The comet assay: Principles and applications

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This project has received funding from the European Union's Horizon 2020 programme: grant agreement 814425.

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Main objectives of the RiskGONE

WP5
Minimum **3** draft TGs will be developed

Deliver pre-validated draft guidance documents for:
Human Hazard assessment

Endpoint	OECD TG	Description
Genotoxicity	TG487 TG476	Micronucleus assay Mammalian cell gene mutation test New *in vitro* guideline for comet assay to detect strand breaks and specific deoxyribonucleic acid (DNA) lesions
Cell Transformation	Guidance documents 214 & 231	Cell transformation assays
Cytotoxicity	TG487 TG432	Relative population doubling (TG487) Colony forming efficiency (CFE) *In vitro* 3T3 NRU Phototoxicity Test

- Refinement of innovative *in vitro* models and mechanistically relevant high-throughput assays for nanosafety eco-toxicological hazard assessment.
- Evaluation and definition of AOPs for ENMs.

3 SOPs

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Main objectives of the RiskGONE

WP6
Minimum **3** draft TGs will be developed and pre-validated

Deliver pre-validated draft guidance documents for:
Eco-toxicological Hazard Assessment

Endpoint	OECD TG	Description
Reproduction	TG211 TG242 TG243	*Daphnia magna* reproduction test (with male induction) *Potamopyrgus antipodarum* Reproduction Test
Multi-generation	TG211	Multi-generation in development
Chronic toxicity (herbicides)	TG238 TG239	Chronic toxicity to water milfoil (*Myriophyllum spicatum L.*)
Genotoxicity	None yet	New Comet assay to detect strand breaks and specific DNA lesions of *Daphnia magna* (or other species) exposed *in vivo*

- Refinement of innovative *in vitro* models and mechanistically relevant high-throughput assays for nanosafety eco-toxicological hazard assessment.
- Evaluation and definition of AOPs for ENMs.

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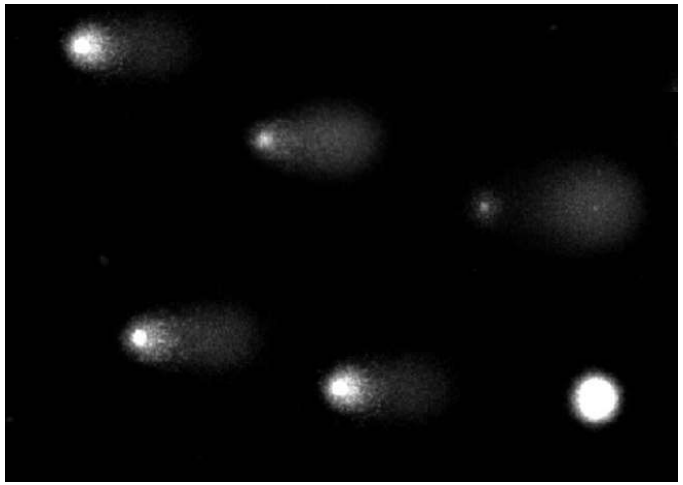
3 SOPs

Comet assay



(single cell gel electrophoresis) – a simple and versatile method for measuring DNA breaks. Widely used in genotoxicity testing, human biomonitoring, ecogenotoxicology, and fundamental research into mechanisms of DNA damage and repair.

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The fraction of the DNA in the tail reflects the frequency of DNA breaks – with the limitation that once all the DNA is in the tail, the assay is saturated. This limit is reached at low levels of damage, in contrast to other assays for DNA damage.

The operating range for the comet assay is quite narrow – from a few hundred to a few thousand breaks per cell; but very conveniently it encompasses the low levels of basal damage in normal cells, and sub-lethal doses of experimentally induced damage.

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The comet assay

Lymphocytes in
1% agarose on
microscope slide

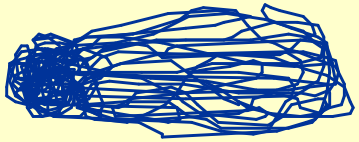
Lysis : Triton X-100, 2.5 M NaCl

Nucleoid; supercoiled DNA

Alkaline incubation:
0.3 M NaOH , 10 mM EDTA

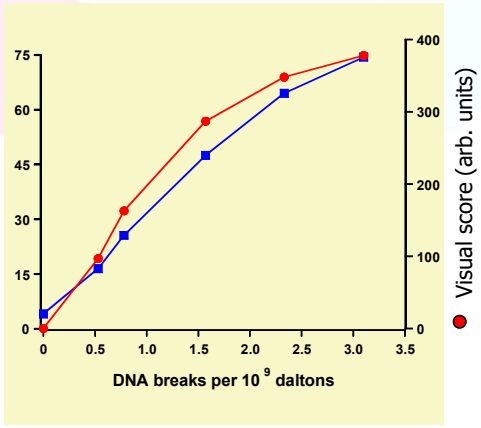
Electrophoresis: 0.8 V/cm, 30 min

Neutralisation, DAPI stain,
fluorescence microscopy



Broken DNA loops extend towards anode, forming tail of comet

% of DNA in tail is related to DNA break frequency



% of DNA in tail
depends on
DNA break frequency

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Isolation cells

Cultured cells: if growing as monolayer, scrape or trypsinise to produce a single cell suspension. Avoid excessive trypsinisation which can cause DNA breaks.

Excessive centrifugation can cause breaks.

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Precoating slides

In earlier protocols, slides were used that were frosted all over; this ensured attachment of the agarose gel, but led to problems of reflection from the frosted surface.

- Now it is recommended to use ordinary slides, precoated with agarose: the gel with the cells then attaches firmly.
- Dip clean slides in a jar of normal melting point agarose held at 55°. Take out, wipe the back, and lay on bench to dry (overnight). Mark the coated side. Slides can then be stacked in boxes for future use.

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Embedding the cells on agarose

- Low melting point agarose is used. It is melted by heating in a microwave oven (avoid boiling!) and then cooled in a water bath set at 37º. It stays liquid at this temperature.
- Centrifuge the required number of cells. Remove supernatant, and resuspend the pelleted cells in the small volume left in the tube (about 50 µl) by tapping hard with your finger.

A few thousand cells are embedded in a thin layer of agarose on a microscope slide.

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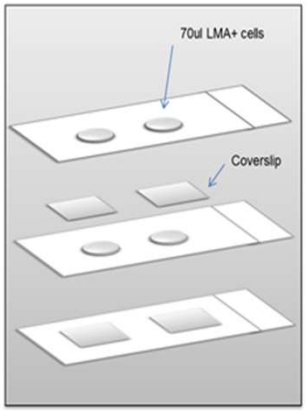
Comet assay miniaturization

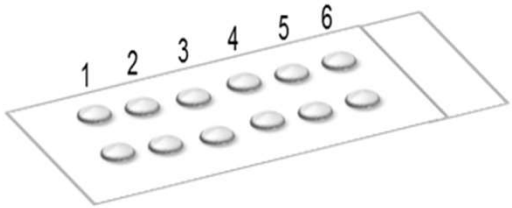
from one gel to 12, 48 or 96 gels

1 gel slide format
100 µl LMA/s 60

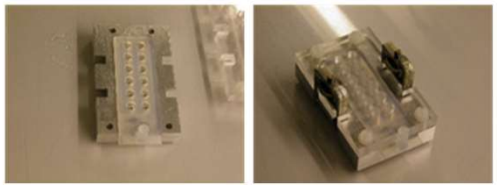
2 gels slide format
5-10 µl LMA/drop



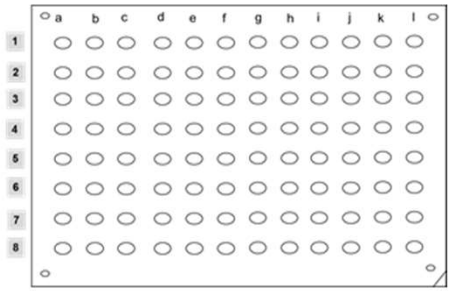
12 gels (6x2) slide format
5-10 µl LMA/drop



Chamber to allow separate incubation



96 or 48 gels GBs film
4-5 µl LMA/drom



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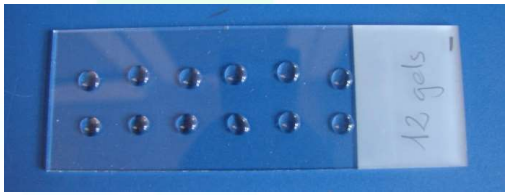
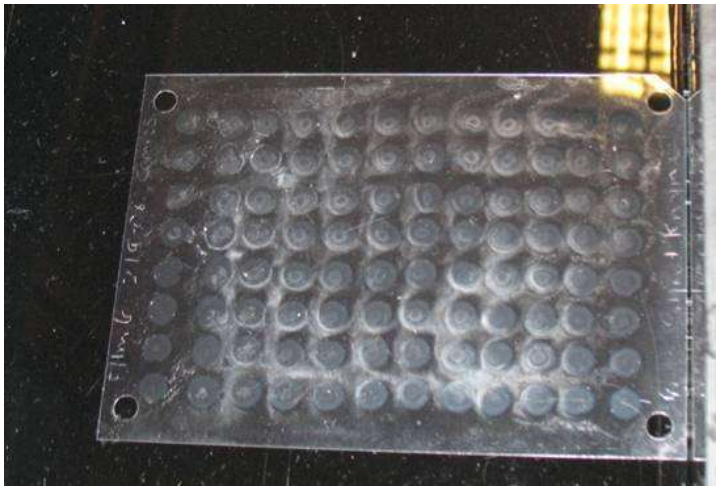
High throughput methods were developed in the 'COMICS' project:



Twelve mini-gels on a standard glass slide…

…or a GelBond film with up to 96 mini-gels.



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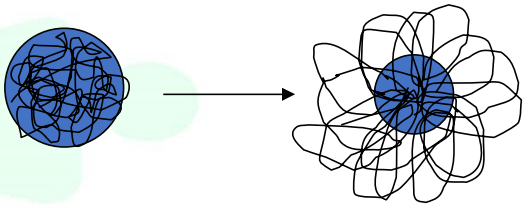
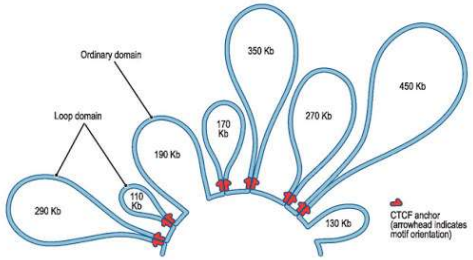
Lysis of cells





- The lysis solution contains detergent (Triton X-100) and 2.5 M NaCl. The detergent breaks down membranes, and allows cytoplasm and nucleoplasm to disperse, leaving DNA as a compact histone-less structure, the nucleoid.

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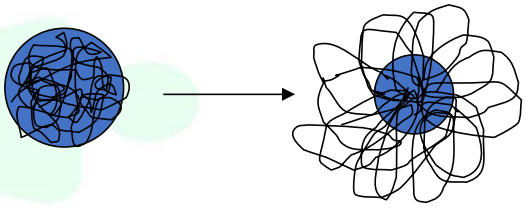
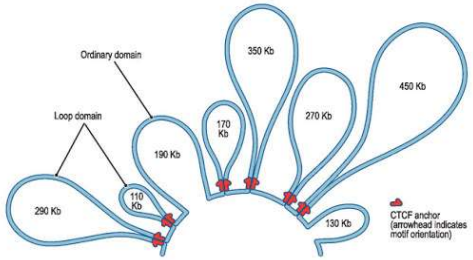
Lysis of cells





- Although the nucleosomes are destroyed by removal of histones, supercoiling is conserved in the DNA loops attached to a structure called the nuclear matrix – unless there is a DNA break in the loop. So, if there is a break in the DNA, the supercoiling in that loop is relaxed. Lysis should be carried out at 4º, for one hour or longer (overnight is OK).

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Alkaline treatment and electrophoresis

- Slides are placed in the electrophoresis tank and cold alkaline solution added to cover the slides: leave for 20 min at 4º.
- The tank should be level, so that all slides are evenly covered.
- Carry out electrophoresis. The critical parameter is the voltage drop across the slides, which should be 1 V/cm. (Most of the voltage drop is across the platform where the layer of alkali is thinnest.)
- If there is more than 1 or 2 mm of solution over the gels, you may exceed the maximum current of the power supply (normally 0.4 or 0.5 A). Remove some solution using a beaker. 300 mA is a popular current – but it is not necessary to have a defined value. The voltage is more important.

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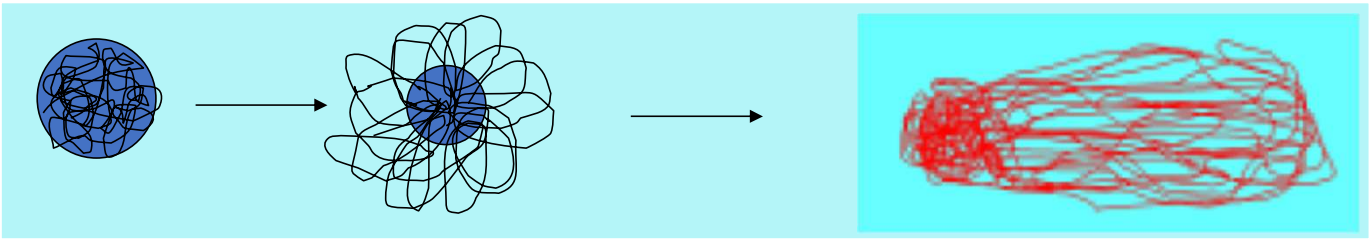
Electrophoresis

Under electrophoresis broken DNA (in relaxed loops) is pulled towards the anode, forming a comet-like tail when stained and viewed under (usually) UV-fluorescence microscopy.

Because only the loops with breaks are able to move, the relative intensity of the tail reflects the frequency of DNA breaks.



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Neutralisation and staining

•Place the slides in a bath of phosphate-buffered saline (PBS) and leave for 5 min. If the slides are to be stored, it is advisable to remove the buffer by washing the slides with water so that crystals do not form.

• To store, first dry the slides by leaving them on a bench overnight. They can be stacked in slide boxes and scored later.

• Either fresh or dried, comets are visualised by staining with one of a selection of stains, including DAPI, ethidium bromide, propidium iodide, Sybr stains, or even silver.

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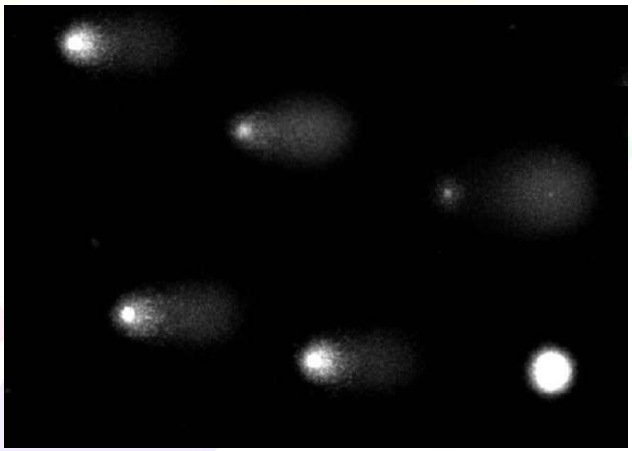
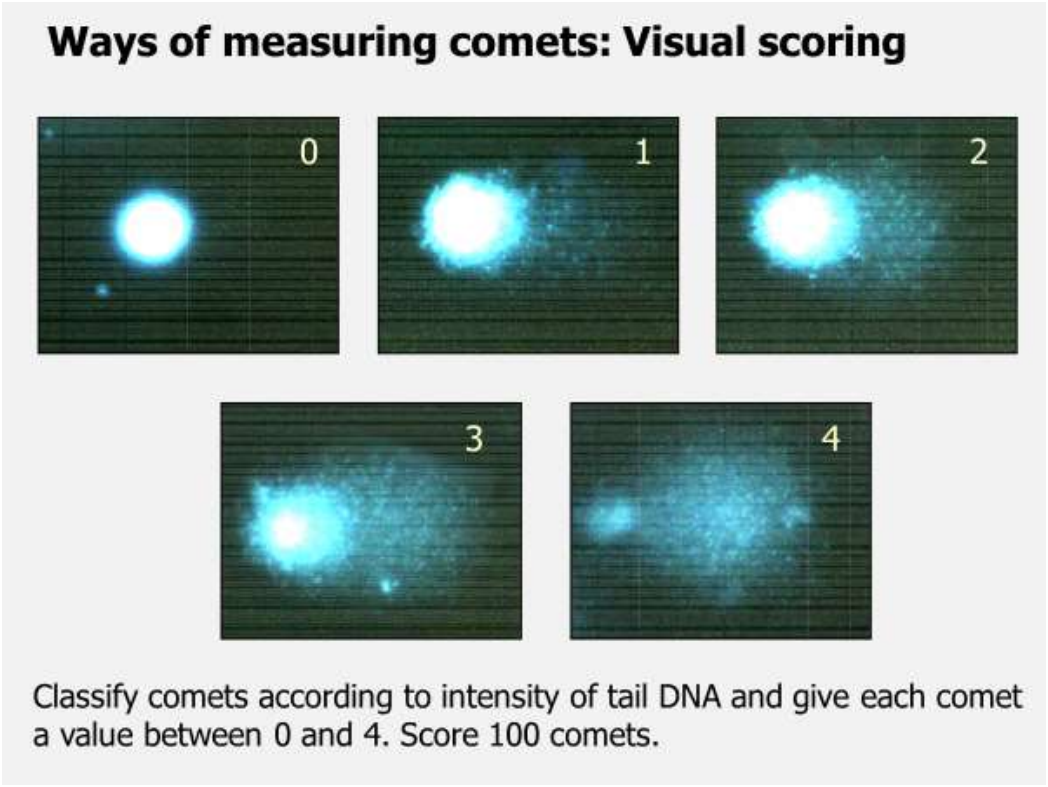


Comet scoring - analysis

The simplest method for measuring comets is by 'visual scoring'.



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Ways of measuring comets: Image analysis

A camera links the microscope to a computer with image analysis software. A comet is selected, and intensity of fluorescence is measured over different parts of the comet. Computer-based image analysis seems to be more objective than visual scoring. But in both, there is a subjective element, as the comets are selected for scoring. If there are too many cells present, comet tails may overlap and be impossible to analyse by computer. So some highly damaged comets may be missed, and damage overall under-estimated.

It is a quantitative assay. Slides are analysed by computerised image analysis

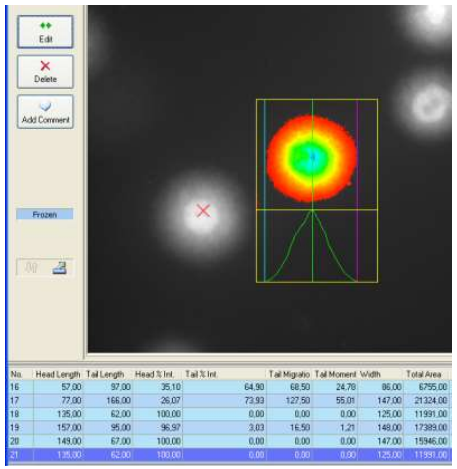

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Image analysis

% DNA in tail?
Tail moment?
Tail length?

- '% DNA in tail' is linearly related to DNA break frequency over wide range of damage.
- 'Tail length' increases only over very low range of breaks.
- 'Tail moment' combines these measures and is non-linear.
- 'Tail moment' has no recognised units and is impossible to visualise.

USE % DNA IN TAIL!

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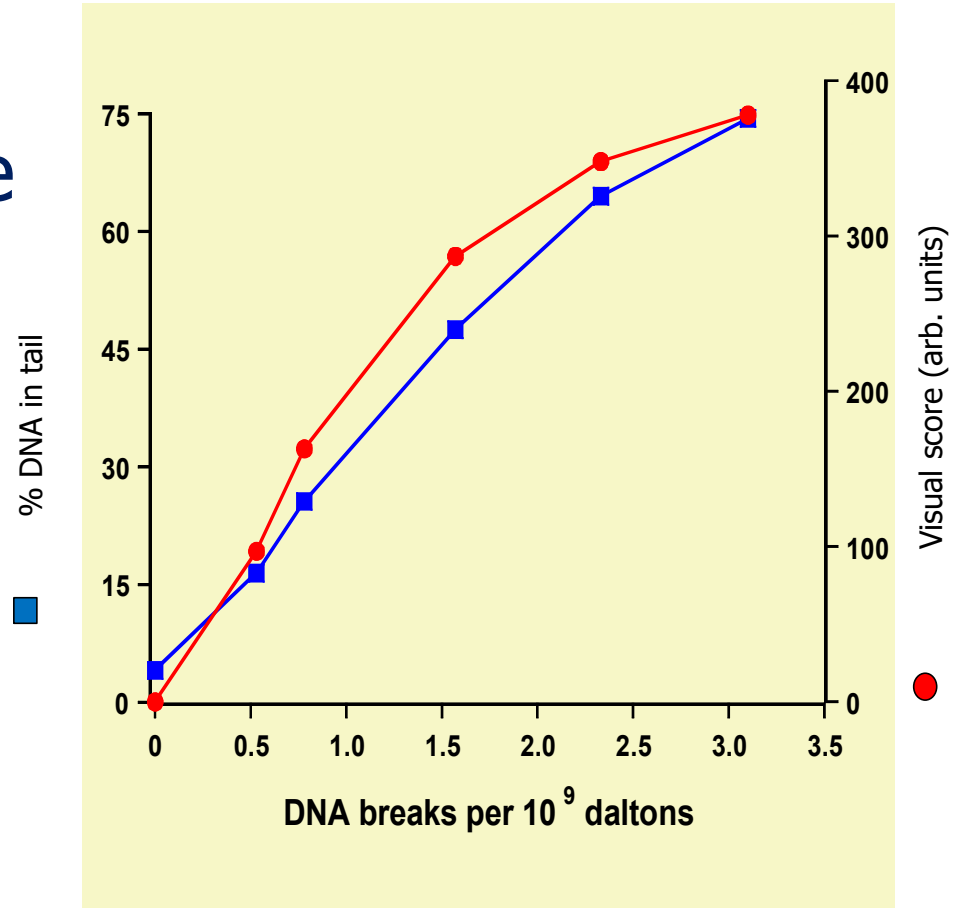
Calibration of the comet assay against X-ray damage

Doses of X-rays up to 10 Gy: it is known that 1 Gy induces 0.31 break per 10^9 Dalton.

The comet assay is saturated at around 10 Gy, equivalent to 3 breaks per 10^9 Dalton



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Some history

1970s: Theory of 'nucleoids' developed by Peter Cook and colleagues: lysis of cells with non-ionic detergent and high concentration of NaCl leaves DNA as supercoiled loops attached to the nuclear matrix. If the supercoiling is relaxed, DNA loops spill out to form a halo.

1984: Östling and Johanson: electrophoresis of agarose-embedded, lysed cells (nucleoids); tail described in terms of relaxed supercoiling. pH <10.

1988: Singh *et al.:* Lysis with 2.5 M NaCl, Triton X-100, Na sarcosinate (sarkosyl), followed by alkaline incubation and electrophoresis. pH>13.

1990: Olive *et al.:* Lysis in weak alkali (pH~12) followed by electrophoresis.

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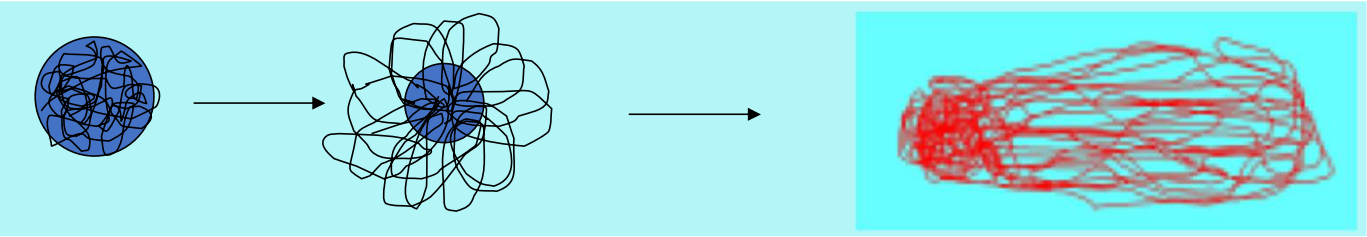
DNA loops or fragments?

Rather than thinking in conventional terms, of migration of fragments according to size, a better analogy (as Östling and Johanson understood) is with the nucleoid assay (the work of Peter Cook and others in the 1970s). Nucleoids are formed by lysis in high salt and Triton X-100. The nucleoid contains supercoiled loops, and if loops are broken, they spill out, forming a 'halo'.



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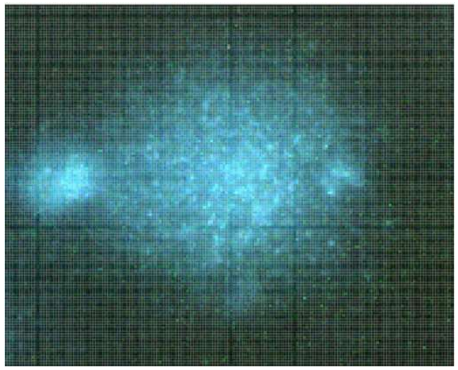
Apoptosis and the comet assay



Do 'class 4' or so-called 'hedgehog' comets represent apoptotic cells? Not necessarily.

- Cells damaged with e.g. H_2O_2 can appear as class 4, but if incubated to allow repair, they produce undamaged comets. Yet apoptosis is irreversible.
- Apoptosis involves fragmentation of DNA to pieces so small that they will disappear from the gel.
- The comet assay may detect cells as they start apoptosis.
- Singh has described a simple assay for apoptosis (essentially cell lysis in the gel with no electrophoresis)

*[Singh (2000) Exp. Cell Res. **256**, 328-337]*

See also Lorenzo Y, Costa S, Collins AR, Azqueta A. The comet assay, DNA damage, DNA repair and cytotoxicity: hedgehogs are not always dead. Mutagenesis. 2013 Jul;28(4):427-32. doi: 10.1093/mutage/get018. Epub 2013 Apr 29.

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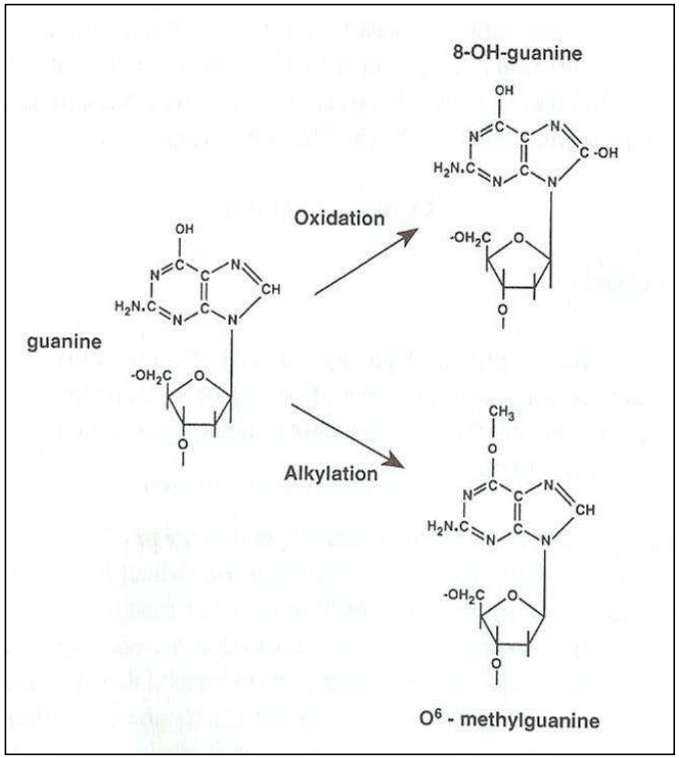
What we can measure by the comet assay

- Single strand breaks, double strand breaks
- Small base changes such – oxidation and alkylation
- DNA repair



The base damage is repaired by base excision repair pathway (BER)

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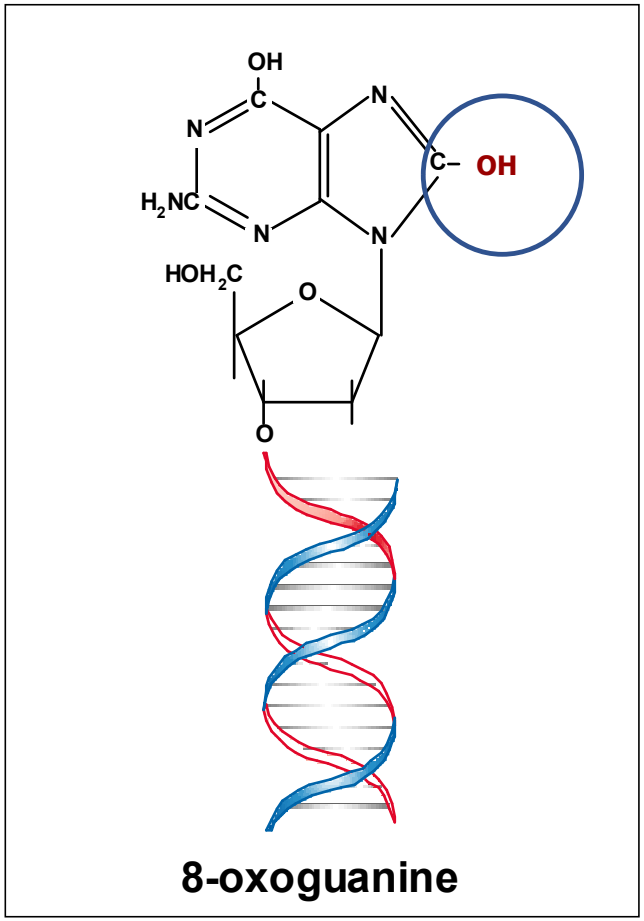
Oxidation:

8-oxoGua or 8-OH-Gua

Results from attack by reactive oxygen; potentially serious for the cell because the base-pairing properties of guanine are altered so that A, rather than C, is likely to be inserted in the daughter strand during replication.

8-oxoguanine – potentially mutagenic, easily measured; a popular biomarker.
Recognised by Fpg.



8-oxoguanine

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DNA repair

- Strand break (SB) rejoining – insertion of missing nucleotide and ligation.
- Base excision repair (BER) (oxidised or alkylated DNA) – a specific glycosylase removes the base, leaving an AP site, which is then cleaved. A DNA polymerase fills the gap; finally, ligation.
- Nucleotide excision repair (NER) (bulky adducts, UV-induced pyrimidine dimers) – a repair protein complex removes an oligonucleotide containing the damage; gap-filling by DNA polymerase is followed by ligation.

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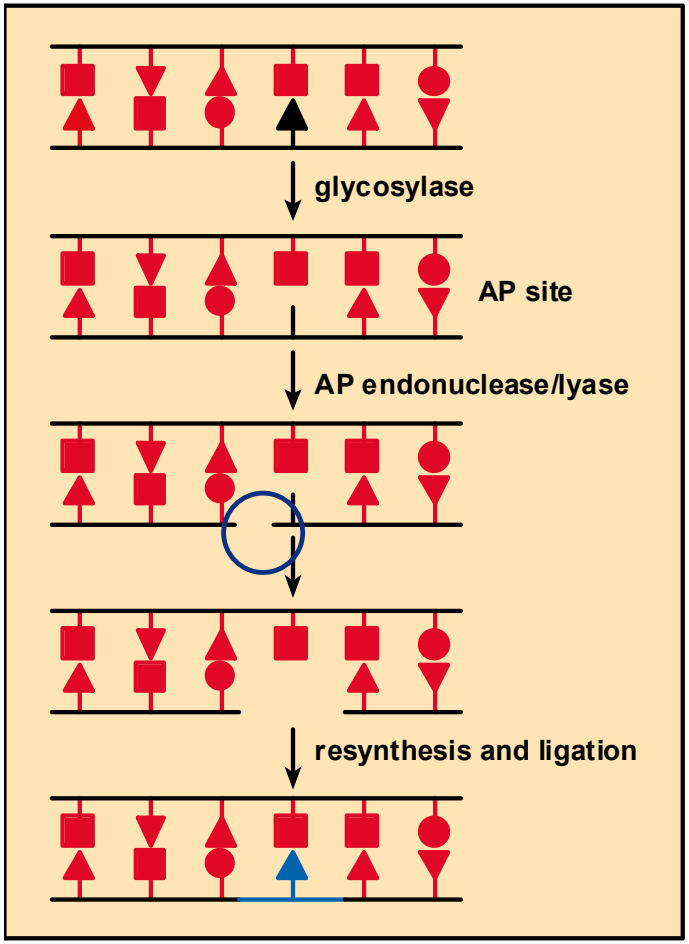
Base excision repair

Correction of small base changes, e.g. oxidation, alkylation.

First step is removal of damaged base by a glycosylase. These enzymes are more-or-less lesion-specific. For example, there is a human enzyme, hOGG1, that removes 8-oxoguanine. The glycosylases often have an associated AP lyase activity which completes the DNA breakage. This may be very slow. An AP endonuclease speeds up the process.



Simplified version

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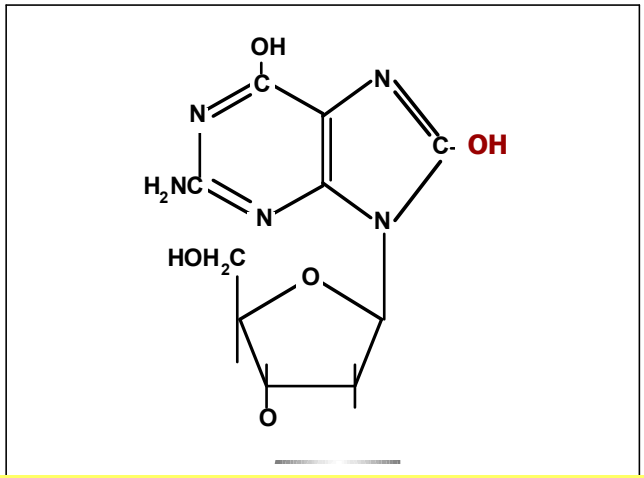
Measurement of specific DNA lesions

To measure altered bases, we introduce an additional step into the comet assay procedure, digesting the nucleoid DNA with a lesion-specific endonuclease, to convert the damaged bases to breaks (*Collins et al., 1993, Dusinska and Collins, 1995).*

An example of base damage: 8-oxoguanine, the result of attack by reactive oxygen. This is a common lesion, potentially dangerous because it mis-pairs with A instead of C and so can lead to mutation.



8-oxoguanine – potentially mutagenic, easily measured; a popular biomarker. Recognised by Fpg.

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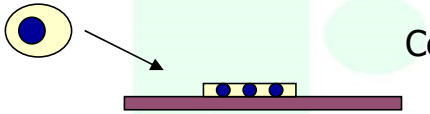
Measuring specific lesions in DNA



Cells embedded in agarose on microscope slide

Lysis: Triton X-100, 2.5 M NaCl

Nucleoid; supercoiled DNA

± Digestion with lesion-specific endonuclease

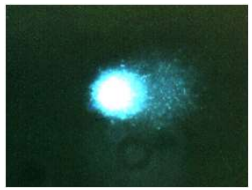
Alkaline incubation: 0.3 M NaOH, 10 mM EDTA

Electrophoresis: 0.8 V/cm, 30 min

Neutralisation, staining, fluorescence microscopy

The frequency of damaged bases is given by the increase in DNA breaks in the presence of the specific endonuclease

This assay allows us to estimate the level of base damage in the cells



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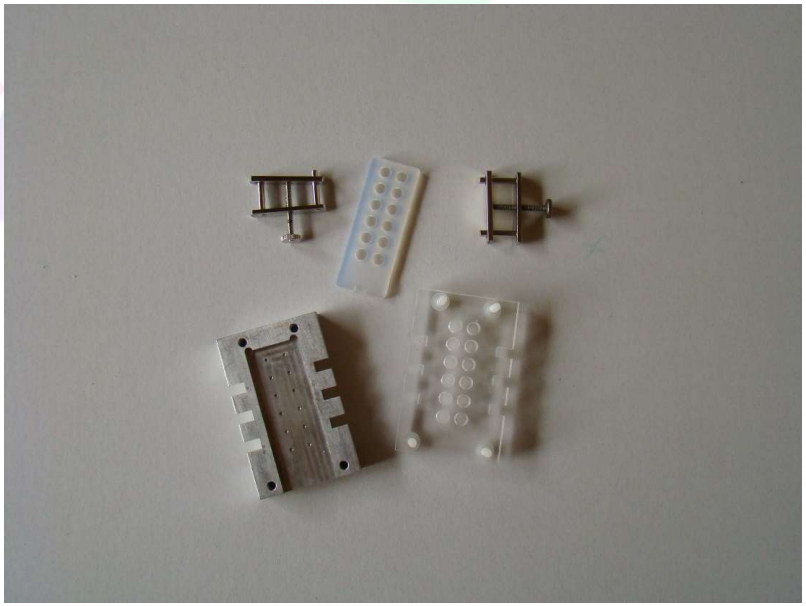
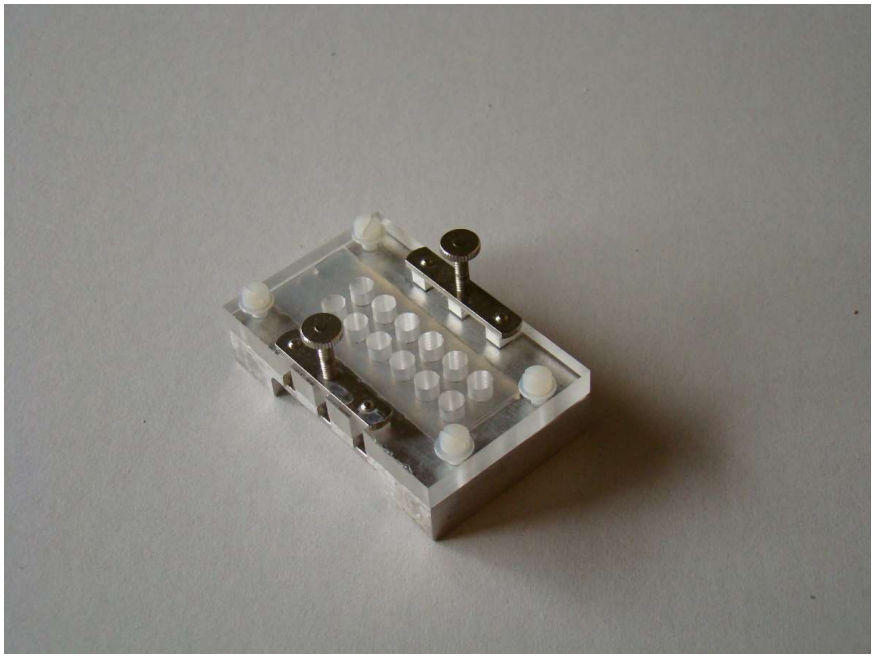
For the 12-gel system, a chamber device (now commercially available) that allows individual treatment of gels with reagents or enzymes was developed





The slide is clamped under a gasket with holes matching the gel positions.

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The enzymes; repair glycosylases (endonucleases)

- Formamidopyrimidine DNA glycosylase (FPG) recognises 8-oxoguanine (probably its main substrate in vivo) and ring-opened purines (fapy- adenine, fapy-guanine)
- Endonuclease III detects oxidised pyrimidines
- AlkA detects 3-methyladenine (but is not very specific)
- T4 endonuclease V detects UV-induced pyrimidine dimers
- Uracil DNA glycosylase (misincorporated U)
- UvrABC (Bulky adducts, helix distortions) – Not yet working satisfactorily
- OGG1 – specific for 8-oxoGuanin

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THANK YOU!

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