# The analysis of DNA interstrand crosslinks and their repair by the modified Comet assay.

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**Abstract**

DNA interstrand crosslinks (ICLs) are an extremely toxic form of DNA damage that cells experience upon exposure to natural metabolites. Moreover, ICLs are the cytotoxic lesion produced by a range of clinically important anticancer agents. Therefore, improving our understanding of ICL induction and processing has important implications for biology and medicine. The sensitive detection of ICLs in mammalian cells is challenging but has been aided by the development of a modified form of the single-cell gel electrophoresis (SCGE) assay, also known as the 'Comet assay'. Here we describe this method and how it can be used to sensitively monitor the induction and removal of ICLs in single mammalian cells.

## 1. Introduction

DNA interstrand crosslinks (ICLs) are amongst the most cytotoxic form of DNA damage a cell will experience (1,2). The study of the cellular response to ICLs has substantial implications, from basic biology, to a better understanding human disease and in drug development. For example, defects in the ability to respond to and repair ICLs is associated with the inherited syndrome Fanconi anaemia, a devastating condition associated with bone marrow failure, developmental abnormalities and an increased risk of cancer (3). Conversely, a number of widely-used cancer chemotherapeutic drugs (platinum agents, mitomycin C, and others) exert their antitumour effects through the induction of ICLs, where alterations in the cellular response to and repair of drug-induced ICLs is associated with drug resistance in malignant

disease (2). These two examples highlight the importance of studying ICL repair, and the identification and characterisation of the cellular processes involved.

The detection of ICLs in cellular DNA is challenging, particularly under conditions where the genomic ICL frequency is pharmacologically or physiologically relevant. Since the 1970s, alkaline elution-based methods have been extensively applied to the detection of ICLs in human cells (4,5). This method has been invaluable in characterising the kinetics of DNA crosslinking reactions in cells, providing insights into the kinetics of ICL removal and helping to identify the cellular pathways that promote ICL repair (for example see reference (6)). The alkaline elution approach, however, suffers from a number of drawbacks and limitations, not least the large number of cells required and the requirement to radiolabel the cells prior to assay. Moreover, alkaline elution involves analysis of the pooled material from a population of cells and is therefore not suitable for single-cell studies where variation within a population can be assessed. More recently, a modified version of the single-cell gel electrophoresis (SGCE) assay has become a powerful tool to examine ICL induction and processing (7). This assay is now more commonly known as the 'Comet' assay on account of the analysed cells microscopic resemblance to icy cosmic bodies, having both a head (nucleus) and tail of broken DNA. This radioactive labelling-free method requires far fewer cells than alkaline elution, is more sensitive, can be applied to the analysis of single cells and is suitable for the analysis of material obtained during *in vivo* experiments and clinical studies, including in drug development (8-10).

In outline, the 'classical' version of the Comet assay involves embedding cells in agarose (upon microscope slides), lysing and deproteinising the cells and then submitting the slides to

conventional submarine electrophoresis (11). During the electrophoresis, broken DNA fragments are able to migrate from the supercoiled mass of the nucleoid producing the Comet 'tail', while the unbroken fraction of the genome is retained within the nucleoid or Comet 'head'. Under neutral electrophoresis conditions, DNA double-strand breaks are detected, whereas under denaturing (alkaline) conditions the nucleoid unwinds such that DNA single-strand breaks, double-strand breaks and alkali-labile sites are all revealed (12). In order to detect the presence of ICLs, the alkaline Comet assay has been modified such that, following treatment with the known (or suspected) ICL-inducing agent and embedding the cells in agarose, cells are exposed to a calibrated, fixed dose of ionising radiation. This induces single-strand breaks and alkali-labile sites into the DNA. The presence of covalent ICLs retards the migration of the broken DNA from the nucleoid, reducing tail length and intensity. The reduction in tail length and intensity is related to the frequency of ICLs in the genome in a linear manner (7,10). In experiments designed to examine the repair of ICLs, post-treatment incubation in drug-free media allows the ICL repair to be followed. This is observed and quantified as a gradual restoration of the Comet tail as the covalent links between the DNA strands are removed as the ICLs are incised. This method was pivotal in establishing that the XPF-ERCC1 structure-selective endonuclease plays a key in during 'unhooking' step of ICL processing, which is required release the covalently linked strands of the DNA double-helix and initiate repair (13,14).

Here, we describe a detailed, updated protocol for the modified version of the Comet assay that can be applied to assess ICL induction and repair kinetics across a broad range of mammalian cell systems.

The following method has been successfully applied to the detection of ICLs, and their repair, in a wide variety of mammalian cell lines, including human and rodent cell lines (Note 1). In our experience (unpublished observations), the method is also compatible with cell synchronisation methods to follow ICL removal in specific phases of the cell cycle.

## 2. Materials

2.1 Chemicals and gel reagents

- Cell culture Media as required by the cell lines used (*see* **Note 2**).
- Frosted-end standard glass microscope slides (VWR cat # 631-1551).
- 24 x 40 mm coverslips (VWR cat # 631-0135).
- Agarose, Type 1-A, low EEO, 1% in $H_2O$ (Sigma cat # A0169) (*see* **Note 3**).
- Agarose, Type VII: low gelling temperature (LGT) 1% in PBS (*see* **Note 4**) (Sigma cat# A9045).
- Propidium iodide 10 mg/mL stocks in $H_2O$ (Merck cat # 81845). Alternatively, SYBR Gold can be used as the DNA stain at 1:10,000 in $H_2O$ (ThermoFisher Scientific cat# S11494) (*see* **Note 5**).
- Lysis Buffer: 30 mM NaOH, 1M NaCl, 0.1% *N*-Lauroyl Sarcosine (Sigma cat# L-9159) (made fresh).
- Alkali Buffer: 30 mM NaOH, 2 mM EDTA. Make 10 x stock, store at 4$^{o}$C.
- Neutralisation Buffer: 1 M Tris-HCl, pH 7.5.

2.2 Equipment

- Large flat-bed electrophoresis tank (e.g. 15 x 25 cm BioRad cat #1704404). (*see* **Note 6**).
- Microscope to collect images and analyse data (e.g. Zeiss Observer Z1 microscope with Zen Pro software).

2.3 Software for Comet analysis

- Analysis software (e.g. OpenComet software, OpenComet, GNU General Public License version 1.3 http://www.cometbio.org/ used as a plugin for ImageJ image analysis software https://imagej.nih.gov/ij/download.html).

## 3. Methods

3.1 Slide and cell preparation

1. Pre-coat slides with 1% Type 1-A low EEO agarose in $H_2O$ by pipetting 1 mL of molten agarose onto the centre of the slide. Allow to set and dry overnight at room temperature (*see* **Note 3**).

2. Seed cells in recommended cell culture media. As an example, for U2OS osteosarcoma cells, a line commonly employed in DNA damage and repair studies, we routinely seed 5 x $10^5$ cells in 2 mL in 6 well plates (*see* **Note 7**).

3.2 Cell treatment and sample processing

All procedures are carried out on ice where practicable.

1. Treat cells and incubate for desired times (*see* **Note 8**).

2. Where ICL repair removal is under assessment, remove ICL-inducing treatment agent and allow cells to repair in drug-free media for the desired time(s) (*see* **Note 8**). An example list of samples and controls to include can be found in Table 1.

3. Melt 1% Type VII low gelling temperature (LGT) agarose in PBS in a microwave and keep at 40$^{o}$C in a water bath (*see* **Note 9**).

4. Harvest cells using standard methods. For each sample transfer approximately 2 x $10^4$ cells to wells of a 24-well plate to a final volume of 0.1-0.5 mL. Prepare duplicate 24-well plates. One plate will be irradiated, one plate will not be irradiated (see Table 1). As an example, for U2OS cells, resuspend the cells treated in step 2 (2 x $10^5$ cells) in 2 mL media, allowing for 0.2 mL aliquots per sample (being approximately 2 x $10^4$ cells). The cell number, and therefore final density, is important to ensure a sufficient number of cells are present in each imaged region. However, if cells are too densely packed data collection becomes difficult due to Comet tails overlapping with other cells/tails.

   NB: Prepare sufficient samples to produce duplicate slides for all samples.

5. Irradiate the plate containing the controls and samples as listed in Table 1 (in duplicate). Plates should be kept on ice to limit any unwanted repair or further degradation of the DNA (*see* **Note 10**).

6. To each sample well of the 24 well plate add 1 mL of 1% Type VII (LGT) molten agarose, mix, and pipette 1 mL cells onto the pre-coated slides. Place a 24 x 40 mm coverslip over the cells and allow the agarose/cell mix to set (*see* **Note 11**). It is advisable in this step to add the agarose to small batches of samples at a time to allow adequate processing time and ensure the agarose does not set prematurely before the samples can be added to slides.

7. Once set (1-3 minutes depending on room temperature), remove coverslip and place slides in a shallow tub (lunch box) on ice.

8. Add ice-cold lysis buffer to the tub containing the slides ensuring that all slides are immersed in buffer (*see* **Note 12**).

9. Incubate (on ice) for 1 hr in the dark.

10. Carefully remove lysis buffer using a vacuum pump. Take care not to disturb the gels.

11. Wash slides twice with ice-cold alkali buffer 2 x 30 minutes ensuring slides remain completely immersed and protected from light.

3.3 Slide electrophoresis

1. Transfer slides to the electrophoresis tank aligning all slides with the same direction/polarity (e.g. frosted ends to anode). Ideally all samples in the same experiment should be run at the same time in the same tank. If this is not possible, controls must be included in each separate tank (this will include replicates of samples 1 and 4 from Table 1).

2. Add alkali buffer to completely cover slides, but no more, as an excess of buffer will alter electrophoresis conditions (*see* **Note 13**). Electrophorese, in the dark, for 15 minutes at 15V for 15 cm tank (1 V/cm/min) (*see* **Note 6**).

3. Remove electrophoresis buffer and flood each slide with 1-2 mL neutralisation buffer and leave for approximately 10 minutes.

4. Rinse slides twice with 1-2 mL $H_2O$ for 10 minutes. Slides can be kept in a moist environment at 4°C (with Milli-Q $H_2O$ or PBS) until stained or allowed to air dry.

3.4 Sample staining

1. Re-hydrate slides with 1 mL Milli-Q water for 30 minutes.

2. Flood each slide twice with 1 mL of 2.5 µg/mL propidium iodide solution in $H_2O$ and incubate for 2 x 5 minutes at room temperature in the dark (*see* **Note 14**).

3. Rinse off DNA stain with Milli-Q water once and replace and leave water on slides for 30 minutes.

4. Dry slides for analysis. Store in slide box until image analysis. Slides will remain readable indefinitely and can be re-stained if necessary (*see* **Note 15**).

3.5 Data acquisition and analysis

1. Collect multiple fields of view to enable acquisition of data from at least 50 cells chosen at random per sample following the software's protocol. Alternatively, tiled images can be obtained. Expected results shown in Figure 1.

2. The degree of interstrand crosslinking (ICL) present following drug treatment can be determined by comparing the length of the Comet tail (tail moment) of the irradiated drug-treated samples with that of the irradiated control (untreated) and the un-irradiated control (untreated) samples (see Figure 2A, (7)). The frequency of ICLs is proportional to the decrease observed in the tail moment when the irradiated drug-treated sample is compared with that of the irradiated control (untreated) sample. This is, in effect, a measurement of the retardation of the mobility of the DNA due to the presence of ICLs. This decrease in tail moment (DTM) is calculated using the following formula:

$$DTM(\%) = \left[1 - \frac{(TMdi - TMcu)}{((TMci - TMcu) + (TMdu - TMcu))}\right] \times 100$$

DTM = Decrease in Tail Moment (%)

TMdi = Mean Tail moment of the drug treated irradiated sample

TMci = Mean Tail moment of the irradiated control

TMcu = Mean Tail moment of the un-irradiated control sample

TMdu = Mean Tail moment of the drug treated un-irradiated sample

3. ICL "unhooking" can also be measured (Figure 2B and formula below). Unhooking is a function of the release of the covalent linkage of the two DNA strands produced by the incision of the ICLs during their repair. Unhooking is determined by measuring the decrease in tail moment observed seen immediately after drug treatment and comparing this with the crosslinks still present at post treatment times. Figure 2B shows that over a period of time, ICLs are "unhooked" showing repair of induced ICLs. This can be calculated *via* the following formula (13):

$$\%\ unhooking\ at\ T1 = \left[\frac{(\%\ DTM\ at\ T1 - \%\ DTM\ at\ T0)}{(\%\ DTM\ T0)}\right] \times\ 100$$

Where DTM is calculated via the formula above (3.5.2) for each time point. Over time, if the ICL is repaired you would expect to see these lesions being "unhooked" and hence and increase in % unhooking. This is also a measure indicating the repair of the ICLs is progressing. A decrease in the speed of unhooking, or failure to unhook indicates a repair defect.

**Notes**

1. This method has been successfully applied to a variety of mammalian cells, including human, mouse and Chinese hamster ovary, from either cells in culture or clinically derived/ primary animal cells.

2. This method can be used on adherent or suspension cell lines. Optimal standard conditions for growing cells should be followed. No variation or special culturing conditions are required in this method.

3. Low EEO agarose (low electroendosmosis) is used to provide a medium of adhesion for the cell-agarose suspension added to the slides in step 8. This process aims to stop the added cell-agarose mix detaching during processing. If preferred, poly-lysine slides can be used as they will also allow the adhesion of the cell-agarose suspension.

4. Type VII agarose is made up in PBS, not $H_2O$, to avoid hypotonic lysis of the cells when the cell-agarose mix is made.

5. Some DNA stains are more sensitive than others. While propidium iodide is satisfactory, other stains might produce a stronger signal. The choice of DNA stain used will be determined by the fluorescent filters available on the microscope to be used for analysis.

6. Any electrophoresis chamber can be used. The conditions for electrophoresis must be optimised to ensure adequate “tail” length in the IR-treated samples. Excessive

electrophoresis can make data acquisition difficult as the length of the comet can confuse the acquisition software.

7. Cell number should be kept the same between experiments to ensure similar treatment conditions. The number of cells is important as when cell-agarose suspensions are added to slides, an optimal number of cells must be used to permit analysis. Slides where cells are at a low-density, or cells that are overlapping (at high-density), present challenges during data acquisition.

8. To study ICL formation adequate incubation time for the drug-specific lesions should be allowed. As a positive control cisplatin, 10 µM 4 hours will induce a significant number of interstrand crosslinks. Treatment time and repair time is drug and cell type dependent.

9. Type VII agarose will set at 37°C, keeping the agarose at temperatures much greater than 40$^{o}$C will result in exposure of cells to excessive temperature when preparing agarose-cell mixes (step 8).

10. Determine the ionising radiation dose required empirically, for most irradiators (we have primarily used a caesium 137 source) and for all mammalian cell lines an irradiation dose of 10 Gy has proven optimal.

11. The coverslip will “flatten” the cell-agarose mix and should cover the entire volume of the mix.

12. Adding and removing buffers to the slides should be done with great care. If the cell-agarose gel is disturbed, detaching it from the slide, reposition the gel on the slide and avoid disturbing it again. When slides are being dried (step 3.3.4) ensure the gel is over the slide and it will dry in place allowing staining and analysis.

13. The amount of buffer added should be noted and the same volume used for each experiment under the same electrophoresis tank conditions. This will aid reproducibility of the data obtained.

14. Extended periods of staining can make cells too “bright”. Staining conditions must be optimised if this becomes a problem. Alternatively, SYBR Gold DNA stain can be used in a 10-minute room temperature stain at the manufacturer’s specified concentration.

15. Slides can be dried in an oven at 37°C (~3-4 hr), 65°C (~2 hr) or at room temperature overnight.

**References**


1. Dronkert, M.L. and Kanaar, R. (2001) Repair of DNA interstrand cross-links. *Mutat Res*, **486**, 217-247.
2. McHugh, P.J., Spanswick, V.J. and Hartley, J.A. (2001) Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance. *Lancet Oncol*, **2**, 483-490.
3. Kim, H. and D'Andrea, A.D. (2012) Regulation of DNA cross-link repair by the Fanconi anemia/BRCA pathway. *Genes & development*, **26**, 1393-1408.
4. Ewig, R.A. and Kohn, K.W. (1977) DNA damage and repair in mouse leukemia L1210 cells treated with nitrogen mustard, 1,3-bis(2-chloroethyl)-1-nitrosourea, and other nitrosoureas. *Cancer Res*, **37**, 2114-2122.
5. Ross, W.E., Ewig, R.A. and Kohn, K.W. (1978) Differences between melphalan and nitrogen mustard in the formation and removal of DNA cross-links. *Cancer Res*, **38**, 1502-1506.
6. Papadopoulo, D., Averbeck, D. and Moustacchi, E. (1987) The fate of 8-methoxypsoralen-photoinduced DNA interstrand crosslinks in Fanconi's anemia cells of defined genetic complementation groups. *Mutat Res*, **184**, 271-280.
7. Spanswick, V.J., Hartley, J.M., Ward, T.H. and Hartley, J.A. (1999) Measurement of drug-induced DNA interstrand crosslinking using the single-cell gel electrophoresis (comet) assay. *Methods Mol Med*, **28**, 143-154.
8. Hartley, J.A., Spanswick, V.J., Brooks, N., Clingen, P.H., McHugh, P.J., Hochhauser, D., Pedley, R.B., Kelland, L.R., Alley, M.C., Schultz, R. *et al.* (2004) SJG-136 (NSC 694501), a novel rationally designed DNA minor groove interstrand cross-linking agent with potent and broad spectrum antitumor activity: part 1: cellular pharmacology, in vitro and initial in vivo antitumor activity. *Cancer Res*, **64**, 6693-6699.
9. Puzanov, I., Lee, W., Chen, A.P., Calcutt, M.W., Hachey, D.L., Vermeulen, W.L., Spanswick, V.J., Liao, C.Y., Hartley, J.A., Berlin, J.D. *et al.* (2011) Phase I pharmacokinetic and pharmacodynamic study of SJG-136, a novel DNA sequence selective minor groove cross-linking agent, in advanced solid tumors. *Clin Cancer Res*, **17**, 3794-3802.
10. Hartley, J.M., Spanswick, V.J., Gander, M., Giacomini, G., Whelan, J., Souhami, R.L. and Hartley, J.A. (1999) Measurement of DNA cross-linking in patients on ifosfamide therapy using the single cell gel electrophoresis (comet) assay. *Clin Cancer Res*, **5**, 507-512.
11. Olive, P.L., Banath, J.P. and Durand, R.E. (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. *Radiat Res*, **122**, 86-94.
12. Fairbairn, D.W., Olive, P.L. and O'Neill, K.L. (1995) The comet assay: a comprehensive review. *Mutat Res*, **339**, 37-59.
13. De Silva, I.U., McHugh, P.J., Clingen, P.H. and Hartley, J.A. (2000) Defining the roles of nucleotide excision repair and recombination in the repair of DNA interstrand cross-links in mammalian cells. *Mol Cell Biol*, **20**, 7980-7990.
14. Bhagwat, N., Olsen, A.L., Wang, A.T., Hanada, K., Stuckert, P., Kanaar, R., D'Andrea, A., Niedernhofer, L.J. and McHugh, P.J. (2009) XPF-ERCC1 participates in the Fanconi anemia pathway of cross-link repair. *Mol Cell Biol*, **29**, 6427-6437.

# Table 1: List of samples to be included for each cell line being tested

| Sample Number | Description | IR 10 Gy | Plate |
|---|---|---|---|
| 1 | Non-drug treated control | No | Plate 1 |
| 2 | Drug treated[1] | No | |
| 3 | Drug treated + Repair[2] | No | |
| 4 | Non-drug treated control | Yes | Plate 2 |
| 5 | Drug treated[1] | Yes | |
| 6 | Drug treated + Repair[2] | Yes | |

1. Drug treatment to induce crosslinking. Eg. CDDP 50 μM, 4 hour. Multiple, varied, concentrations can be tested to measure an effective drug dose to induce crosslinking (as calculated in section 3.5.2).

2. Repair times will be cell type dependent. Taking measurements for 8, 16, 24, 36 and 48 hours post treatment should give an indication of “unhooking” (being repair time as calculated in section 3.5.3).

In step 4, two 24-well plates are prepared. Both plates will contain the same samples (as listed above). One plate to be irradiated (plate 2 in this example), one plate will not be irradiated.

Both plates must be kept on ice until slides are made.

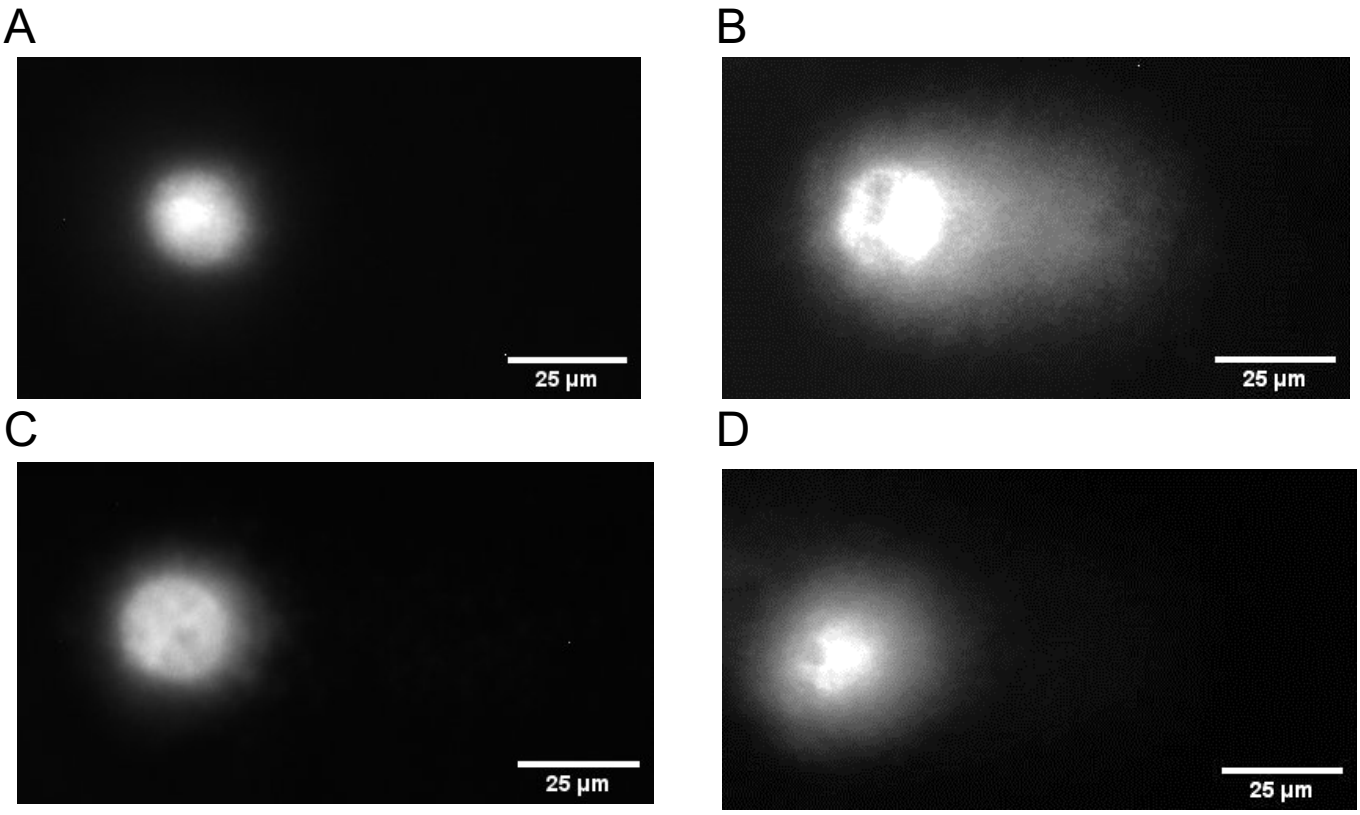
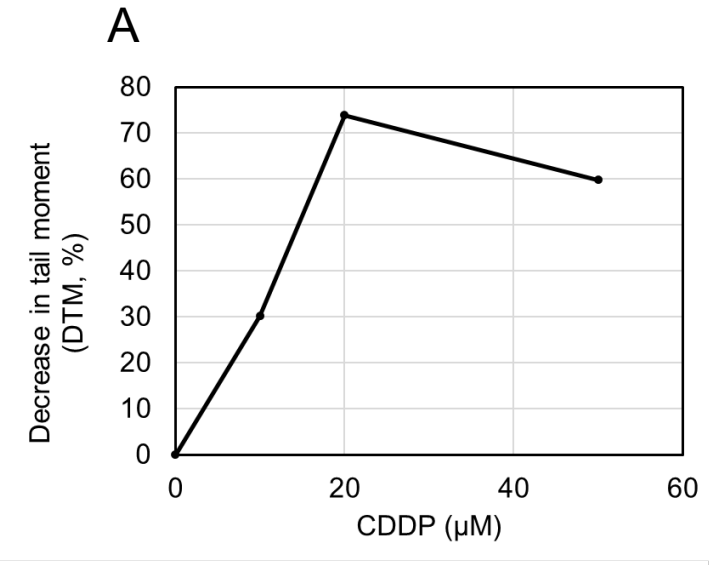



**Figure 1: Comet assay assessment of ICL formation.** U2OS osteosarcoma cells as assessed by modified Comet Assay. **A** Untreated, unirradiated cells have intact DNA that remains concentrated within the nucleoid and only a Comet 'head' is observed. **B** Untreated, irradiated cells have damaged DNA following exposure to 10 Gy ionising radiation (IR) this results in the formation of a 'tail' of DNA containing breaks and alkali labile sites that migrate from the nucleoid during electrophoresis. **C** Cisplatin treated (50µM, 4 hour) unirradiated cells show no visible significant Comet tail – if present this would be a measure of DNA single and double-strand breaks and alkali-labile sites induced by the treatment or as a by-product of the repair of the lesion. **D** Cisplatin treated (50µM, 4 hour) irradiated cells have multiple ICLs that impede DNA migration during electrophoresis and the Comet tail decreases in size in a drug concentration-dependent manner (compare D with B).



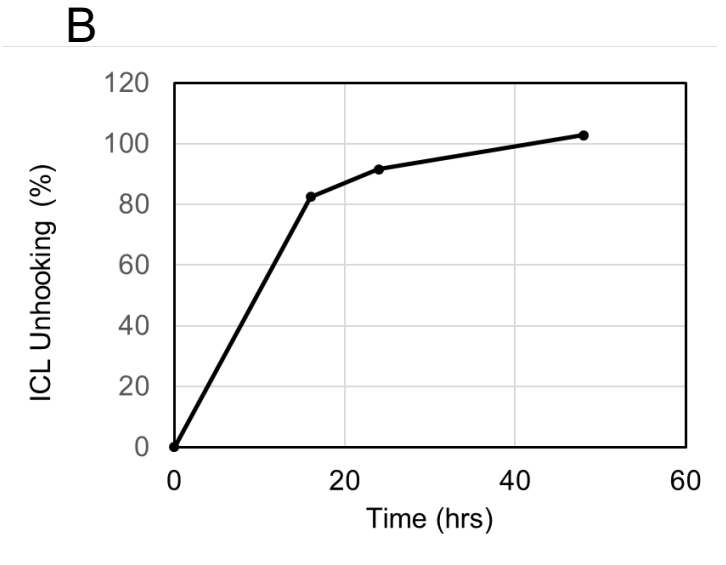




**Figure 2. Examples of data obtained: A.** percentage decrease in tail moment indicating the increase in ICLs as a function of increasing drug concentrations of Cisplatin (CDDP). **B.** Unhooking kinetics as measured by the modified Comet assay. The Comet tail of a given drug treatment (Cisplatin 50 μM, 4 hour) will increase, over time, indicating repair ("unhooking") of ICLs has occurred.