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Meeting Report
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The next three decades of the comet assay: a report of the 11th International Comet Assay Workshop

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Abstract

The International Comet Assay Workshops are a series of scientific conferences dealing with practical and theoretical aspects of the Comet Assay (single-cell gel electrophoresis)—a simple method for detecting DNA strand breaks. The first paper describing such an assay was published over 30 years ago in 1984 by Swedish researchers O. Ostling and K. J. Johanson. Appropriately, the theme for the 2015 meeting was looking to the future: 'The Next 3 Decades of the Comet Assay'. The programme included 25 oral and 43 poster presentations depicting the latest advances in technical developments as well as applications of the comet assay in genotoxicity testing (*in vitro* and *in vivo*) and biomonitoring of both humans and the environment. Open discussion sessions based on questions from the participants allowed exchange of practical details on current comet assay protocols. This report summarises technical issues of high importance which were discussed during the sessions. We provide information on ways to improve the assay performance, by testing for cytotoxicity, by using reference samples to reduce or allow for inter-experimental variation, and by standardising quantification of the damage, including replicates and scoring enough comets to ensure statistical validity. After 30 years of experimentation with the comet assay, we are in a position to control the important experimental parameters and make the comet assay a truly reliable method with a wealth of possible applications.

Introduction

The International Comet Assay Workshops are a series of scientific conferences dealing with practical and theoretical aspects of the Comet Assay, aimed at both experienced and new users. The meetings began as a satellite to the 2nd International Conference on Environmental Mutagens in 1995. So far, all have been held in Europe, which perhaps reflects the fact that, while the Comet Assay has mixed North American/European parentage, more users of the assay are in Europe than anywhere else (1).

The first paper on this single-cell gel electrophoresis assay was published over 30 years ago, in 1984, by the Swedish researchers O. Ostling and K. J. Johanson (2). The 11th International Comet Assay Workshop (ICAW 2015), entitled 'The Next 3 Decades of the Comet Assay', was held at Hof Van Liere in Antwerp. It was hosted by Drs Sabine Langie and Gudrun Koppen from the Environmental Risk and Health unit at VITO (http://icaw.vito.be/). The meeting included discussion forums, a women-in-science session, talks by recognised experts and allocated poster sessions. Specific sessions included: the history of the comet assay, occupational exposure and biomonitoring, ecogenotoxicology in plants and the aquatic environment, clinical applications, statistical issues, genotoxicity of chemicals and nanomaterials, regulatory toxicology and new applications

of the comet assay, as well as novel modifications and improvements. There were 25 oral and 43 poster presentations depicting the present and especially the future of the Comet Assay. Fourteen per cent of the 97 participants came from outside Europe (e.g. Brazil, Mexico, Russia and Japan). The largest European contingent was from Turkey (14%), followed by France, Germany and the UK (each representing 10%), then Norway, Italy and Belgium at 8%. It is noteworthy, in view of the women-in-science session, that there were equal numbers of male and female speakers in this workshop. The major issues under discussion and highlights of the meeting are summarised in this report. A full list of abstracts was published by Frontiers (http://www.frontiersin.org/events/ICAW_2015_-_11th_International_Comet_Assay_Workshop/2557/topic) and presentations can be consulted on the ICAW 2015 website (http://icaw.vito.be/presentations).

Current and future applications

The single-cell gel electrophoresis, or comet assay, is a method for assessing DNA damage and repair quantitatively, at the level of single cells. A major advantage of the assay is that DNA strand breaks and some base modifications can be measured using dyes, rather than by radioactive labelling of cells. The assay requires nucleoids (supercoiled cellular genomic DNA) but not proliferating cells, and the assay is therefore applicable to any cell line or tissue from which a single-cell suspension can be obtained, including differentiated cells. It can be used in a broad range of *in vivo*, *in vitro* and *ex vivo* applications.

A questionnaire, completed by 97 participants, gives a good overview of the various application fields (Figure 1). As expected the majority used the standard alkaline comet assay, for *in vitro* and *in vivo* genotoxicity testing (37 and 28%, respectively) as well as human biomonitoring (24%). The keynote speaker Günter Speit presented an overview of how the comet assay evolved into a useful tool for genotoxicity testing and biomonitoring purposes (3). The extended use of the comet assay and the increasing need for genotoxicity testing of chemicals led to the development of the *in vivo* mammalian alkaline comet assay protocol, now recommended by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (4) and the European Food Safety Authority (EFSA) (5). The protocol was approved in 2014 by the OECD for the testing of chemicals; Guideline TG 489 (6). The *in vivo* comet assay was reported to be more sensitive than the *in vivo* UDS test and *in vivo* micronucleus test (7). For the *in vitro* comet assay, validation studies are ongoing under the auspices of the Japanese Center for the Validation of Alternative Methods (JaCVAM) in collaboration with the European Centre for the Validation of Alternative Methods (ECVAM), as was presented by Hajime Kojima at ICAW 2015 (8). As indicated in the OECD guideline, the *in vivo* alkaline comet assay is especially relevant for assessing genotoxic hazard: the response reflects conditions such as *in vivo* absorption, distribution, metabolism and excretion of the genotoxic chemicals, and also the capacity of cells for DNA repair. These factors will of course vary among species and tissues and depend on the types of DNA damage.

Among the ICAW 2015 participants, 19% used the comet assay for *in vitro* genotoxicity testing of nanoparticles (Figure 1). Nowadays, it is the most used assay in *in vitro* assessments and is second only to the micronucleus assay in *in vivo* experiments (9–11). The *in vivo* version of the assay is a very useful tool since it can detect DNA damage induced by secondary mechanisms such as oxidative stress resulting from inflammation.

The use of the comet assay in DNA repair studies (reviewed in (12)) has increased greatly during the last 10 years, with 157 and 188 publications in 2014 and 2015, respectively (source: PubMed—using search terms 'single cell gel electrophoresis' AND 'DNA repair'). An updated overview of the use of the comet assay in DNA repair studies over the past three decades was provided by Sabine Langie (13). At ICAW 2015, 16% of the participants used the most straightforward approach for measuring DNA repair capacity which is to induce DNA damage in cells and subsequently monitor the rate at which these lesions are repaired/removed over time. Alternative

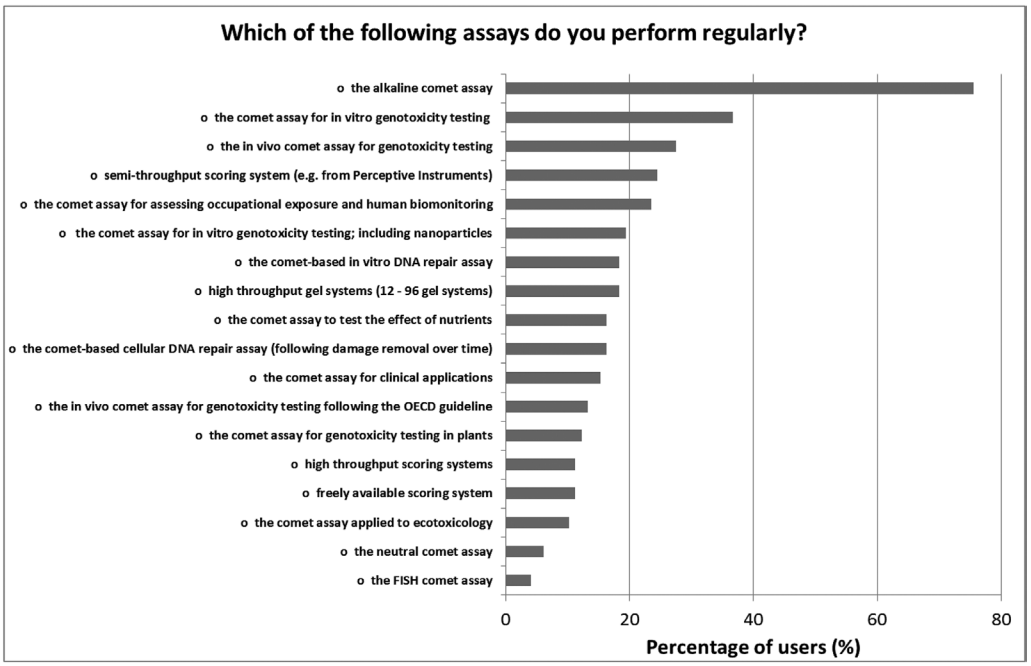

Figure 1. Comet assay applications and modifications, as reported by ICAW 2015 participants.

in vitro comet repair assays have been developed (see Assessment of DNA repair using the comet assay section) and were reported as being used by 18% of the ICAW 2015 participants (Figure 1).

The FISH (fluorescent *in situ* hybridisation) comet assay had a limited popularity among the ICAW 2015 participants (4%) (Figure 1), though it offers the possibility to study gene-specific DNA damage and repair. It makes use of fluorescent probes of up to 300 nucleotides that are sequence-specific and can be detected against the background DNA staining. New applications of the FISH comet assay, e.g. studying formation and repair of interstrand DNA cross links or region-specific hypomethylation, have been reviewed recently (14).

In the field of ecogenotoxicity, the use of the comet assay has greatly increased (15–17). This is particularly true for studies on plants. Twelve per cent of the ICAW 2015 participants reported having regularly applied the comet assay to plants (Figure 1). The recent protocol improvements (described in more detail in Plant comet assay section) (18) and the work towards a medium-throughput version (19) open up the prospect of its increased use to study genotoxicity in plants. In theory, any tissue, of any eukaryotic species, can be studied using the comet assay, the only limitation being the nuclear genome size (if much less than 1 pg of DNA per cell, the DNA is not visible in a standard fluorescence microscope).

Innovations in the comet assay used for human biomonitoring

The comet assay has been extensively used in >300 environmental and occupational human biomonitoring studies (20). In addition to conventional exposure assessments (presentations at ICAW 2015 (21,22)), topics discussed at ICAW 2015 indicate that human biomonitoring has recently shifted its focus to for instance indoor air quality (23), physical activity (24) and occupational exposure to antineoplastic drugs (25,26) as environmental/life style factors that can affect an individual's level of DNA damage and capacity for DNA repair. Also the use of non-invasively collected samples, such as saliva, was discussed as an important issue, especially in the context of longitudinal cohorts that involve young children (27–29). The novel modification of the comet assay for the detection of global DNA methylation levels (30,31) and the application of this methodology in several biomonitoring studies (32) were highlighted.

DNA damage observed in different cell types within the individual may vary in the case that the genotoxic effect is local or the cell population is heterogeneous with differing sensitivities. Blood is often used in human biomonitoring, as it needs no or hardly any preprocessing, and it can be used fresh or frozen. A finger-prick sample can provide sufficient white cells. Furthermore, the background levels of strand breaks in cells from untreated blood samples are low. Mononuclear cells (monocytes and lymphocytes) are isolated from the blood and used in the assay, but also whole blood is a valid matrix. The leukocytes fraction of whole blood contains mainly neutrophils (60–75%) and lymphocytes (20–30%). These cell populations have different half-lives of, respectively, hours to days vs. weeks to years, and may show different sensitivities to exposure. Inclusion of a whole blood cell differential count was proposed to control for differences caused by different leukocyte composition (20).

During the ICAW 2015 workshop, Ceretti *et al.* (27), Vannini *et al.* (29) and Grindel *et al.* (28) presented the use of leucocytes from saliva as an alternative for assessment of DNA damage. This seems promising, as saliva samples can be collected quickly and non-invasively. Nevertheless, there are still some challenges related to this matrix. The authors reported unreliable sampling of the oral leukocytes, which led to: (i) low and (from subject to subject) very variable cell counts, and (ii) the preponderance sometimes of epithelial buccal mucosa cells rather than leucocytes. The use of the comet assay for analysis of DNA damage in tissues such as epithelial cells has recently been reviewed (33). Lens and corneal epithelial cells collected in clinical applications (e.g. cataract) clearly represent an invasive procedure. Tear duct, buccal and nasal epithelial cells can be sampled using, respectively, a capillary, a cheek scraper or a cytobrush. Single epithelial cells must be obtained and dissociated and such procedures may induce varying amounts of DNA damage among the isolated cells. In the review (33), the different protocols in use for cell collection and the subsequent comet assay are described.

Spermatozoa, from either rodents or humans, need more aggressive lysis for breakage of disulphide (S–S) bonds in DNA protamines (protocol details in (34)). This results in rather high background DNA damage. It was reported by Baumgartner and colleagues (35) that human sperm were more sensitive to DNA damage induced by a variety of chemicals *in vitro*, compared to human lymphocytes. However, different electrophoresis conditions were used for the two cell types (affecting both the background and the induced level of DNA damage) and therefore the sensitivities should not be directly compared.

Current and new developments for genotoxicity testing

The use of the comet assay in the genotoxicity testing of chemicals and nanomaterials was discussed at ICAW 2015 and key points are summarised in this section. Different cell lines (i.e. V-79, Caco-2, TK-6, SHSY5Y, H295R, A549, BEAS-B2 and Cos-1 fibroblast-like kidney cells), as well as human lymphocytes, and human and dog whole blood (depending on the compound evaluated and the purpose of the study) have been used in *in vitro* testing. The use of the comet assay in the 3D skin model was discussed in detail. Moreover, the use of root cells of *Allium cepa* to evaluate the genotoxicity of nanomaterials was also presented. Regarding *in vivo* genotoxicity studies, liver, kidney, heart, colon and testis of rats were the organs described. The new OECD guideline was discussed.

Several important aspects of *in vitro* and *in vivo* genotoxicity evaluation were discussed, such as low pH of the cellular environment increasing the genotoxic potential of chemicals (36) as well as optimised methods on how to freeze and process liver (37) and colon samples (38).

Furthermore, the role of the comet assay in the genotoxic evaluation of nanomaterials was discussed; the comet assay and the micronucleus test are the most used techniques at research level (9,11). Nevertheless, the assay has not been taken into account by the OECD Working Party on Manufactured Nanomaterials (WPMN) as an appropriate test. It is worth mentioning that the last report of the WPMN is from 2014, the year in which the OECD guideline for the *in vivo* alkaline comet assay was published (6).

In vitro comet assay and *in vitro* skin model

While the *in vitro* comet assay is not yet formally recognised in regulatory toxicology, it is recommended as an appropriate test for use in the technical guidance documents of REACH. The assay is also widely used for screening novel cosmetics (where only *in vitro* testing is allowed) and pharmaceuticals. It was suggested during the ICAW 2015 workshop that the *in vitro* comet assay could be further upgraded as a genotoxicity testing tool, by increasing the range of lesions detected (using specific enzymes and DNA repair

synthesis inhibitors; see below), integrating the various steps of the assay into an automated comet slide processing unit, and developing simple and rapid scoring methods aside from the already existing fully automated scoring systems nowadays used (e.g. using labelled DNA probes to double-stain the comets, or scanning the whole field of comets using a plate reader with integrated imaging software).

During the ICAW workshop, Kerstin Reisinger presented the development and standardisation of the use of the 3D skin models for *in vitro* genotoxicity testing (39). Cosmetics Europe aims to establish and validate new methods for genotoxicity testing in the human 3D skin models (using the micronucleus test and comet assay), to improve the predictivity of the assay and decrease false positive results (40,41). These cellular models have the advantage of allowing topical application of the compounds, evaluation of formulations and poorly soluble compounds, as well as measurement of local toxicological effects in target cells. Reconstructed 3D human epidermal skin models are considered more relevant than cell lines because of their morphological resemblance to human skin, with the presence of a functional stratum corneum acting as an absorption barrier for added chemicals and with metabolic capacity (42–45). DNA damage is an important toxicity endpoint as it provides an early prediction of mutagenic and carcinogenic potential. Apart from a low specificity, another shortcoming of the current test strategy for compounds with a dermal route of exposure is the lack of *in vitro* assays that specifically evaluate the genotoxic potential in the skin. The comet assay is currently being validated for this 3D skin model in a joint effort between Cosmetics Europe and the German Federal Office for Risk Assessment (BfR). In her presentation, Kerstin Reisinger showed a high degree of reproducibility within and between laboratories when coded compounds were tested in ring trials. It was furthermore observed that the reconstructed skin models mimic the native human skin metabolic capacity (46).

In vivo comet assay

The OECD recently approved guidelines for the *in vivo* alkaline comet assay applied to the genotoxicity testing of chemicals (6), after 10 years of testing and international validation organised by JaCVAM in collaboration with ECVAM (47,48) and the Interagency Coordinating Committee on the Validation of Alternative Methods (8). In order to reduce the use of laboratory animals in safety testing, the comet assay can be performed as a specific site-of-contact *in vivo* test, minimising animal toxicity burden. In the same animal, it is possible to combine analysis of micronuclei in bone marrow, with comet analysis of multiple tissues (49). The guidelines provide recommendations on various statistical issues such as the use of median vs. mean values and the recommended number of comets to be scored. It is expected that the OECD guideline protocol will substantially increase the use of the comet assay in chemicals testing, probably replacing other assays such as UDS (7). TG 489 does not include germ cells. However, at ICAW 2015, it was described by Sharma *et al.* how mouse testicular cells could be used to test a variety of compounds when combined with an automated scoring system (50).

Plant comet assay

During the ICAW 2015, Caroline Lanier reviewed the use of the comet assay on plants during the last two decades (51). The comet assay, in its neutral version, was used for the first time on plant tissues 20 years ago (52). The alkaline version was developed on broad bean (*Vicia faba*) roots by Koppen and co-workers a few years later (53,54). Since then, most of the researchers using the comet assay for ecogenotoxicity have developed their own protocol depending on the studied species (16,18). Indeed, these species can differ strongly in terms of anatomy, size, physiology and genome size. The protocols which are in use vary with respect to factors such as the nucleus isolation method, optional filtration of the cells, lysis buffers, concentration of agarose and electrophoresis conditions. Thus, efforts have recently been made to develop a standardised method. Pourrut and co-workers (18) identified the key steps of the comet assay on plant models and proposed an optimised protocol to increase its reliability, including nucleus isolation by chopping, no filtration and no lysis, and controlled laboratory conditions (e.g. use of a non-actinic lamp, stable temperature). This protocol has been shown to be robust and reliable on the most commonly used plant models (*Arabidopsis thaliana*, *A. cepa*, *Nicotiana tabacum*, *V. faba*), as well as on cultivated crops (*Lycopersicon esculentum*, *Miscanthus* × *giganteus*, *Triticum easivum*, *Zea mais*) or on wild species (*Lolium perenne*, *Trifolium repens*).

Because of the large variation in plant genome sizes (16), unwinding and electrophoresis times need to be adapted, aiming at a minimal DNA migration in the control samples as well as a maximal sensitivity of the assay in the plants exposed to a reference treatment (preferably ionising radiation). Alternatively, calibration tests can be done in soil, sediment or water matrices using well-known mutagens (e.g. EMS, MMS) or H_2O_2 in a liquid matrix (18). It is recommended to perform such calibration test for each plant species since the plant genome size could influence sensitivity to DNA damage. As highlighted by Einset and Collins on X-irradiated plants (55), larger genomes are more sensitive to DNA damage, resulting in higher % DNA in tail.

At ICAW 2015, promising results were shown in the development of automated scoring for the plant comet assay, based on the Imstar Pathfinder™ system (19). Optimisation of the plant comet protocol to reduce background (reducing plant debris) and to increase nuclei density, combined with slight modifications of the image analysis software allowed the use of the Imstar Pathfinder™ system with the same reliability as manual scoring systems. Moreover, new developments in the use of plant comet assay to study DNA repair and biomonitor aquatic environments were presented, respectively, by Angelis and co-workers (56) and Mukherjee and co-workers (57,58).

Technical aspects and critical parameters

ICAW 2015 participants were asked to indicate any technical problems they were experiencing (Figure 2A), and the technical aspects they considered important when performing the comet assay (Figure 2B). Fifteen per cent of ICAW 2015 participants were encountering problems with gels detaching from glass microscope slides, although various guidelines have been published to avoid this (59). Using GelBond® Films excludes the problem completely, as gels stick firmly to the hydrophilic side of this film. Other issues that seem to be raised at every workshop include: 'Can I detect double strand breaks with the neutral comet assay', 'Can I study apoptosis or mitochondrial DNA damage' or 'Do I report my data as % DNA in tail or tail moment'? These topics have been reviewed before (60), and will only briefly be touched upon in this report. Other questions, such as avoiding 'edge effects' and comet tails going in different directions in minigels (12- or 96-gel system), the importance of lysis time, pH of the unwinding/electrophoresis solution, and other aspects of electrophoresis (voltage/current/temperature/circulation) have received more attention recently, and were discussed in talks and discussion sessions of ICAW 2015.

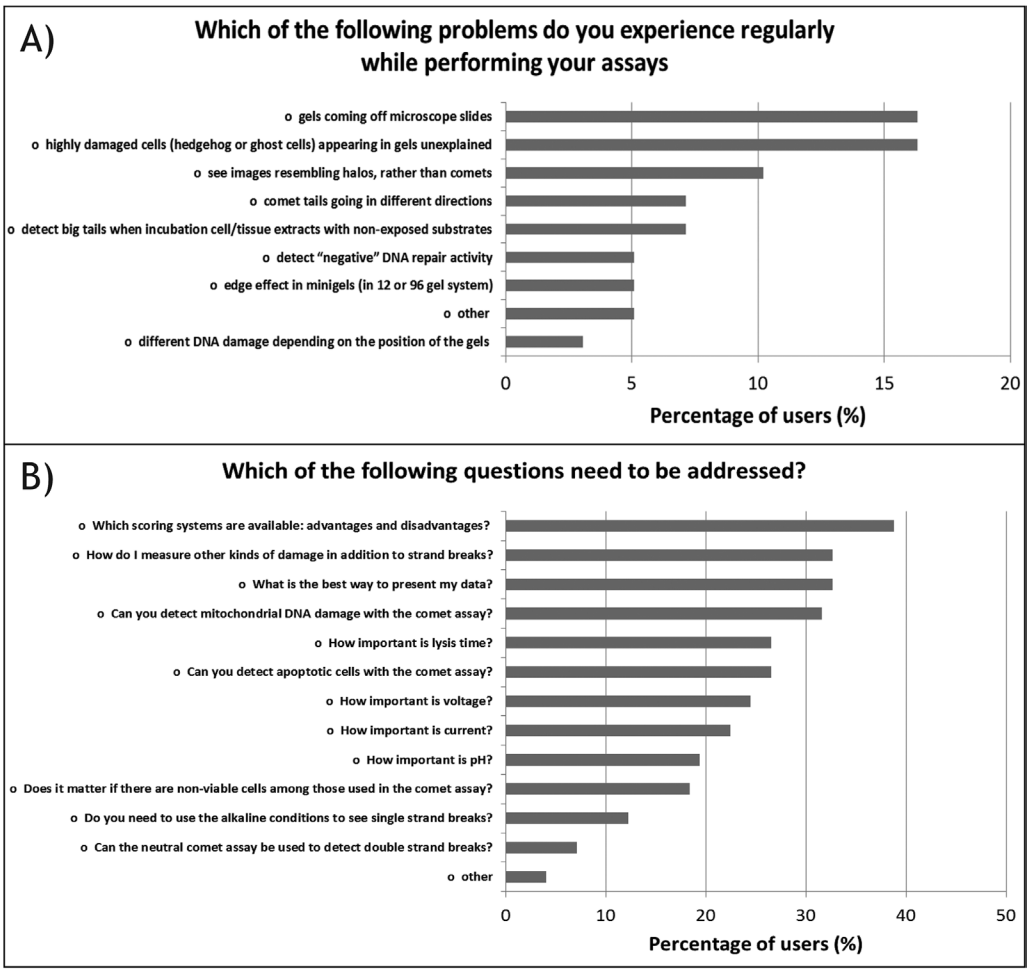

Figure 2. Technical aspects reported as significant by ICAW 2015 participants. Types of problems experienced regularly (A); issues which should be addressed (B).

Viability testing

Eighteen per cent of ICAW 2015 participants were worried about the use of non-viable cells in the comet assay (Figure 2B). When using the comet assay for *in vitro* as well as *in vivo* genotoxicity testing, it is crucial to assess whether cells are viable until comet analysis. Cell death can cause 'secondary' DNA damage, thus possibly leading to a false positive classification of a chemical as genotoxic. In the *in vitro* comet assay, it is recommended that concentrations of a test chemical producing high (>30%) mortality should be avoided (61), although some experts allow a loss of viability of 50% (e.g. (62)).

It is generally accepted that the truest measures of viability are based on the ability of cells to divide or to survive to form clones (63); proliferation and cloning efficiency assays are therefore most appropriate for checking the viability of cells. It should be emphasised, however, that viability is in itself not necessarily required for all comet assay applications. If frozen cell samples are thawed with their DNA still intact, it does not matter whether or not they might, if given the chance, survive to proliferate. However, in human biomonitoring studies, in particular, a cytotoxicity assay, such as the trypan blue assay, can be carried out to check for poor handling of the cell sample, which could be responsible for high levels of damage (rather than environmental or occupational exposure to genotoxic agents).

Regarding the *in vivo* comet assay, toxicity may also be responsible for an increase in DNA migration. Thus, the examination of toxicity markers—when a sign of genotoxicity is seen—is critical for the interpretation of results. The OECD Guideline TG 489 proposes the organ/tissue histopathological examination as a relevant measure of toxicity (6).

Lysis time

Twenty-seven per cent of ICAW 2015 participants had questions regarding the importance of the duration of the lysis step (Figure 2B). The lysis time is not believed to be critical (although there are exceptions to this, as discussed below). Nevertheless, it has been suggested that it should not be <1 h. Overnight lysis is often used since it is very convenient from a practical point of view. Enciso and co-workers (64) studied different durations of lysis (from no lysis to 1 week) with control HeLa cells and HeLa cells treated with two concentrations of methyl methanesulfonate (MMS) or H_2O_2. It seemed that the alkaline treatment before the electrophoresis, or even the electrophoresis period, is all that is necessary to lyse the cells. Prolonged lysis up to 24 h increased the DNA damage levels detected and thus the sensitivity of the assay, which was important in cells treated with either MMS or H_2O_2. The DNA damage level in control HeLa cells was not affected by the time of lysis.

The same study was performed applying the comet assay in combination with formamidopyrimidine DNA glycosylase (Fpg) to HeLa cells treated with photosensitiser Ro 19-8022 plus light (to induce 8-oxoguanine lesions). A minimum lysis period of at least 5 min was

needed to allow the enzyme to access the DNA, but the same DNA damage levels (i.e. Fpg-sensitive sites) were detected after 5 min or 1 h of lysis. The net level of Fpg-sensitive sites increased up to 24 h, but there were no differences between 24 h and 1 week of lysis.

Taking these results into account, a constant time of lysis should be used to compare results from different assays in a series of experiments or between different laboratories, for a specific genotoxic agent. However, it seems that the optimum lysis time may vary according to the type of DNA lesion induced.

Detection of specific DNA lesions

More than 30% of ICAW participants were interested in ways to detect genotoxic lesions other than strand breaks and alkali-labile sites (ALS) (Figure 2B). The classic concept of ALS includes abasic sites, apurinic or apyrimidinic, deoxyribose moieties, or masked DNA breaks that can be rapidly denatured under weak alkaline conditions. Aside from ALS that are products of reaction with DNA, ALS are also present in repetitive DNA regions, and might be interpreted as a constitutive part of the chromatin structure (65).

The usefulness of DNA repair enzymes (with endonuclease or lyase activity) to detect specific DNA lesions was discussed at the workshop, acknowledging that some base modifications are highly mutagenic, whereas others are not. Most used so far by comet researchers has been Fpg, which detects oxidised purines, including 8-oxo-7,8-dihydroguanine (8-oxoGua), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPyGua), 4,6-diamino-5-formamidopyrimidine (FaPyAde) and other ring-opened purines. Furthermore, Fpg also recognises alkylation damage, namely alkylation of N7 guanine: methylated guanine leads to time- and pH-dependent instability of the purine resulting in slow imidazole ring opening; this lesion is recognised by Fpg producing strand breaks (66,67). Though, people should keep in mind that it has been suggested that high concentrations of methylating agents can cause oxidative stress as well and will thus be recognised as oxidative DNA damage by Fpg (68). Such mechanisms may be exploited for the specific detection of certain DNA adducts.

The broad substrate specificity and high sensitivity of Fpg is at the expense of specificity; the human counterpart of Fpg, the DNA glycosylase oxoguanosine 1 (hOGG1), is more specific for 8-oxoGua (69). New and genetically engineered enzymes should prove useful for even more detailed identification of specific lesions. An important point, however, is that successful use of DNA repair enzymes depends on careful titration of a batch of enzyme to determine the appropriate concentration range, ensuring that specific lesions are detected and cleaved quantitatively (i.e. with saturating enzyme kinetics) while avoiding non-specific nucleolytic activity which can occur at high concentrations of crude enzyme extract.

Electrophoresis

Several ring trials have been conducted with the *in vitro* comet assay. Considerable inter-laboratory variations in results were disclosed in the trials carried out in the ESCODD (70) and ECVAG projects (71) in which identical cell samples were distributed, although in a trial of silver staining in which laboratories scored an identical set of slides (70) agreement was good. Efforts have been made to identify protocol parameters that may explain such variations. It is clear that the electrophoresis step is particularly important. Electrophoresis is a physical/chemical process in which 'loose ends' in denatured and charged DNA are pulled out from the nucleoid in an electric field. One-fifth of the ICAW participants had questions on the importance of voltage, current and pH of the electrophoresis solution (Figure 2B).

The voltage makes the difference

The formation of the tail follows physical laws, implying that the tail magnitude (i) is linearly related to the voltage potential (V/cm); (ii) is linearly related to the duration of electrophoresis; (iii) is reduced in a more dense matrix (higher agarose concentration); and (iv) is dependent on the temperature. Both (iii) and (iv) are more complex functions, whereas (i) and (ii) are basically linear. The electric current during comet assay electrophoresis has traditionally been specified as 300 mA. However, although the current through an electrophoresis tank is determined by the applied voltage, the depth of the electrophoresis solution and also its conductivity, the current itself is in fact unimportant. Striving to maintain a current of 300 mA, as is often done, therefore reflects a misconception (60). The volume of the electrophoresis solution during electrophoresis may be tripled, so that the current will increase to ~900 mA. The tail magnitude is, however, unchanged provided that the voltage per cm is kept constant. In other words, the voltage is the driving force for DNA migration. In the recently published *in vivo* comet assay TG 489 protocol (6,60), the importance of the voltage is highlighted but there is still a reference to a 'starting current of 300 mA', which is misleading.

Based on these considerations, the electrophoresis conditions should be under careful control. A good approach in future publications is always to specify the applied voltage (V/cm) (measured on the platform of the electrophoresis tank) and the time of electrophoresis, or to normalise the comet assay results for these parameters. The latter can be done via linear normalisation in relation to a set of standard samples which are to be run in each lab (see Reference samples for quality control section). Furthermore, the agarose concentration should be standardised, and the temperature should be controlled.

Influence of pH on strand breaks detected

The belief that running electrophoresis at neutral pH allows detection of only double-strand breaks (DSBs) is, we hope, no longer widespread. This belief implies that alkaline conditions are needed to reveal single-strand breaks (SSBs). This fallacy (discussed in several reviews) (60,72) arose from the comparison of the comet assay with other methods for measuring DNA breaks, namely alkaline sucrose gradient sedimentation, alkaline elution and alkaline unwinding. The comet assay, however, depends on the presence of a SSB or DSB, to relax the supercoiled DNA and allow the migration of the loop containing that break during electrophoresis, and this will occur whether the pH is neutral or high (alkaline). A high pH will provoke the unwinding of the two strands and also the conversion of some ALS into breaks, and this feature accounts for differences seen in results from alkaline and neutral assays. Collins and co-workers (73) reported similar degrees of DNA migration at neutral and alkaline conditions of comet analysis of cells exposed to either MMS or H_2O_2.

Adjusting sensitivity by altering electrophoresis conditions

Using a standard protocol, the maximum and saturating level of DNA lesions is limited to a few thousand lesions per cell. This is because at that level of damage, all supercoiling is destroyed and virtually all DNA is in the tail. As discussed above, less electrophoresis (i.e. lower V/cm and/or its duration) reduces DNA migration, which can be exploited to measure higher levels of DNA lesions; in other cases, the assay is optimised to achieve maximum sensitivity (at the expense of dynamic range) (74).

Anomalous comets

Hedgehogs, ghosts and clouds

So-called hedgehog comets, ghosts or clouds are comets with almost all their DNA in the tail (>80%) and with a small but clear head. They are classified as class 4 when using the visual scoring system (going from class 1 to 4 with, respectively, little and strong DNA migration). These comet types are sometimes referred to as an indicator of cell death or even apoptosis. However, mitochondrial DNA molecules, which are much larger than apoptotic fragments, diffuse through the agarose gel during the lysis step, and are not detectable after electrophoresis (75). Lorenzo and co-workers published a critical review presenting theoretical and empirical arguments against the cell death or apoptosis interpretation (76). Hedgehog comets *may* be produced during apoptosis, at a very early stage before extensive DNA degradation, but such comets should not be regarded as indicators of apoptosis.

The OECD guidelines for the *in vivo* Mammalian Alkaline Comet Assay advise that the frequency of hedgehogs should be determined and separately documented, but should be excluded from data analysis as they reflect DNA breaks resulting from cytotoxicity or may indicate poor tissue quality. As previously concluded by Lorenzo *et al.* (76), our considered opinion is that this interpretation of 'hedgehog' comets is misguided. They should not be excluded from analysis of genotoxicity, as they clearly fall within the continuum of damage levels detectable with the comet assay. Researchers should be aware of the fact that some image analysis systems may be unable to score this type of comet or may need manual selection of the hedgehog comets. A solution could be to assign a fixed value, e.g. 90% DNA in tail, to all such comets.

On the edge

A potential source of anomalous comets are so-called 'edge effects': comets with unusual intensity, size, shape and even direction of tails appearing at the edge of the gel compared with normal comets located in the centre of the gel. When using conventional large gels (spread over the surface of a microscope slide), avoiding scoring of the comets at the edges of the gels is enough to solve this problem. Nevertheless, in the case of minigels (i.e. 12 gels/slide or 96 gels/Gelbond film formats), the edge occupies a high proportion of the whole gel. Azqueta and co-workers (59) tested the effect of allowing minigels to dry before lysis (after setting the gels) and after electrophoresis. To avoid the edge effect it is recommended to place the gels in the lysis quickly and to fix the gels in ethanol just after neutralisation (59). Avoiding the production of the anomalous comets is crucial but the problem is non-existent if the recommendations in (59) and (77) are followed.

Scoring comets: image analysis and quantification of damage

After electrophoresis and neutralisation, gels can be fixed in ethanol and dried in air, after which the samples may be stored at room temperature. Gels can be stained with various fluorescent dyes, and viewed in a wet state (either fresh from neutralisation or after dry storage) by fluorescence microscopy. It has become common to speak about the scoring of comets, though, sometimes reports mention scoring of nucleoids since undamaged cells do not form a comet during electrophoresis. When we talk about scoring comets in this manuscript, we mean scoring of both the nucleoids with a comet shape and undamaged nucleoids (class 0 by visual scoring).

The scoring of comets can be a serious bottleneck. In the early days of the comet assay, a simple analysis system based on visual scoring was devised, and this is still in use in some laboratories; comets are categorised as class 0, 1, 2, 3 or 4 according to the shape and size of the tail and an overall score for 100 comets is calculated. It has proved to be a reliable method, with acceptable inter-laboratory and inter-scorer reproducibility (after basic training). It has the advantages of speed and low cost, and gives results that are comparable with more sophisticated (and expensive) image analysis systems (78). A range of semi-automated image analysis systems are available, requiring the operator to select, focus and click on individual comets for analysis—still a time-consuming procedure. Fully automated (but expensive) image analysis systems have recently been developed, with automatic selection and focusing of comets and therefore much faster scoring: analysis of 100 samples by 'manual' methods might take 1 or 2 days, whereas an automated system would take 2–4 h for the same samples (and the number of comets per sample may then be several hundred). In addition to increased speed, the automated systems analyse in an unbiased way without subjective selection from the operator.

Twelve per cent of the ICAW participants reported that they use high throughput scoring systems, implying automatic selection and focusing of comets (Figure 1).

Which is the appropriate comet descriptor?

Recently, Møller and co-workers (79) comprehensively summarised the issue of the most appropriate comet assay descriptor. This issue was also raised during the ICAW 2015 discussions. Percentage DNA in the tail can be seen as a good way of describing the migration during electrophoresis. In case another descriptor is used (e.g. tail moment, i.e. tail length × % DNA in tail), per cent DNA migration should also be reported. These descriptors are semi-quantitative and depend on the assay conditions. In certain cases, it is important to know the actual amount of damage, in terms of numbers of lesions per cell or per amount of DNA. The usual and most reliable method is to prepare a standard curve by treating cells with ionising radiation (doses ranging typically from a fraction of a Gy up to 10 Gy). The frequency of DNA breaks induced by radiation is well established: 1 Gy of X- or γ-irradiation introduces 0.31 breaks (single, double and alkali-labile sites) per 10^9 Da of cellular DNA, which is close to 1000 breaks per diploid mammalian cell (80). In the ECVAG ring trials irradiated cell samples were distributed and proved to increase inter-lab comparability of the comet results considerably (81,82).

Reference samples for quality control

An essential aspect is the use of assay controls. The issue raised at the ICAW 2015 was the use of reference standards to be used in different laboratories. Reference samples may be prepared by treating a batch of cells (either cultured cells or, human/animal lymphocytes) with an appropriate DNA-damaging agent to induce strand breaks, such as X-rays (producing a defined lesion frequency), EMS, MMS or H_2O_2. For oxidised DNA, Ro 19-8022 (or similar photo-activated compounds) plus light is used to produce 8-oxoguanine lesions for detection with Fpg. A suitable level of damage in reference standards is 40 to 50% tail DNA. Aliquots can then be frozen in culture medium with serum and 10% DMSO to maintain viability. An aliquot is thawed for each experiment. Reference standards are particularly useful in human biomonitoring when many samples have been collected and further analysis takes place on different days. A considerable deviation (e.g. 3 SDs of the mean) from the expected % tail DNA for the reference samples would indicate a technical problem with the assay. For this control charts should be made, and

the Nelson rules on the number of points on the same side of the mean, or continually increasing or decreasing, can be used to identify a deviation (83). If the deviation is moderate, however, it is a simple matter to normalise the test results by using a correction factor based on actual/expected reference values (20). Untreated reference standards should also be prepared and frozen. In general, untreated reference cells should have a background level of breaks corresponding to 5% tail DNA or less. Under normal conditions, a higher background (>10%) suggests poor control of comet assay parameters and a need to modify the assay conditions.

Reference cells can be embedded in separate gels or—even better—in the same gel as the cells under test. This latter option requires that the two cell types are distinguishable during scoring. A system for internal reference cells was developed by Zainol *et al.* (84) based on bromodeoxyuridine-prelabelled cells that could be identified on the basis of their fluorescence signal after immunostaining, allowing specific identification during scoring of mixtures of the stained and the unstained tester cells. This involves extra treatment steps, however, and it is costly due to the requirement for antibodies. Another approach is to use cells from an organism with a different genome size compared with the experimental human or rodent cells. Certain species of fish fit this requirement, and a feasibility study has been successfully carried out using turbot erythrocytes (fish erythrocytes—in contrast to human—contain DNA) (85). Discrimination of the cell type is performed based on the total fluorescence intensity using standard comet scoring software. The total staining signal from each cell—whether damaged or undamaged—is related to the cell's DNA content. So far, fish erythrocytes are less suitable for use as reference standards since freezing causes excessive DNA damage after prolonged storage time (85).

In addition to reference samples, William Barfield (83) suggested in his presentation at ICAW 2015 that good historical controls, e.g. in the *in vivo* comet assay, should comprise at least 10—preferably 20—experiments conducted under comparable experimental conditions.

Also at ICAW 2015, the correct terminology for control groups in population or ecotoxicological studies was also discussed. It was agreed that the concept 'negative control group' should be avoided, (i) because of potential confusion with untreated reference samples (described above), and (ii) because we can never be sure that the control group for a particular disease or exposure is truly negative, i.e. not suffering from some other disorder or exposed to some other agent. The term 'control' or 'unexposed' or 'untreated' group is therefore more appropriate.

Number of comets and final 'score' of sample/individual

It has been shown (in *in vitro* experiments) that increasing the number of scored comets per sample results in a lower inter-sample variation and thereby increased statistical power (86). One hundred to two hundred comets scored per treatment or individual may be advised (regardless of the number of gels it is derived from), but this depends on the level of damage and the magnitude of the differences to be detected. In human biomonitoring studies, power calculations with comet data are almost never presented. Experience is that the required number of individuals needed to detect significant differences can vary according to the specific type of exposure studied. A comprehensive power analysis, using data from multiple human biomonitoring cohorts, should be performed before formulating any recommendations on the number of comets to be scored for such studies. In the *in vivo* comet assay protocol, which recommended five animals per dose, 200 comets per animal are advised to be scored on two slides per tissue. For plant tissues, as mentioned above, Pourrut *et al.* (18) recommended to score at least 150 comets per sample (e.g. 50 comets on three slides per sample) and at least three samples per treatment (18). Hartmann *et al.* (87) did some power calculations and reported that groups of four or five animals provide comparable statistical power following analysis of, respectively, 150 cells/animal (derived from three gels) or 100 cells/animal (derived from two gels). Smith *et al.* (88) showed that scoring 150 comets (derived from three gels) per tissue for six animals allowed detection of 2- to 3-fold increases over control in rat tissues to be detected with 80% probability. At ICAW, Anoop Kumar Sharma presented (50) power analysis based on the testing of 11 compounds (originally reported in (89)) and showed that scoring 200 comets (derived from two gels) in 4 and in 6–7 animals, allowed, respectively, a 2.5- and 2-fold increase to be detected in treated compared to control animals with 80% probability.

The median of the scored comets is calculated for each gel, to reduce the influence of anomalously high values, since the comets are rarely normally distributed. In fact, calculating the median is a simple way of describing or rather scoring the distribution (descriptor scores) (90). In the *in vivo* comet assay TG 489 OECD guideline, it was decided to calculate the median for two gels per sample, and the average of both median scores, giving the 'mean of medians' for a specific test condition.

At ICAW 2015, William Barfield presented the statistical approach followed in the JACVAM *in vivo* comet trials. The distribution of the comet descriptor score from a group was normalised (using a suitable transformation) in case the distribution was not normal. Trend analysis techniques, followed by *post hoc* comparison, were used to compare the control with increasing doses of the test compound (83). During the ICAW workshop, it was also mentioned that due to the nesting of different animals in a dose group, 'dose' should be entered as random effect in the mixed effect statistical model (89).

Assessment of DNA repair using the comet assay

The simplest way to measure cellular DNA repair is to treat cells with damaging agent and monitor the kinetics of removal of the damage. For human studies this is impracticable as it requires prolonged incubation, repeated sampling, and must be carried out on freshly isolated cells. Alternative *in vitro* repair comet assays have been developed (reviewed by (12)). In those assays a cell/tissue extract of an individual/organism is made, and the activity of repair enzymes in the extract is assessed by incubating it with substrate DNA, i.e. gel-embedded nucleoids containing specific lesions [e.g. oxidised purines induced by the photosensitiser Ro 19-8022 plus light to study base excision repair (BER), or pyrimidine dimers induced by UV(C) to study nucleotide excision repair (NER)]. The experimental modulation of DNA repair in animals is an interesting genotoxicity endpoint; a comet-based *in vitro* repair assay to study BER was optimised with extracts from rodent tissues (91), including extra measures to reduce the often reported non-specific nuclease activity.

The ECVAG trials already identified the incubation step of cell extracts with the substrate cells as a major source of inter-laboratory variation in the modified comet assay for BER. During the ICAW 2015 open discussion session, the issue of a negative net DNA repair activity arose. A few recommendations to avoid this issue, related to the enzyme incubation step, are: (i) optimise the protein concentration of the extract, (ii) use appropriate gel concentration,

and (iii) reduce non-specific enzyme activity with aphidicolin. It is advisable to run protein dilution curves for each cell or tissue type before starting a study. This is especially important when preparing extracts from tissues. A too low or too high protein concentration could for instance lead to 'negative' DNA repair or non-specific incision activity. More details and recommendations may be found in the Supplementary Materials of (92). While optimising the protein concentration, the concentration of the low melting point agarose should be taken into account as well. It not only affects the migration of the DNA through the gel as observed in the ECVAG trial (82), but can also affect the enzyme incubation step in the *in vitro* repair assay as discussed by Langie *et al.* (91).

Aphidicolin has been known for many years to inhibit the DNA polymerase involved in NER, while incision at lesions continues, leading to an accumulation of incomplete repair sites as DNA breaks (93). Aphidicolin can be used in this way to measure the initial rate of DNA repair after treatment with damaging agent, by the accumulation of incision sites (94–96). In addition, pulsing with aphidicolin during a prolonged incubation of treated cells allows monitoring of the kinetics of repair. However, another activity of aphidicolin is the suppression of non-specific nucleases, and this has proved useful in the *in vitro* repair assay, especially with extracts from animal tissues (91).

Concluding remarks

After 30 years of use in genotoxicity research, the comet assay is now becoming more widely used also in human biomonitoring. New tissues are being explored for analysis, such as saliva and epithelial cells. The ComNet project, now incorporated in a COST Action (hCOMET; http://www.hcomet.org/), aims to create a large database of human biomonitoring comet assay results. Pooled analysis of the data will allow definitive assessment of the impact of host factors (smoking, age, age, nutrition, occupational exposure), and standard protocols for human biomonitoring will be elaborated.

The use of the comet assay in ecogenotoxicity has been limited by a lack of standardised protocols or adaptation of modified protocols for animal and plant models. In this field there is certainly a need for inter-laboratory calibration and validation trials to be carried out.

In recent years, the comet assay is acquiring an increasingly important role in the field of genotoxicity testing. Among others, Cosmetics Europe aims to establish and validate the human 3D skin comet assay, to decrease false positive results in *in vitro* testing of novel cosmetics and pharmaceuticals. The test is recommended and in the meantime further validated for use in the technical guidance documents of REACH. Furthermore, the *in vivo* comet assay is recommended by ICH (4), EFSA (5) and OECD (6). In the field of nanogentoxicity the comet assay is the preferred genotoxicity test.

Following three decades of experimentation with the comet assay, it is expected that a better control of important experimental parameters should be within reach. Progress has been made in establishing optimised protocols, but further ring trials are still needed. In the meantime we advise all comet users to follow as closely as possible the recommendations discussed in this report and summarised in Box 1. It is likely that more extensive—or even complete—automation of the comet assay protocol might contribute to better standardisation and less variation between experiments and laboratories. Processing and analysing large numbers of samples are now feasible, with one major remaining bottleneck: scoring of samples. Automated scoring is still too expensive for most comet users. New high throughput methods have been developed and others are likely to come.

The next ICAW will take place at the University of Navarra, Pamplona, in northern Spain, on August 28–31, 2017. We look forward to meeting you there!

Box 1: Summary of recommendations with respect to technical aspects and assay parameters.

- Use proliferation and cloning efficiency assay for checking viability of cells (for the *in vitro* comet assay).
- Use concentrations of test chemical producing viability of at least 70%.
- Use a constant lysis time for a given set of samples.
- Control the electrophoresis conditions, in particular the V/cm at the location of samples. This includes using a power supply which does not restrict the output current.
- Include 'hedgehog' comets in the analysis. In case image analysis systems do not detect them, a fixed value of % DNA in tail should be assigned to such comets.
- Use % DNA in the tail as the descriptor. In case another descriptor is used (e.g. tail moment), it is preferable to report % DNA in the tail as well.
- Score at least 100 comets per individual/animal/plant may be advised (regardless the number of gels it is derived from), but this can be increased depending on the level of damage and the magnitude of differences to be detected.

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