining of X-ray-induced SBs by mouse leukocytes was studied by Gudkov et al. (2009). The natural ribonucleosides guanosine and inosine were present during the repair period, and SBs were measured after irradiation. Both ribonucleosides increased the repair rate. Moreover, in the presence of the repair inhibitor nicotinamide (prior to the irradiation and during the repair process), repair was slower and ribonucleosides did not induce any effect.

2.2.3 The challenge assay in animal studies

Although this approach is not ideal for application in *in vivo* animal studies, the lack of a good alternative makes it very common. Gover et al. (2001) studied the repair of DNA lesions induced by different concentrations of the fungicide mercuric chloride in leucocytes of rats. The comet assay was performed in whole blood at different times after an oral administration of a single dose. The level of the DNA damage decreased from 48 h and reached the control level at 2 weeks after the treatment. Very similar studies have been done to check the effects of the insecticide JS-118 (Zhang et al., 2010) and of copper sulfate (Saleha Banu et al., 2004) in mice.

This approach has also been applied to DNA repair in organs. Cells from the liver, kidney and bone marrow of mice were used to check the effect of the intraperitoneal administration of (MMS), a known genotoxic compound, and acetaminophen, an analgesic drug (Oshida et al., 2008). The level of DNA damage found at 4 h was less than at 24 h in all the organs and with both compounds. According to the authors this decrease can be due to detoxification, repair of the lesions induced by the treatment, or cell turnover.

The effect of intraperitoneal administration of the phytochemical feluric acid on repair of the DNA damage induced in lymphocytes by whole body γ -irradiation of mice was studied by Maurya et al. (2005). The disappearance of the induced SBs was faster in animals which received feluric acid compared to controls.

In these studies the challenging agent is given to the animals and repair occurs in physiological conditions (inside the animal). The assay has also been applied in animal studies where the challenge occurs *ex vivo*. Miranda et al. (2008) studied the protective effect of intragastric administration of aqueous extracts from Yerba mate tea in mice over a period of 60 days. After this period cells were isolated from liver, kidney and bladder and embedded in agarose before treating them with H₂O₂. There was an enhancement of DNA repair in liver cells.

2.2.4 The challenge assay in human studies

As explained before, the challenge assay presents many inconveniences when used in humans, but there are several studies that use this approach to measure the DNA repair capacity of individuals.

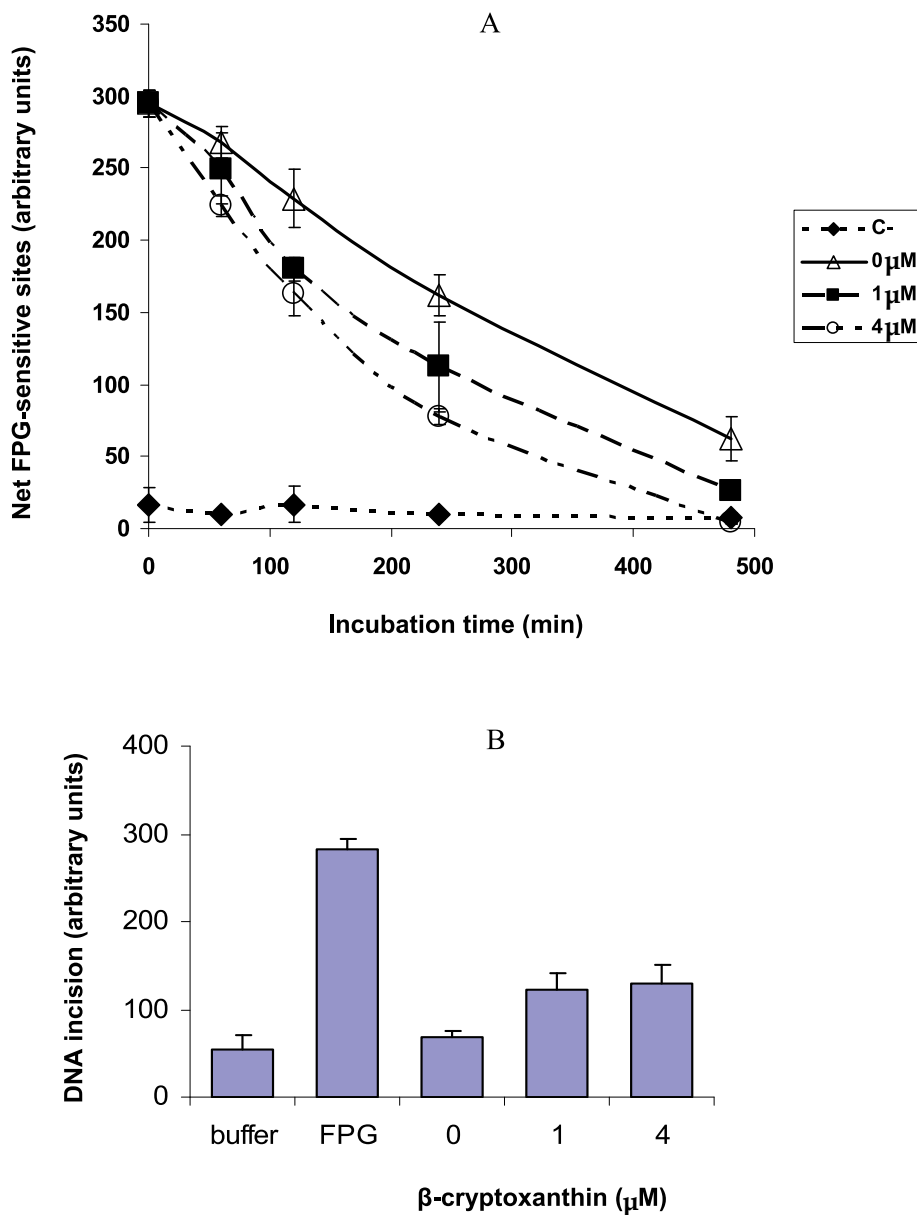


Fig. 3. Effect of β -cryptoxanthin on BER in Caco-2 cells: “challenge assay” (a) and *in vitro* repair assay (b). From Lorenzo Y, Azqueta A, Luna L, Bonilla F, Dominguez G, Collins AR (2009) The carotenoid β -cryptoxanthin stimulates the repair of DNA oxidation damage in addition to acting as an antioxidant in human cells. *Carcinogenesis* 30 (2):308-314, by permission of Oxford University Press.

In a case-control study the DNA repair capacity of lymphocytes from 44 healthy donors and 38 patients with squamous cell carcinoma of head and neck (before treatment) was measured (Palyvoda et al., 2003). Repair of γ -ray induced lesions showed a high variability between individuals ($t_{1/2}$ from about 10 min to more than 1 h). Lymphocytes from patients showed lower repair rates and a higher amount of non-repaired damage after the incubation period.

This approach has also been used to monitor DNA repair in relation to occupation, environment or lifestyle. The repair rates of stimulated lymphocytes from 10 nuclear power plant workers chronically exposed to low doses of ionizing radiation and 10 controls were assessed (Touil et al., 2002). The interindividual variation in the rates of repair of γ -irradiation-induced DNA damage was high but there were no significant differences between groups. In a similar study, the repair rate of lymphocytes from 104 asbestos-exposed workers and 101 control workers was studied (Zhao et al., 2006). Lymphocytes from asbestos-exposed workers showed slower repair of H_2O_2 induced damage.

The challenge assay has also been applied in nutritional studies to check the influence of phytochemicals or whole foods on DNA repair. To study the effect of lutein, lycopene and β -carotene, 8 healthy volunteers were given supplements daily during 1 week in a cross over study with a wash-out period of 3 weeks (Torbergsen & Collins, 2000). Lutein did not have any effect on the DNA repair rate, but lycopene and β -carotene apparently accelerated the rejoining of the SBs. However, an increase in the level of SBs in non-irradiated cells during approximately the first 4 h of the incubation period was seen. This could be due to the oxidative stress that lymphocytes suffer from sudden exposure to atmospheric oxygen. This transient increase was less pronounced in lymphocytes taken after lycopene or β -carotene supplementation so the apparent acceleration of repair could be explained by an antioxidant protection exerted by the presence of carotenoids. In another study (Astley et al., 2004) healthy volunteers followed a dietary intervention with a mixed carotene capsule, a daily portion of cooked minced carrots, a portion of mandarin oranges, a vitamin C tablet or a matched placebo (about 10 volunteers per group). Only the lymphocytes from individuals taking mixed carotene capsules showed an improvement in rate of repair of H_2O_2 induced DNA damage.

As explained above, crucial information is lost when just one time of recovery is used in the challenge assay, and – especially if starting levels of damage are not the same – it can be misleading to compare repair rates on the basis of residual damage.

2.3 Measuring NER by inhibiting DNA synthesis

Many years ago, inhibitors of the DNA polymerase species that participate in NER were employed to block repair synthesis after UV(C) irradiation of cells in culture: the earlier steps of repair continue, leading to an accumulation of DNA breaks which normally occur as very transient repair intermediates. Aphidicolin, or cytosine arabinoside in combination with hydroxyurea, are equally effective as inhibitors. As methods for measuring DNA breaks, alkaline unwinding and alkaline elution were used. The principle was then combined with the comet assay, and used as early as 1992 (Gedik et al., 1992), detecting the accumulation of breaks in HeLa cells irradiated with 0.5 Jm^{-2} of UV(C) and incubated for just 5 min.

This approach was adapted by Speit et al. (2004) as a way of enhancing the detection of damage done to DNA by a range of different agents (benzo[a]pyrene diolepoxide [BPDE],

bischloroethylnitrosourea, and MMS). It is particularly useful in the detection of 'bulky adducts', which are repaired by NER, but are not recognised by T4endoV, and so are not amenable to the enzyme-modified comet assay. (The bacterial enzyme complex, uvrABC, detects bulky DNA adducts as well as UV-induced pyrimidine dimers. Many efforts have been made to incorporate this enzyme complex to the comet assay but, until now, it seems to detect only a small fraction of the available lesions [Dusinska & Collins, 1996]). This inhibitor-based incision assay can be used as a simple and sensitive method to measure repair capacity, reflected in the rate of accumulation of breaks. Incision is generally considered to be the rate-limiting step of NER. Before the introduction of the comet assay, the accumulation of incision events was used to investigate the molecular defects in the disease xeroderma pigmentosum (Squires et al., 1982) and to characterise DNA repair-defective mutant cell lines (Stefanini et al., 1991).

2.3.1 Applications of the inhibitor assay for NER

This assay has not been widely used but it has considerable potential, particularly in human studies. Actually it seems that in the case of freshly isolated lymphocytes, the DNA breaks present as NER intermediates persist long enough to be detected with the comet assay without using aphidicolin or cytosine arabinoside (Collins et al., 1995; Green et al., 1994). Repair synthesis is unable to proceed due to the lack of enough DNA precursors (dNTPs). If deoxyribonucleosides are added to the medium, breaks are no longer detected. However, it seems wise to include aphidicolin or cytosine arabinoside to ensure that DNA resynthesis is completely blocked and so to be sure of detecting all the breaks.

Cipollini *et al.* (2006) treated lymphocytes with 1.5 Jm⁻² of UV(C) and observed breaks accumulating to a maximum at about 60 min with then a decrease. This decline can be due to eventual completion of repair even with the low concentrations of precursors, or to a synthesis of DNA precursors induced as a response to the DNA damage. Experimental variation and inter-individual differences in kinetics were seen in 4 subjects. This could be explained by individual differences in the precursor pool size rather than differences in the repair capacity. The characterisation of several UV-sensitive rodent mutant cell lines included the measurement of their ability to carry out incision after irradiation with 0.1 Jm⁻² of UV(C) (Collins et al., 1997).

The assay has been used to look for effects of *in vivo* exposure to different genotoxic agents by looking for an enhanced level of breaks when lymphocytes are incubated with DNA synthesis inhibitors. Crebelli et al. (2002) found a higher level of breaks (with cytosine arabinoside) in aluminium workers compared with controls; while Speit et al. (2003) did not detect such a difference between smokers and non-smokers.

The best use of the assay in human biomonitoring is probably as an *ex vivo* assay, i.e. treating the subjects' lymphocytes with UV(C) (or some other agent whose damage is repaired by NER) and incubating them with inhibitor *in vitro*. The Kirsch-Volders group recently carried out a pilot study with 22 subjects, treating peripheral blood mononucleated cells with BPDE for 2 h with and without preincubation with aphidicolin (Vande Loock *et al.*, 2010). They quantified repair capacity as the amount of SBs induced by BPDE with aphidicolin, minus the SBs induced by aphidicolin (a very small amount) and by BPDE alone - reckoning that this equates to the incision activity of the NER enzymes. (UV(C) is a cleaner agent to use, since it does not directly induce significant levels of SBs; all the SBs detected are NER intermediates.)

As a biomonitoring assay for human studies, the inhibitor assay for NER is still in the development phase. A comparison of results from this assay and from a UV challenge assay and an *in vitro* NER assay would be very informative.

2.4 Measuring BER and NER with an *in vitro* assay

2.4.1 Practical details

The comet assay has been modified to measure the excision repair activity in an extract of cells (or a nuclear extract). In this *in vitro* approach a substrate, in the form of agarose-embedded nucleoids derived by lysis of cells containing a specific lesion, is incubated with the extract whose excision repair activity is to be measured by the comet assay (Collins et al., 2001; Gaivão et al., 2009; Langie et al., 2006) (Figure 4). The nature of the DNA lesion in the substrate defines the repair pathway that is measured. Substrate containing 8-oxoG is used to measure the BER activity of 8-oxoG DNA glycosylase (OGG) in the extracts tested (Collins et al., 2001); if substrate contains bulky adducts or cyclobutane pyrimidine dimers NER is measured (Gaivão et al., 2009; Langie et al., 2006). More recently an assay for cross-link repair has been developed (Herrera et al., 2009). In all cases the enzymes contained in the extract will carry out the initial steps of repair by recognizing the lesion and introducing a break at or near its site. The rate of accumulation of breaks, assessed by alkaline electrophoresis, is a measure of the repair capacity of the cells.

The substrate nucleoids should contain a high level of specific base damage so that the enzymes in the extract have an excess of lesions to work on. This level should be more than enough to saturate the comet assay but the background level of breaks as well as other unwanted lesions should be very low. To reach this equilibrium is not always an easy task and as a result it is not always possible to produce substrate with the desired lesion.

Furthermore breaks do not continue to increase indefinitely but reach a saturation. This means that the longer the incubation, the less difference will be detected in activity between different extracts - so the time of incubation is crucial.

The description of the assay is divided into 5 steps: preparation of cells for the substrate, preparation of cells for the extract, preparation of the substrate nucleoids, preparation of the extract and incubation of the substrate with the extract.

Preparation of cells for the substrate for BER and NER: A substrate to measure BER is prepared by treating the cells with the photosensitizer Ro 19-8022 plus visible light to induce oxidized purines, mainly 8-oxoG. For NER, cells are irradiated with UV(C) to induce cyclobutane pyrimidine dimers (they can also be treated with BPDE or oxaliplatin to produce bulky adducts or cross-links respectively but we do not have experience with such treatments). Non-treated cells should be used to prepare a control substrate. The cell type used for substrate is not important.

After treatment cells are slowly frozen in aliquots and kept at -80°C (for months or even years).

Preparation of cells for the extract: Extract is normally prepared from lymphocytes or cultured cells (recently animal tissue has been successfully used [Langie et al., 2011] in this assay but this will not be covered in this article). In the order of 5-10 million cells are needed to perform about 6 determinations of repair activity.

Cells are washed, spun, suspended at 10^7 per 100 μ l in extraction buffer and aliquots flash-frozen in liquid nitrogen before storage (for at least months) at -80°C. In fact there are three alternative methods to prepare cells for making extract: (1) direct preparation in extraction buffer, as above; (2) preparation of a dry pellet of the cells, snap-frozen and kept at 80°C (the

rest of the extraction being done on the day of the assay); (3) cells frozen slowly in freezing medium to maintain viability, with complete extraction procedure carried out on day of experiment. Each method gives comparable results but experience shows that with method (2) the presence of residual supernatant when the pellet is thawed presents problems.

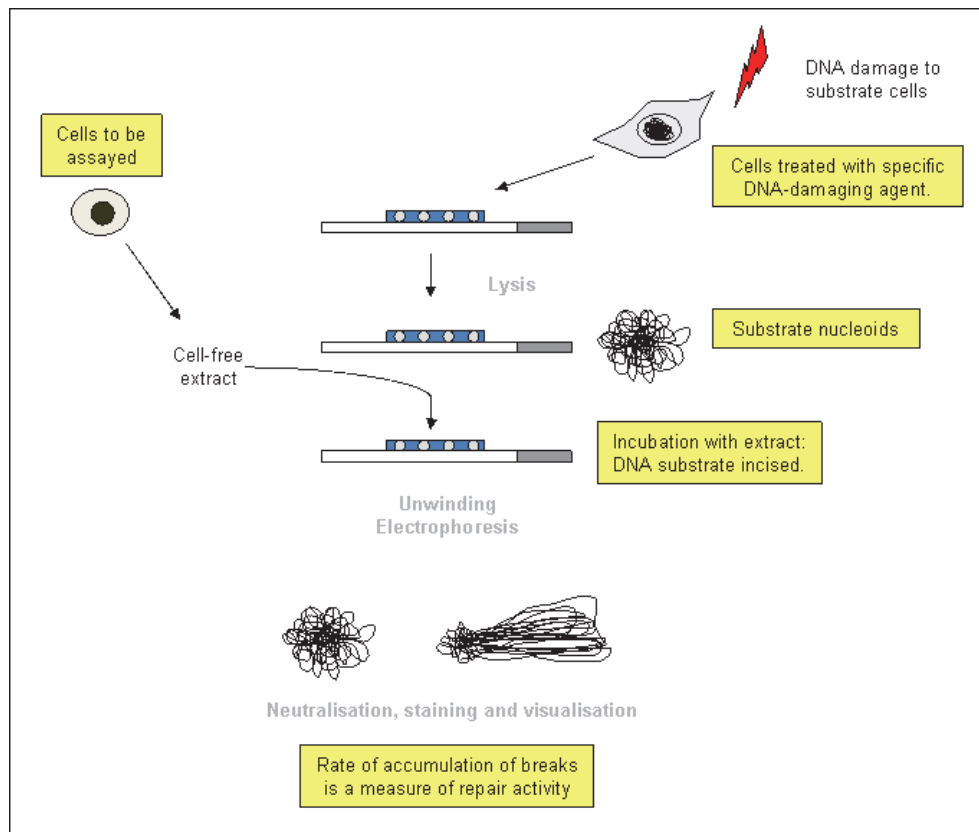


Fig. 4. Scheme of the *in vitro* repair assay

Preparation of the substrate nucleoids: Cells are thawed and embedded in agarose on a microscope slide as explained before (see section 2.1. "The comet assay"). Then slides are placed in the lysis solution for at least one hour to produce nucleoids. Control cells, without any induced damage, should always be included to check for unspecific nuclease activity in the extracts.

Preparation of the extracts: On the day of the experiment, after preparing substrate gels, an aliquot of the cells frozen for preparing the extract is thawed and kept on ice. Triton X-100 is added (final concentration 0.2%) to destabilize the membranes and complete lysis. After centrifugation at high speed to remove nuclei and cell debris, the supernatant is then diluted 4-fold in reaction buffer. All the procedures should be done on ice to avoid loss of activity.

Incubation of the substrate with the extracts: Substrate gels should be washed with the reaction buffer before the incubation with the extracts. Extracts are incubated with the substrate gels

for between 10 and 30 min at 37°C in a humidified atmosphere. The optimal time should be established in each laboratory. If the 2 gels per slide format is used about 45 µl of extract is added on the top of the gel and a cover slip (or a Parafilm square) is placed on top. After that the standard comet assay protocol is followed.

Substrate nucleoids should be also treated with a buffer control (extraction buffer + Triton + reaction buffer) and with enzymes to test the level of DNA damage contained in the nucleoids. FPG is used for BER substrates and T4endoV for NER. Therefore the standard experiment should include substrate for BER or NER and substrate without damage, incubated with extracts, the buffer control and FPG or T4endoV.

To express the results the values of the activity of the extracts and controls obtained from the substrate without damage, representing the non-specific activity, should be subtracted from the values obtained from the BER or NER substrate.

It is crucial that the number of cells to prepare the extract is the same in all the samples. Counting is always time consuming and not accurate; it is useful to measure the protein concentrations in the extract residues left after an experiment, and to express repair activity relative to the protein concentration, thus allowing for variation in cell numbers. However, this correction is valid only over a narrow range, since repair activity deviates from linearity at high concentrations. Therefore, care must be taken to work with similar and appropriate protein concentrations or cell densities in all extracts.

2.4.2 Applications of the *in vitro* DNA repair assay

The *in vitro* DNA repair assay has been used in some cell culture and animal studies but it is mostly used in human biomonitoring. It is a very useful tool in this type of study since it can be used on extracts prepared from lymphocyte samples and stored frozen until a batch of extracts are ready to be measured at the same time.

This approach does not measure the whole process, but only the initial step of repair.

2.4.3 Cell culture studies with the *in vitro* assay

Very few examples of the *in vitro* repair assay applied to cell culture studies can be found in the literature. Ramos et al. (2010a) evaluated the incision activity of extract from Caco-2 cells treated for 24 h with water extracts of *Salvia* species, rosmarinic acid and luteonil-7-glucoside on nucleoid substrate containing 8-oxoG. All extracts from treated cells showed an increase. It was significant in the case of one of the *Salvia* species and with luteonil-7-glucoside. In the same way, Ramos and colleagues showed an increase in the BER activity of extract from Caco-2 cells incubated with ursolic acid (a triterpenoid) while the incubation with luteolin (a flavonoid) had no effect (Ramos et al., 2010b). The effect of β-cryptoxanthin in BER was also assessed in extracts from HeLa and Caco-2 cells incubated for 2 h with different concentrations of β-cryptoxanthin, on HeLa nucleoids. A significant increase in BER was shown by extracts of cells treated with β-cryptoxanthin, even at very low concentration (Lorenzo et al., 2009). They also incubated β-cryptoxanthin with nucleoids to check whether β-cryptoxanthin present in the extract could directly induce breaks in the nucleoids, but did not find any effect. The increase in the incision activity of Caco-2 cells incubated with β-cryptoxanthin is shown in Figure 3b.

The effect of STI571, the most used drug in the treatment of chronic myeloid leukemia, on NER was assessed using the *in vitro* DNA repair assay (Sliwinski et al., 2008). STI571 inhibits the activity of the BCR/ABL oncogenic kinase, and so 3 different cell lines were used: human

myeloid leukemic cells expressing BCR/ABL, human lymphoid leukemia cells also expressing BCR/ABL, and human lymphoid leukemic cells which do not express BCR/ABL. Extracts were prepared after treating the cells with STI571 for 2 h, and incubated with UV-treated nucleoid DNA. The NER activity of extract from BCR/ABL cells showed a drastic and highly significant decrease after treatment with the drug – in contrast with the control cells, not expressing BCR/ABL, in which the drug did not induce any change in NER activity.

2.4.4 Animal studies with the *in vitro* assay

The *in vitro* repair assay has been mostly used on humans; applications in animal studies are extremely limited. Obtaining sufficient lymphocytes from rodents to prepare extract is not easy.

Recently Langie et al. (2011) have optimised the assay to measure the BER in extracts from rodent tissues. Various attempts have been made before, but they were hampered by the high non-specific nuclease activity present in the extracts. Langie et al. successfully measured the incision activity of extracts from liver and brain from C57/BL mice. Optimisation of the protein concentration in the tissue extract as well as the use of aphidicolin were the key steps to get rid of the non-specific activity. The assay was validated by using tissues from BER deficient OGG1 knockout mice where a low activity was found, significantly lower than with wild-type mice. In the same paper the assay was used to determine the effect of aging on the incision activity of extracts from mouse brain and the effect of diet on the incision activity of extracts from mouse liver. The BER activity of brain decreases with age, and in liver it is induced under dietary restriction.

2.4.5 Human studies

The *in vitro* repair assay has been widely used to measure BER and NER activities in human lymphocytes. The BER capacity of lymphocytes from 86 workers in a plastics factory, exposed to styrene, and 52 controls was studied (Vodicka et al., 2004a). The incision activity on HeLa nucleoids containing 8-oxoG was higher in styrene-exposed workers. The same group carried out a similar study to determine the effect of the occupational exposure to different xenobiotics from a tire plant on the DNA repair capacity of the workers (Vodicka et al., 2004b). No differences in repair activity were reported in lymphocytes of 15 workers with a high risk of exposure, 11 with a low risk and 12 employed in checking and quality control. In the same way the BER repair capacity was determined in 61 exposed workers from an asbestos cement plant and 21 controls (Dusinska et al., 2004). Females exposed to asbestos showed a decrease in their BER capacity compared with non-exposed ones but there were no differences between exposed and non-exposed males.

This approach has also been widely used in nutritional intervention studies. BER activity was measured in lymphocytes from 6 subjects before and after the intake of coenzyme Q₁₀ during 1 week (Tomasetti et al., 2001). A significantly higher OGG1 activity was seen after the supplementation. Very recently a nutritional intervention study has been published, where not only BER but also NER in UV-treated nucleoids was measured (Brevik et al., 2011). A randomized parallel study with 3 groups (a high phytochemical group with a high intake of a variety of antioxidant-rich plant products, a kiwifruit group supplemented with three kiwifruits per day and a control group without supplementation) and 8 weeks of intervention was carried out. BER activity was measured in lymphocytes from 23, 25, and 21 subjects from each group respectively, while NER was measured in lymphocytes from 13, 11

and 12 subjects from each group. BER showed an increase in both supplemented groups, being significant in the high phytochemical group, while NER showed significant decreases in both these groups. The control group did not show any changes.

It is important to include in *in vitro* repair experiments an incubation of extract with an undamaged substrate, to check for possible non-specific nuclease activities in the extract. It is not always clear in publications whether this has been done.

2.5 Following DNA repair at the level of the gene (FISH-comet assay)

The special feature of the comet assay is the ability to study DNA damage in individual cells. By combining fluorescent *in situ* hybridization (FISH) and applying labelled probes to particular DNA sequences, an even finer level of resolution can be achieved. Fig 5 illustrates general principles of the comet assay combined with FISH.

Depending on the target sequence, different probes are applied to comets. The most widely used FISH probes are centromere, telomere and ribosomal DNA repeats, short interspersed repetitive elements (SINEs) and long interspersed repetitive elements (LINEs). Those repetitive probes produce strong signals and are often commercially available. Other popular commercially available non-gene-specific probes are chromosome arm- or band-specific painting probes (DNA from microdissected chromosomes) and whole-chromosome painting probes (DNA from flow-sorted chromosomes).

Probes for specific DNA sequences can consist of PCR products, cDNAs or genomic DNA cloned in cosmids, P1 artificial chromosomes, bacterial artificial chromosomes (BACs) or yeast artificial chromosomes. Large unique probes that are not commercially available can be prepared for FISH using standard molecular biology techniques. Another useful design of probe is the 'padlock probe'—a linear oligonucleotide designed so that the two end segments, connected by a linker region, are complementary—in opposite orientations—to adjacent target sequences (Larsson et al., 2004). On hybridization, the two juxtaposed probe ends can be joined by a DNA ligase, circularizing the padlock probe and leaving it physically catenated to the target sequence. The reaction requires a perfect match between the probe ends and the target sequence and therefore, it is stable and extremely specific. The crucial feature of these probes when applied to comets is that the reaction steps are performed at 37°C so that there is no tendency for the agarose to melt or become unstable.

When analysing FISH-comets results, visualization and scoring depend entirely on direct observation. In most cases it is not possible to score the signals automatically because of the complexity of the preparations (for instance, the occurrence of signals in the same cell in different optical planes). Figure 5 illustrates different appearances of the signals: a linear array or separate spots.

An important question related to FISH signal visualization, is how many signals to expect. Based on our hypothesis that DNA organisation in comets reflects the DNA loop organization in living cells, it is reasonable to expect that the number of signals detected in comets will be related to the number of signals observed on chromosome spread preparations. Our results with chromosome 16 probes confirmed this hypothesis: twice as many signals were observed in the alkaline version of the assay relative to the number seen under neutral conditions (Shaposhnikov et al., 2008). This can be explained by DNA denaturation in alkaline comets since each strand of DNA will act as a target for the FISH probe. Furthermore, the average numbers of signals seen per cell corresponded closely with the numbers expected according to the gene copy number in a random interphase cell population.

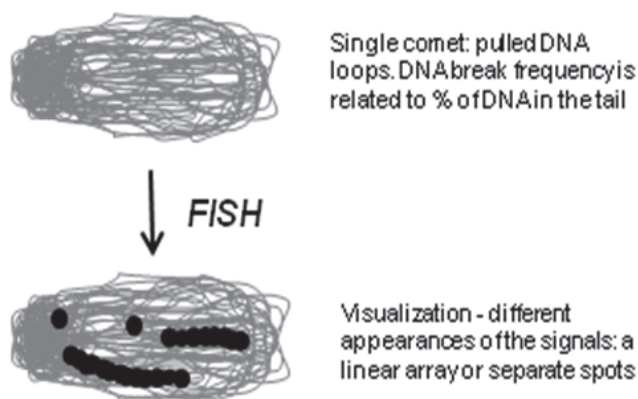


Fig. 5. General principles of the FISH-comet assay

Santos et al. (1997) published the first successful results of combining FISH with the (neutral) comet assay. Their aim was to investigate how centromeric and telomeric DNA behaves under electrophoresis. Probes to all centromeres, all telomeres, as well as chromosome-specific centromere and telomere DNA, and 3 segments of the gene *MGMT* (coding for the repair enzyme O6-methylguanine DNA methyltransferase) were used. Telomere probes were seen mostly over the comet head, consistent with their attachment to the nuclear membrane. The signals from the much larger centromere DNA (1000 kb in size) appeared as long strings of dots, extending well into the comet tail. *MGMT* gave signals that were found in the head as well as the tail, the 3 segments generally forming a linear array.

2.5.1 Gene-specific DNA repair

The FISH-comet assay can be used to monitor gene-specific DNA repair by following the 'retreat' of the gene-specific signals from tail to head during the incubation period. Thus it is possible to compare the kinetics of overall genomic and gene-specific repair.

McKenna et al. (2003) examined the repair of γ -ray-induced SBs in human cells. Using a probe for the *TP53* gene, they found that the number of signals increased immediately after irradiation (most being in the comet tails), and decreased over the first 15 min at which point most were in comet heads. By 60 min, the normal, lower number of signals was restored, while in contrast the % tail DNA (representing total DNA) was still elevated. Thus *TP53* repair was faster than total genomic repair.

We studied the repair of the *DHFR* gene (coding for dihydrofolate reductase), *MGMT*, and the *TP53* gene using a different approach (Horvathova et al., 2004). Probes were designed for each end of the gene and detected using antibodies giving different coloured signals so that the gene had red and green ends after hybridization. After H_2O_2 -treatment, Chinese hamster ovary (CHO) cells gave comets with about 50% of the DNA in the tail. Almost all *DHFR* probe signals were in comet heads, whereas we had expected them to have a similar distribution to total DNA. The probable explanation is that a 'matrix associated region' or MAR is present in this gene, and this prevents the DNA from escaping from the head. For the *MGMT* gene, CHO cells were treated either with H_2O_2 or with Ro 19-8022 and light to create 8-oxoG residues (FPG-sensitive sites). In contrast to the *DHFR* result, signals

appeared over tail DNA - though they were predominantly green dots, while almost all red dots were located over the head. Thus one end of the gene appeared to be attached to the matrix. Green signals were restored to the head region of the comets with similar kinetics to the total DNA, indicating similar time courses for total DNA repair and repair of *MGMT*. H_2O_2 -treated human lymphocytes, hybridized with *TP53* probes, gave signals of both colours in the tail; after 20 min incubation, virtually all *TP53* signals were in the head, while the % of total DNA in the tail had decreased by only about one-third. Thus, the region of DNA containing *TP53* was apparently repaired significantly faster than genomic DNA overall. Kumaravel et al. (2005) also reported preferential repair of *TP53*, after ionising radiation or H_2O_2 treatment.

We recently used padlock probes and rolling circle amplification (RCA) to investigate the repair of two DNA repair genes, 8-oxoguanine-DNA glycosylase-1 (*OGG1*) and xeroderma pigmentosum group D (*XPD*), and the housekeeping gene for hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) (Henriksson et al., 2011). The repair rates of these genes after H_2O_2 damage were compared with the repair rates of *Alu* repeats and of total genomic DNA. The signals were mainly detected in comet tails. The *HPRT* gene showed rapid repair compared to total DNA, and after approximately 10 min the *HPRT* gene signals were almost completely absent, whereas the mean % tail DNA, indicating total DNA damage, decreased from 67 to 43% over 2 h (consistent with the slow repair of SBs by lymphocytes, as described by Torbergson and Collins, 2000). *HPRT* and *XPD* were repaired more rapidly and *OGG1* more slowly than *Alu* repeats.

3. Conclusion

The comet assay has proved to be remarkably versatile. Far from being just another way of measuring DNA breaks, it can give quantitative information about base damage if lesion-specific endonucleases are included in the protocol, and by extension it can be used to monitor the cellular repair of such damage (the challenge assay). The NER pathway for helix distortions and bulky adducts can be blocked at the repair synthesis stage by DNA polymerase inhibitors, and this leads to an accumulation of SBs - readily measured with the comet assay. A more biochemical approach to DNA repair is exemplified by the *in vitro* repair assay, in which a cell extract is incubated with a specifically damaged DNA substrate - again leading to an accumulation of DNA breaks - repair intermediates, for which the comet assay is ideally suited as a detector. These different approaches have found application in cell culture studies (e.g. investigating inhibitors and enhancers of repair, and repair mutant phenotypes), in animal experiments, and in human biomonitoring (particularly in relation to occupational exposure, and nutrition). Finally, DNA repair has been examined at the level of specific genome regions, using fluorescent *in situ* hybridisation with probes recognising different genes; it is clear that the rate of repair varies greatly between genes - as it does between people.

4. References

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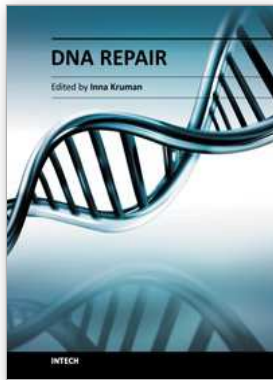
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