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Biological effects of contaminants: Assessing DNA damage in marine species through single-cell alkaline gel electrophoresis (comet) assay

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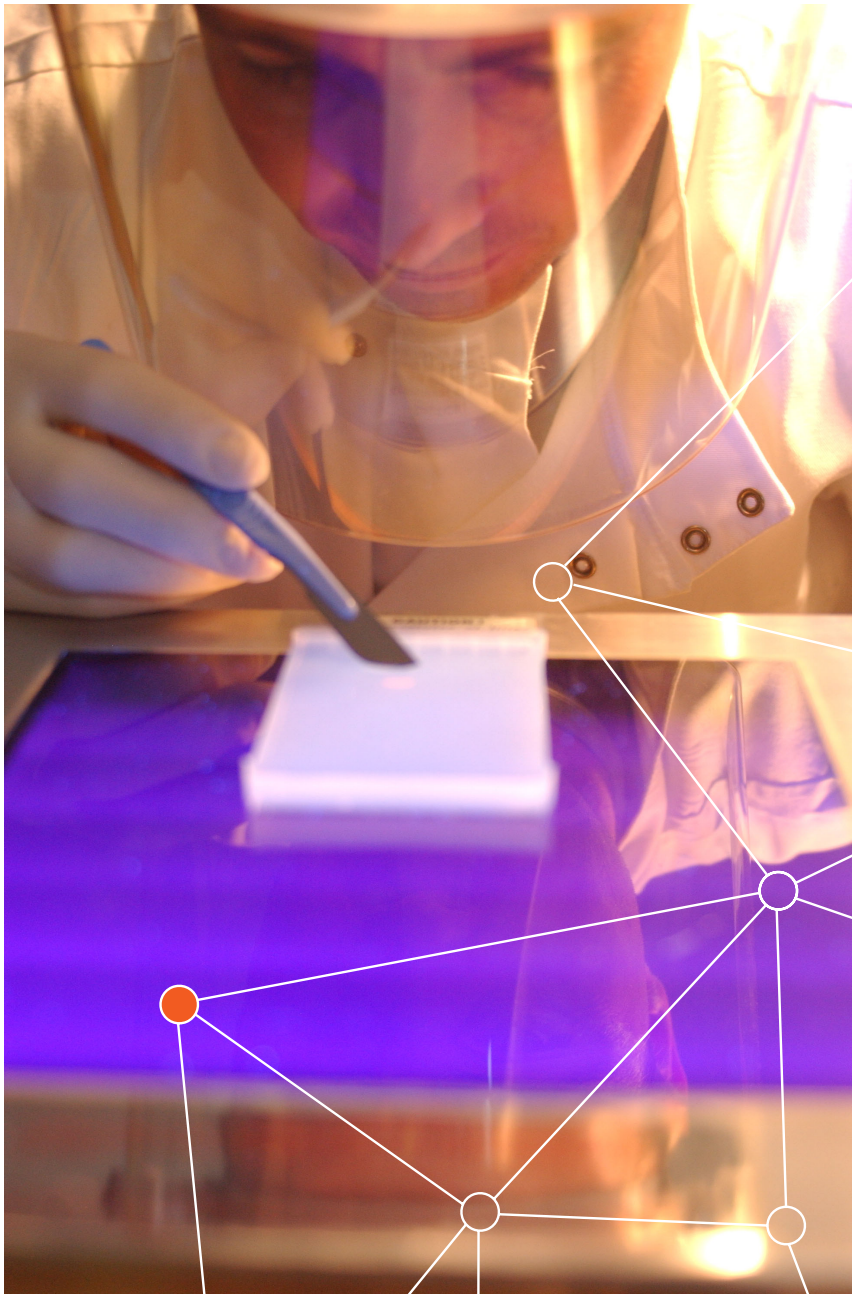

ICES INTERNATIONAL COUNCIL FOR THE EXPLORATION OF THE SEA
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Editors

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Correspondence about the details of any method or procedure should be directed to the author(s).

This series presents detailed descriptions of methods and procedures relating to chemical and biological measurements in the marine environment. Most techniques described have been selected for documentation based on performance in ICES or other intercalibration or intercomparison exercises: they have been carefully evaluated and demonstrated to yield good results when correctly applied. They have also been subject to review by relevant ICES working groups, but this is not to be construed as constituting official recommendation by the Council.

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Contents

Abstract

Single cell gel electrophoresis (SCGE or comet) assay allows quantification of DNA damage in individual cells and is an ideal tool for use within biological monitoring programmes. Comet assay can be used on a range of cell types including somatic, reproductive (gametes) or circulatory cells in many different species including both marine bivalves and flatfish. The assay can be employed with simple equipment available in most laboratories, is sensitive to environmentally relevant levels of DNA damage (Frenzilli and Lyons, 2009), accurately demonstrates a linear dose response to exposure (Collins *et al.*, 1996), and can be adapted for use on most nucleated cell types. This document concentrates on the simplest and most repeatable method of comet assay in circulatory cells of species commonly used in marine biomonitoring programmes both for chemical and biological effects. This manuscript describes standardized assay procedures and recommends the minimum level of information required when reporting comet assay results.

Keywords

Comet assay, single cell gel electrophoresis, DNA damage, genotoxicity, biological effects, bivalves, flatfish, integrated monitoring.

1 Introduction

1.1 DNA damage

DNA damage is a physical abnormality in the structure of DNA that results in reduced DNA integrity. It occurs endogenously as part of normal cellular activity as well as being induced by interaction with environmental chemicals (Kruszewski *et al.*, 2012), many of which have direct or indirect effects on genetic material. In the case of organic pollutants, genotoxicity generally requires metabolic activation leading to the production of by-products or metabolites that can directly or indirectly react with the DNA.

Genetic lesions can be discriminated into several types, including primary structural lesions such as adducts, strand breakage, base oxidation, and alkylation, and interstrand crosslinks (Geacintov and Broyde, 2010). These lesions are usually repaired by the DNA repair systems but if the repair is incomplete or inefficient, the lesions can lead to genetic mutations. Severe or repeated cases of exposure to genotoxicants are more likely to result in such alterations. These changes then increase the probability of severe effects such as cell death or neoplasia and cause potentially fatal diseases such as carcinoma (Bailey *et al.*, 1996). Genotoxic effects are even more marked when they affect chromosomes. Unlike structural lesions, cytogenetic damages are irreversible and can be observed on chromosome structure or number (clastogenic and aneugenic effects) leading respectively to chromosome and genome mutations. Chromosome breakages can result from an accumulation of DNA strand breaks, whereas number anomalies are observed in cases of a chromosome loss (hypoploidy) or gain (hyperploidy) during cell division, either during mitosis or meiosis.

1.2 Assessing DNA damage in chemical risk assessment and the MSFD

The potential of chemical compounds to cause DNA damage and cancer has resulted in thorough guidelines surrounding genotoxicity evaluation as part of the chemical risk assessment process. Since the 1970s, government policies have progressed to cope with the presence of toxic substances in aquatic systems in order to protect the environment. The development of the Marine Strategy Framework Directive (MSFD) in the European Union (EU) has resulted in a legislative requirement for European waters to have good environmental status (GES) by 2020 (EU framework directive 2008/56/EC) (Lyons *et al.*, 2010). This legislation, alongside other EU initiatives such as the Council Directive (96/61/EC) on Integrated Pollution Prevention and Control, which was adopted in 1996 (O'Malley, 1999), requires EU countries to reduce input and where possible to clean up legacy pollution. This legal weight requires regular monitoring of EU waters for both the level of contaminants and also for the level of contaminant effects, of which DNA damage in marine organisms is a key component. Legal implications aside, public interest surrounding events such as the Deepwater Horizon accident, which resulted in the release of 600,000 tonnes of crude oil into the Gulf of Mexico (Crone and Tolstoy, 2010) have shown that it is crucial to recognize baseline effects and levels of contamination so that in the case of environmental disaster it is possible to quantify the amount of damage.

Guidelines for systematic marine monitoring have been produced by the ICES Steering Group for Integrated Monitoring of Contaminants (Davies and Vethaak, 2012), which recommend that the measurement of DNA damage should be used within a weight of evidence approach to environmental monitoring, forming part of an integrated system which gives an indication of the overall effects of chemicals and chemical mixtures.

The comet assay used synergistically with other molecular and biochemical biomarkers is a key part of the toolbox required to understand the overall issue of chemical effects throughout marine environments.

1.3 The comet assay

The comet assay is used to assess the level of single and double-stranded DNA breakages in a cell. Due to its numerous advantages it has been widely applied in medical research and ecotoxicology. At alkaline pH it allows the measurement of both strand breaks and alkali labile sites (Singh *et al.*, 1988) making possible a sensitive and integrated measurement of genotoxicity for the detection of environmentally relevant levels of DNA damage (Frenzilli and Lyons, 2009). The assay is rapid, simple, and demonstrates a linear dose response to exposure (Collins *et al.*, 1996). It can be adapted for use on most nucleated cells whether they are germinal or somatic. Circulatory cells are widely used because they are already singular and there is no need to isolate cells. However, tissue cells can also be analysed following enzymatic or mechanic dissociation.

Comet assay has been utilized following *in vitro* and *in vivo* exposures to assess the potential of many chemicals to cause DNA damage (Tice *et al*., 2000). The sensitivity and efficacy of the assay to recognize genotoxic compounds has been proven across different cell lines and species. Methods have been approved for use in regulatory testing by the Organization for Economic Cooperation and Development (OECD) (Test Guideline 489) (Burlinson, 2012). In addition, and of more interest to International Council for the Exploration of the Sea (ICES) Techniques in Marine Environmental Science (TIMES) readers, the assay can also be used to assess DNA damage in the environment using two distinct methods; either by directly sampling wild or transplanted animals *in situ* or by exposing laboratory animals to an environmental substrate, usually water or sediment. These can both be done to assess genotoxicity at different locations or over time following an incident. The comet assay can be useful as a standalone test to identify DNA damage caused by significant marine point source contamination, for example environmental DNA damage increases as caused by a fuel oil spill from the MSC Napoli (Lewis *et al*., 2010), or alongside other methods to understand the effects of chronic pollution.

Although the comet assay is suggested as a primary method for measurement of DNA damage, it is worth noting that it is one test of several that have been used successfully on environmental samples. The most widely used of the alternative assays is the micronucleus (MN) assay which provides an efficient measurement of irreversible chromosomal damage in bivalves and fish cells and is the most widely used of the alternative assays (Bolognesi and Fenech, 2012; Bolognesi and Hayashi, 2011). A further assessment of the effects of genotoxic chemicals can be achieved through measurement of DNA adducts in aquatic organisms. These adducts are the result of the covalent binding to the DNA of a chemical agent providing information on both pollutant exposure and damage to the DNA. DNA adduct levels above background have been shown to occur in the event of major point source contamination (Lyons *et al.*, 1997). Levels of DNA adducts in legacy contaminated estuaries have been known to give less sensitive indications of environmental contamination when compared to comet assay (Akcha *et al.*, 2003). However, it is worth noting that although these techniques often correlate they represent different endpoints and as such should be regarded as complementary, rather than directly comparable techniques. As such it is often preferable to use both where required.

1.4 Species for monitoring DNA damage

When choosing a species to study the biological effects of contaminants it is important to take into account several aspects of the biology of the species in question, e.g. wide distribution to allow site comparisons, high abundance and easy sampling, low mobility to guarantee site specificity, clear position in the trophic network, good knowledge about the ecology, physiology of the species, and position in the ecosystem. For standardized marine monitoring there are two phyla that appear to fulfil all of the above. Invertebrate bivalve molluscs, such as oysters and mussels, are sessile, widespread, and filter feeders and are used for chemical biomonitoring programs such as the Mussel Watch Program in the USA and the Réseau National d'Observation in France. Vertebrate marine flatfish, such as *Limanda limanda*, or *Platichyths flesus* are also ideal species since they live in contact with the sediment and are known as a sink for a large number of chemical pollutants. Whether in bivalves or fish, circulatory cells can be easily sampled in a non-destructive way from the haemolymph or blood respectively for DNA damage analysis by the comet assay. With any species the potential influence of the reproductive process or the climate must be considered to better assess the relationship between chemical exposure and biological responses. In the case of mobile organisms migratory movements must also be considered.

There are many examples of successful applications of the comet assay for marine biomonitoring. In dab (*Limanda limanda*) from the eastern part of the English Channel, the comet assay was applied to obtain better insight into the relationship between organic chemical exposure and genotoxic effects in fish. The comet assay discriminates different sampling sites in accordance with their level of chemical pollution (Akcha *et al.*, 2003; Akcha *et al.*, 2004). Significant positive correlations were obtained between the level of DNA strand breaks of dab erythrocytes and the PCB and PCDD/PCDF concentrations in dab liver and muscle (unpublished results). As predicted with PAHs, the correlation was significant only when PAH metabolites were considered rather than PAH content (Dévier *et al.*, 2013). In addition, age and sex have a significant effect on the level of DNA damage in dab as measured by the comet assay (Akcha *et al.*, 2004). The significant interaction between these two factors suggests a complex influence of other factors such as the reproduction status on the extent of DNA damage.

Studies have also been conducted for coastal biomonitoring using bivalves (Klobucar *et al.*, 2008; Rank *et al.*, 2005) and correlations between DNA damage and chemical pollution were observed. Transplanted mussels have been shown to have reduced DNA integrity three days after transplantation to a polluted site. They then continue to maintain the increased level of DNA damage over a full 30 day transplantation experiment, suggesting that the assay is not only sensitive but also reliable over periods of exposure in bivalves (Regoli *et al.*, 2004).

1.5 Methodology

This document describes in detail the *in situ* application of the comet assay for marine biomonitoring. Despite the easy application of the comet assay, some technical points are important to control and adjust to guarantee the quality of the comet data collected in both fish and bivalve cells. Note this document does not include risk assessment of the tasks, which should be completed independently by each user.

2 Test methods

Preferably the whole assay, from cell isolation through to slide fixing, should be completed in one phase (Figure 1). However, if required for logistical purposes, there are several stages at which it is possible to put the assay on hold. Cells can be cryopreserved at -80°C or after isolation they can be maintained at 4°C. Alternatively after cell lysis but prior to alkaline unwinding slides can be held at 4°C. Where these alterations to the standard protocol are used they should be thoroughly verified to ensure that results are not artificially confounded.

Note that DNA damage is caused directly and indirectly (through photo-activation of chemicals) by UV light. Where possible the comet assay should be performed under safelight (i.e. in a dark room) to prevent the formation of experimental error. In the absence of safelight it is important to keep cells out of direct sunlight and to maintain consistent lighting for all samples.

A small amount of preparation is required in advance of running the assay. Buffers should be mixed in advance and chilled to 4°C. Slides need to be prepared one day in advance and also kept cool (e.g. at 4°C). Alternatively Trevigen® pre-prepared slides (Gaithersburg, Maryland, USA) can be utilized as supplied by manufacturer. If a high-throughput, membrane mounted, version of the assay is to be performed it may be necessary to prepare polyester gel-binding membrane of the correct size onto which agarose can later be pipetted (Gutzkow *et al.*, 2013).

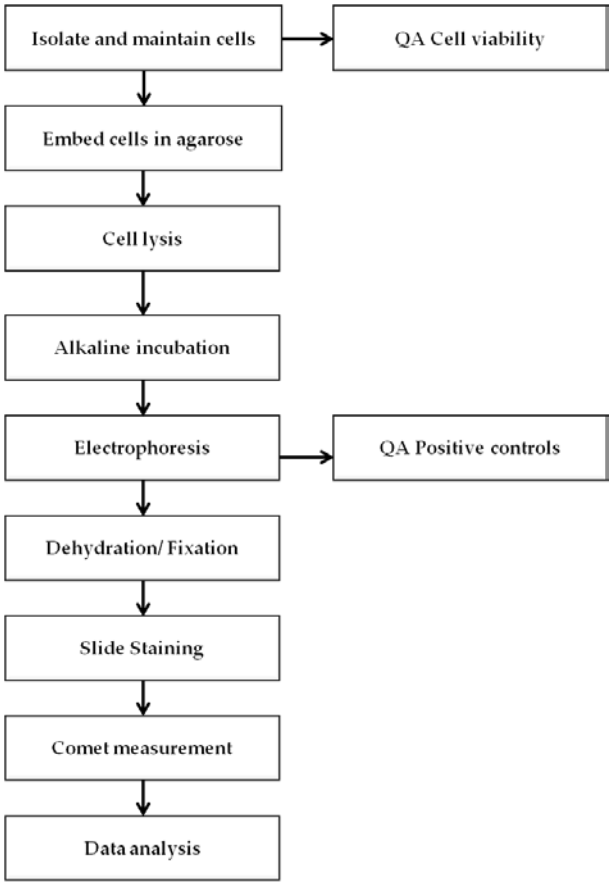

Figure 1. Flow diagram of comet assay process. The section from cell isolation through to slide fixation should be followed in one period, after which slides can be stored before analysis.

2.1 Methods

2.1.1 Sampling

Sampling strategy depends heavily on the species used and the purpose of the sampling. Therefore it is not possible to recommend specific sampling procedures except to say it is important that DNA damage as an artefact of sampling must be avoided. All sampling variables should be recorded and reported with the comet assay data (see section 3.2. Data reporting).

2.1.2 Cell handling

It is important to ensure that cell handling does not result in induction of DNA damage. Preferably the cell is embedded in agarose as soon as possible after isolation and then kept at 4°C in the dark or, alternatively, the cell is maintained in an osmotically balanced solution in the dark at 4°C (Hartl *et al.*, 2010). The duration of this storage should be minimized to prevent artefact formation and methods should be tested for each novel cell type. The present paper describes the methodology for circulatory cells, which are often easier to extract, but the method can be adapted for dissociated tissue cells.

Bivalve haemocytes

To get to the adductor muscle of, for example, a *Mytilus* species mussel, the bivalve is opened with a solid blade scalpel. Hold the mussel posterior edge down and ventral side towards the analyst and insert the blade between the valves, roughly half way down the ventral side, and gradually push the blade in between the valves and down. Once the valves are gaping at around one millimetre the mussel should be drained of seawater and then turned posterior side up, with the scalpel still in place. The 23G needle can then be inserted directly into the posterior adductor muscle (from the ventral side) and up to 1 ml haemolymph withdrawn.

Haemocytes can be used directly in the assay, as detailed below, or can alternatively be cryopreserved for analysis at a later date (Kwok *et al.*, 2013). Briefly, for cryopreservation, haemocytes are extracted into an equal volume of osmolality adjusted Hanks' Balanced Salt Solution (HBSS) with 20% glycerol (end concentration 10%) and incubated at 20°C for 20 min. The sample is then frozen in a BioCision CoolCell® device in the vapour phase of a dry shipper for three hours before being transferred to liquid nitrogen where it can be stored for up to 30 days. Haemocytes are defrosted in a water bath at 37°C for 30 seconds before being added directly to low melting point (LMP) agarose for analysis in the comet assay.

Flatfish erythrocytes

Blood is taken directly from the caudal vein of the organism using a heparinized syringe with a 23-gauge needle. One drop (20 µl) is sufficient to perform the assay. Once the drop is withdrawn, the needle is removed from the syringe and one drop of blood is recovered in 700 µl of either calcium magnesium free saline buffer or in a phosphate saline buffered saline for a direct application of the assay. Blood can also be recovered in a cryopreservation solution to allow a delay for the processing of the samples.

Cryopreservation is commonly required when sampling at sea, in the field, or when a high number of samples are collected at the same time. The freezing medium is made of RPMI 1640 supplemented with fetal calf serum (25%) and dimethyl sulfoxide (DMSO) (20%). Once gently mixed, the blood sample is either stored at 4°C in the dark for direct analysis or brought gradually to -196°C, including a two-hour hold step at -

20°C. Erythrocytes are rapidly defrosted in a water bath at 37°C before embedding on comet slides. Depending on the erythrocyte concentration, a dilution step in phosphate-buffered saline (PBS) may be required before inclusion in low melting point (LMP) agarose.

2.1.3 Cell embedding

The method of embedding varies depending on the slide type used.

For Trevigen® slides:

Trevigen® comet slides (Gaithersburg, Maryland, USA) are pre-prepared for the comet assay and are the simplest to use, albeit at increased cost. To embed cells, add 38 µl of cell suspension to 150 µl of 0.8% LMP agarose and mix gently by pipette. Immediately pipette 50 µl onto the well in the slide and ensure it touches all edges. Make two replicate wells for each sample and allow the slide at least five minutes to set at 4°C in the dark.

For homemade slides (frosted glass prepared with normal agarose):

Frosted glass slides with a smooth window are particularly convenient for the comet assay. One day in advance of running the assay, the first layer, consisting of 0.5% normal melting point agarose, is added by dipping half the slide into molten agarose and allowing it to set at 4°C. This gives better adhesion of the second layer of LMP agarose in which target cells are suspended. The concentration in agarose of this second layer is usually between 0.4 and 0.8% depending on the basal level of DNA fragmentation of the cell types analysed. The higher the % of agarose is, the lower the migration length. However, at concentrations below 0.4% samples can be more difficult to manipulate due to lower melting temperature and increased fragility. At least two slides are prepared for each sample.

For high throughput assays on polyester film:

Recent work has demonstrated that large numbers of samples can be simultaneously processed by using polyester film techniques (Gutzkow *et al.*, 2013). This technique has not been fully tested for environmental sampling but it should be possible to mount cells by adapting the protocol as utilized for Trevigen® slides. As always, potential introduction of artefact DNA damage should be taken into consideration.

2.1.4 Lysis

Once all the slides are set, they are immersed in freshly prepared cold lysis buffer for at least one hour at 4°C in the dark (note: this can be allowed to continue for extended periods if required). This stage allows the digestion of cellular and nuclear constituents including proteins and RNA.

2.1.5 Alkaline unwinding and electrophoresis

After lysis, cells undergo DNA "unwinding", followed by electrophoresis.

DNA "unwinding" is performed by incubating the slides (15 min, in the dark) in freshly prepared electrophoresis buffer in the electrophoresis tank. The pH value is important as it determines the type of damage measured by the comet assay. At alkaline pH (pH ≥ 13), both single strand breaks and abasic sites are measured. Following denaturation, the 3D structure of the DNA is disrupted and all hydrogen bonds are eliminated resulting in DNA in a single-stranded form.

To avoid warming of the buffer during electrophoresis, it is recommended to start with cold electrophoresis buffer and to conduct the electrophoresis in a temperature controlled room. Electrophoresis systems which allow a good buffer circulation may prevent the formation of pH and ionic gradients resulting in a heterogeneous migration pattern. Migration will depend on both electrophoresis time and current voltage.

Electrophoresis is performed at a constant voltage of 0.7 Vcm^{-1}, a starting current of ~300 mA, and at a constant temperature, usually 4°C. Voltage is controlled by power pack and set at a voltage equal to the distance between electrodes in cm multiplied by 0.7 (for example a 30 cm tank would be run at 21 V). In order to achieve the correct starting current the quantity of electrophoresis buffer required should be assessed empirically for each electrophoresis tank. Briefly, place the equivalent number of blank slides in the tank as you are expecting to run. Add electrophoresis buffer until it just covers the slides. Run the electrophoresis tank with voltage fixed at 0.7 Vcm^{-1} and check the current. Adjust the quantity of buffer until the current is 300 mA (± 10). Measure the quantity of buffer in the tank and record for future use. Note, voltage is the driving force behind DNA migration and is therefore the most important variable to control. Utilization of a tank with circulating (and or cooling) allows for greater heterogeneity of results (Gutzkow *et al.*, 2013).

2.1.6 Washing and fixing

At the end of electrophoresis, slides are washed by incubation in Tris(hydroxymethyl)aminomethane (Tris base) 0.4 M pH 7.5 for at least 15 min (three baths of five min each in fresh buffer solution). To further eliminate salts, the slides should be gently rinsed in distilled water prior to fixing. Slides are then fixed by immersion for ten min in absolute alcohol and then air dried. Once fixed, the slides can be stored for several months before analysis.

2.1.7 Slide staining

Slides are stained by a DNA specific fluorescent dye such as Gel Red, SYBR Green, SYBR Gold, or ethidium bromide. The type of dye used for DNA staining is often stipulated within a laboratory but where there are options the authors recommend a 1X solution of SYBR Green I dye. Stains can either applied directly to the section of the slide with embedded cells, or through bath incubation. Staining should consistently saturate the DNA (i.e. the stain should be in abundance). Ensure the dye is compatible with the microscope being used and be aware of chemical safety issues associated with DNA intercalating dyes.

2.1.8 Microscopic analysis

Slides are read using an X40 lens and X10 objective. The slide must always be positioned with comets facing the same direction (usually with the tail heading to the right but this may be dependent on your analysis software). Read the slide using a parallel search pattern (Figure 2) selecting cells for measurement without bias. Count over 50 cells per slide and 100 cells per animal. Ensure that cells are measured from across the entire image. Note, reading the slide should be done with haste as exposure to light can dull fluorescence of the DNA binding dye.

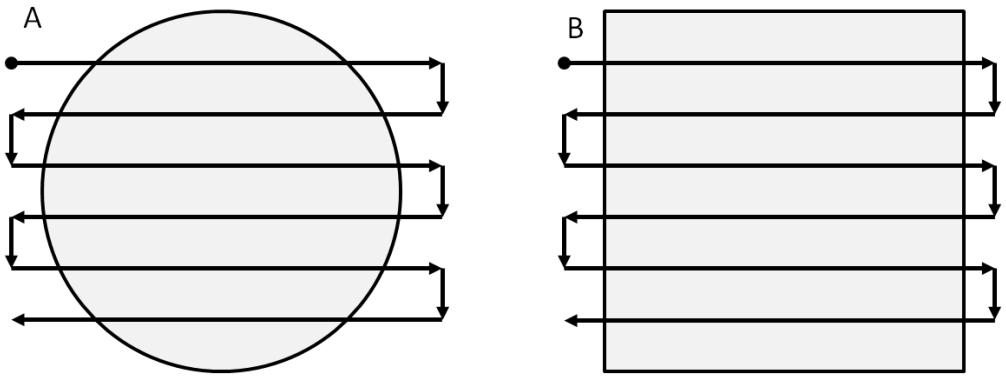

Figure 2. Systematic reading of the comet slide. For either a Trevigen® slide (A) or homemade slide (B) the comet data should be recorded systematically using a parallel search pattern.

2.2 Method modifications

The method described here is suggested for use with flatfish erythrocytes or bivalve haemocytes but the following can be changed to suit different cell types: agarose concentration, denaturation time, electrophoresis duration and intensity. Minor alterations can be used to refine comet shape (Azqueta *et al.*, 2011). Ensure all the parameters of the assay are recorded as defined in section 3.2. Data reporting.

2.3 Quality assurance techniques

One of the key issues with using the comet assay on environmental samples is the difficulty in running a measurable set of positive and negative controls through from start to finish. Therefore, it is especially important to ensure that methods are followed consistently. There are several stages at which quality assurance techniques can be implemented which can be especially helpful when first running the assay, or when diagnosing errors.

2.3.1 Cell viability

Cell viability measures are cited as being important in order to assess the level of apoptotic cells within a sample as apoptosis can mistakenly be measured as damaged cells by the untrained eye. Trypan Blue exclusion assay is commonly cited as being the most appropriate assay for use, but it is not applicable for all cell types and remains subjective. When possible, a previous analysis of cell viability by flow cytometry remains the most sensitive and rapid technique (Ramsdorf *et al*., 2009).

2.3.2 Positive Electrophoresis control

To ensure electrophoresis is running correctly a positive control can be run alongside normal cells. Simply expose isolated cells to 100 mM hydrogen peroxide for 20 minutes or to intense UV light (five minutes UVA at 1800–2000 $W.cm^{-2}$) prior to embedding in agarose and run through the assay alongside normal cells. Damage should be clearly evident in these cells and demonstrate that lysis and electrophoresis are running as required.

2.4 Benchtop Protocol

2.4.1 Cell isolation

Bivalve haemocytes

1. Haemocytes should be aspirated from the adductor muscle of a marine bivalve into a syringe with a 23-gauge needle.
2. Add haemocytes to an equal volume of osmotically balanced solution, such as sterile filtered seawater or HBSS buffer adjusted with 22.2 $g\,l^{-1}$ NaCl (Hartl *et al.*, 2010). Osmolarity should be verified for each new species utilized.
3. Maintain haemocytes at 4°C in the dark prior to embedding in agarose or cryopreservation.

Flatfish erythrocytes

1. Take blood directly from the caudal vein using a heparinized syringe with a 23-gauge needle. One drop (or roughly 20 µl) is sufficient to perform the assay.
2. One drop of blood is recovered either in calcium magnesium free saline buffer or in a phosphate saline buffered solution (around 700 µl) for a direct application of the assay.
3. Maintain blood in osmotically balanced solution at 4°C in the dark prior to embedding in agarose or cryopreservation.

2.4.2 Cell embedding

The method of embedding varies depending on the slide type used.

For Trevigen® slides:

1. Add 38 µl of cell suspension into 150 µl 0.5% LMP agarose (held at 37°C) and mix with pipette tip.
2. Immediately pipette 50 µl on to the prepared slide. Replicate with a second 50 µl from each test individual.
3. Store at 4°C in the dark to set.

For homemade slides (frosted glass prepared with normal agarose):

1. Pipette 38 µl of cell solution into 150 µl 0.5% LMP agarose (held at 37°C) and mix with pipette tip (to give end concentration of 0.4% LMP).
2. Pipette a 20 µl drop of LMP agarose and cell solution agarose onto the layer of pre-laid agarose. For each sample repeat on a second slide.
3. Immediately place a cover slip over the drop and allow to set at 4°C. This ensures even distribution over the slide.
4. Carefully remove the cover slip without removing agarose. If required to protect the cells a further layer of 0.5–0.8% LMP agarose without cell suspension can be layered on top.

2.4.3 Lysis, unwinding and electrophoresis

1. Immerse the slides in pre-chilled lysis buffer for one hour at 4°C
2. Remove slides and wash once in Tris neutralizing buffer.
3. Place the slides in the electrophoresis tank, all facing the same direction, and add pre-defined quantity of chilled electrophoresis buffer (See appendix 1).
4. Incubate for 15 minutes.

5. After the incubation period switch on the electrophoresis tank at 0.7 Vcm^{-1} and 300 mA for 30 minutes. It may be necessary to adjust to volume of buffer slightly to achieve the correct current.
6. After 30 minutes remove slide and wash three times for 5 minutes each time in tris neutralizing buffer.
7. To fix slides, immerse them for ten min in ice-cold absolute ethanol.
8. Air dry at room temperature and store for up to six months before analysis.

2.4.4 Analysis

1. Cover gels with fluorescent stain DNA specific dye such as Gel Red, SYBR Green, SYBR Gold, or ethidium bromide. Ensure the dye is compatible with the fluorescent microscope and following manufacturer's instruction for working concentration.
2. Using fluorescent microscopy at magnification X400 (X10 lens and X40 objective) score 50 comets per slide. It is usually necessary to calibrate the automated software with a microscope micrometer.

3 Data analysis and reporting

Image analysis software generally records several key parameters from each comet and from these calculates data for reporting. Parameters measured include total image intensity, head intensity, tail intensity, head length, and tail length. Calculated data include % DNA in Tail (sometimes also known as tail intensity) and tail moment (Figure 3).

The recommended unit for reporting the comet assay is % DNA in Tail. % Tail DNA is linearly correlated with the number of breaks (Collins, 2004). Moreover, it is less affected by camera setting parameters allowing comparisons from one system to another. Further data (e.g. tail length, tail moment) can be reported where the author deems it useful.

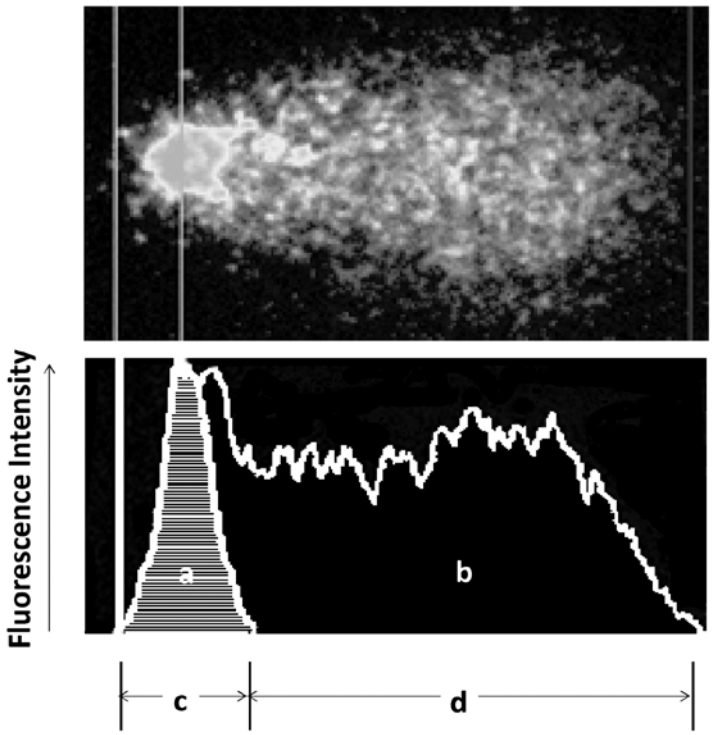

Figure 3. Analysis of comet tails in bivalve haemocytes through image analysis. Measurements: a Head intensity, b Tail intensity, c Head length, d Tail length. Inferred Measures: % DNA in tail = 100*(b/a+b); Tail Moment = d*(b/a+b).

3.1 Statistical analysis of comet assay results

The application of the comet assay for the *in situ* assessment of genotoxicity generally implies sampling of animals in different locations or times. The amount of individuals sampled per site should be large enough to be representative. Another important point is the number of nuclei analysed per individual. Intra-individual variability can be higher than inter-individual variability as previously demonstrated in dab (Akcha *et al.*, 2003). As a consequence, the higher number of nuclei is analysed, the better is the estimation of the mean amount of damage of each individual (experimental unit). The number of nuclei analysed should be at least 50 per slide. Usually two slides are prepared per sample but in some cases it can be useful to increase the number of slides per individual (Wiklund and Agurell, 2003). Once the data are collected, a nested design ANOVA can be used to analyse the mean level of DNA damage of each individual

as the dependent variable. Site location and animal can be considered as factors, with factor "animal" being nested in factor "site" to take into account the hierarchical nature of the data.

Comet assay data does not always follow a normal distribution. Therefore in order to use powerful parametric statistics, it can be necessary to mathematically transform the data (Lovell and Omori, 2008).

3.2 Data reporting

To ensure repeatability of this assay it is crucial that all variables are recorded. Here we suggest the minimum recommended requirements for recording of variables associated with both field sampling processes and the assay itself. The system of minimum reporting required for lab based chemical testing has previously been covered in depth (Tice *et al.*, 2000). However, field work requires slightly different reporting of results mainly due to the associated increase in the number of variables. Any variations from the standardized protocol should also be recorded.

Sampling variables to be recorded:

- Site location (name and coordinates);
- Date and time of sampling;
- Species;
- Tissue;
- Number of animals sampled;
- Time out of water prior to procedure;
- Cell preservation method (if used);
- Time from sample retrieval (blood sampling/ tissue dissection) to lysis.

Comet assay variables:

- Slide type/preparation method (e.g. Trevigen® comet slide);
- LMP Agarose concentration;
- Lysis conditions (pH, time, temperature);
- Alkali incubation conditions (pH, time, temperature);
- Electrophoresis conditions (pH, Vcm-1, mA, time, temperature);
- Slide stain used (type, concentration);
- Quality assurance undertaken.

Comet Scoring Variables:

- Microscope;
- Magnification;
- Image analysis software;
- Mean number of cells scored per animal.

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Appendix 1. Buffer recipes

Lysis solution –2.5 M NaCl, 0.1 M Na_2EDTA, 0.01 M Tris base, 1% N-sarcosinate, 10% DMSO, 1% Triton X-100, adjust pH to 10.

- Add NaCl, Na_2EDTA, Tris base, N-sarcosinate and 8 g NaOH to 800 ml of distilled water (dH2O) and mix. Make up to 900 ml, adjust pH to 10 and chill to 4°C. On the day of the assay add DMSO and Triton X-100.

Alkaline electrophoresis solution - 0.3 M NaOH, 0.001 M EDTA.

- Mix in dH2O, chill to 4°C.

Tris neutralization buffer - 0.4 M Tris base.

- Mix in dH2O, adjust pH to 7.5, chill to 4°C.

Cell maintenance buffer to store bivalve haemocytes for latent analysis- HBSS or synthetic seawater.

- Hanks balanced salt solution adjusted with 22.2 g l^{-1} sodium chloride and sterile filtered. Alternatively, use sterile filtered synthetic seawater.

Calcium magnesium free saline buffer - 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 500 mM NaCl, 12.5 mM KCl, 5 mM EDTA.

- Mix in dH2O and adjust pH to 7.4.

Phosphate buffered saline (PBS) - 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4.

- Mix in dH2O and adjust pH to 7.4.

Low melting point (LMP) agarose.

- 0.5% LMP agarose in cell maintenance media (use the same cell maintenance media as required for species being analysed). Melt 0.5 g LMP agarose per 100 ml media on hotplate or in a microwave, aliquot into microcentrifuge tubes and maintain in a hot block or water bath at 37°C.

Standard agarose.

- 0.5% agarose in cell maintenance media (use the same cell maintenance media as required for species being analysed). Melt on hotplate or in a microwave. Allow to cool slightly and use before setting.

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