

## CHAPTER 6

# *Development and Applications of the Comet-FISH Assay for the Study of DNA Damage and Repair*

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### **6.1 Introduction**

The single-cell gel electrophoresis (SCGE) assay was first reported by Östling and Johanson<sup>1</sup> in 1984 as a technique for visualising the migration of DNA containing strand breaks in individual agarose-embedded cells under electrophoretic conditions. A few years later, Singh *et al.*<sup>2</sup> used a similar method, but modified it slightly to use highly alkaline (pH > 13) conditions that encourages unwinding of DNA around a strand break. In both studies, the underlying principle of the assay is that when DNA is subjected to an electric current, DNA containing strand breaks will migrate through an agarose gel due to relaxation of the DNA supercoils, whilst unbroken DNA remains immobile. Following the staining of DNA with a fluorescent DNA-specific dye, the resulting image can be visualised and resembles a comet, with undamaged DNA forming a “head” and damaged DNA forming a “tail”, an observation that has led this technique to be more commonly called the Comet assay.<sup>3</sup>

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Since then, it has become a widely accepted and versatile method for measuring a variety of DNA lesions in individual cells.<sup>4-6</sup> Its versatility stems from various modifications to the original Comet assay that have been developed in order to measure different types of DNA damage, including single- and double-strand breaks, crosslinking and oxidative damage, as well as DNA breaks associated with replicating DNA and DNA repair (reviewed in Collins<sup>6</sup>). One further modification is the combination of fluorescent *in-situ* hybridisation (FISH) with the Comet assay, in which fluorescently labelled probes are hybridised to a specific gene sequence, region or chromosome. Thus, the localisation of a particular DNA sequence within an individual comet can be visualised, thereby providing information about DNA damage in and around the probed region, compared to overall DNA. Further information about whether or not DNA breakage occurs within the probed region is obtained by observing changes in the number of hybridisation signals in the comet.

This review discusses the methods for employing the Comet-FISH assay in the laboratory and summarises the various studies that have successfully used this approach, examining the different applications and uses of this versatile technique. The potential and limitations of the assay are also considered and the importance of these limitations on accurate data interpretation is emphasised.

## 6.2 The Comet-FISH Assay Procedure

Several slightly different protocols exist for the Comet assay, but all follow the same basic premise.<sup>7</sup> Cells under investigation are embedded in an agarose gel on microscope slides and are subsequently lysed by placing the slides in a high-salt solution (usually containing detergent). Following this lysis step, which typically occurs for 1 to 24 h at 4 °C, the slides are then placed into an electrophoresis tank filled with buffer and are subjected to DNA unwinding and electrophoresis. The lysis and electrophoresis may be carried out under alkaline<sup>8</sup> or neutral<sup>9</sup> pH conditions depending on whether the investigator wishes to examine single-strand breaks, alkali-labile sites and/or double-strand breaks. Modifications that allow analysis of crosslinking DNA damage<sup>10,11</sup> or that employ restriction enzymes<sup>12,13</sup> can also be incorporated as these depend on the production of DNA breaks for indirect measurement. Depending on the damaging agent being used, cells may be treated in culture (*e.g.* with chemicals<sup>14</sup>) prior to collection for slide preparation, or alternatively can be treated on the slides immediately after they are embedded in agarose (*e.g.* with radiation<sup>15</sup>).

In the standard Comet assay, the cells are finally stained with a DNA-specific dye such as ethidium bromide or propidium iodide to allow visualisation under the fluorescent microscope. However, in the Comet-FISH assay, this staining step is omitted and instead the cells on the prepared slides are subjected to an extra hybridisation protocol. This can be performed following either the alkaline or neutral version of the Comet assay<sup>16</sup> and the steps involved in this procedure are discussed below and summarised in Table 6.1.

**Table 6.1** Steps in the Comet-FISH assay. This gives an overview of the sequential steps involved in the Comet-FISH assay. For further reading and information, a detailed account of the Comet assay procedure is provided by Olive and Banáth,<sup>7</sup> whilst a discussion of the Comet-FISH assay procedure can be found in Rapp *et al.*<sup>16</sup>

Step	Conditions	Notes
Comet assay	Standard Comet assay conditions <i>Slide preparation</i> : 1–2 h <i>Lysis</i> : 1–24 h <i>Unwinding &amp; Electrophoresis</i> : 1 h <i>Neutralisation</i> : 20 min	<ul style="list-style-type: none"> <li>• Cells may be treated prior to slide preparation or on slides if protocol allows.</li> <li>• Specific times for each step may vary from protocol to protocol, but should be kept constant between experiments.</li> </ul>
Alkaline denaturation	Immerse slides in 0.5 M NaOH / 1 M NaCl for 30 min at room temperature.	<ul style="list-style-type: none"> <li>• This step can be omitted if a heat codenaturation step is used for hybridisation (see hybridisation step below)</li> </ul>
Slide dehydration	Sequential immersion of slides in 70%, 85%, 100% v/v ethanol for 5 min each. Leave slides to air dry on tissue paper.	<ul style="list-style-type: none"> <li>• Slides can be stored until ready for hybridisation.</li> <li>• Dehydrated slides can be stored for several weeks at room temperature.</li> </ul>
Probe preparation	Probe mixture prepared according to manufacturer's protocol for <i>in-situ</i> hybridisation. Probe is denatured at specified temperature in heating block. Alternatively, heat codenaturation can be used.	<ul style="list-style-type: none"> <li>• Rapid drying (<i>e.g.</i> at 37 °C) may cause gels to crack</li> <li>• Approximately 10 µl total hybridisation volume is needed for a 22 × 22 mm gel.</li> <li>• Probe should be labelled with suitable fluorophore for visualisation on user's imaging system.</li> <li>• If heat codenaturation hybridisation is being performed place slide on hotplate at correct temperature (typically 75–80 °C) and add probe followed quickly by coverslip. Leave for 2 mins, then remove slide and place in humidified dark box overnight at 37–42 °C</li> </ul>
Hybridisation	Probe is applied to agarose gel and coverslip applied on top. Hybridisation occurs in a humidified dark box overnight at 37–42 °C	<ul style="list-style-type: none"> <li>• Slides should be prewarmed prior to probe application to aid efficient hybridisation.</li> </ul>

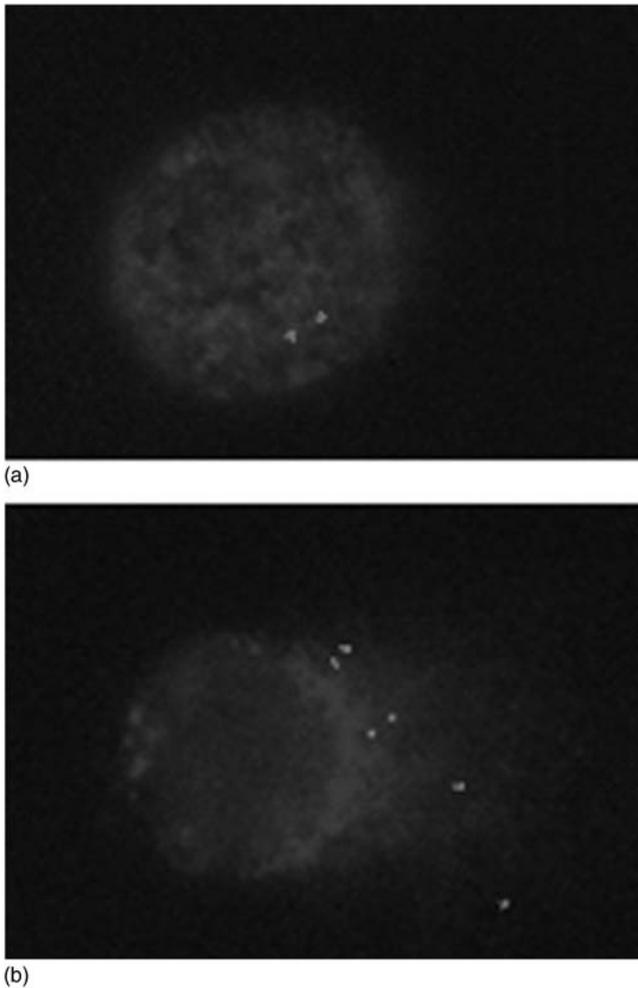
**Table 6.1** (Continued).

Step	Conditions	Notes
Posthybridisation washes	Slides washed in sequential washing solutions. Typical wash solutions would be :- (i) 2X SSC/50% formamide, pH 7.0 (3 × 5 mins at 45 °C) (ii) 2X SSC, pH 7.0 (1 × 5 min at 45 °C) (iii) 2X SSC/0.1% Igepal pH 7.0 (1 × 5 min at 45 °C)	<ul style="list-style-type: none"> <li>• Slides should be delicately treated to ensure gels do not dislodge from slide.</li> <li>• Excessive shaking should be avoided.</li> </ul>
Counterstain	Add suitable counterstain and place coverslip on top. Store slides in dark box at 4 °C until viewing	<ul style="list-style-type: none"> <li>• Counterstain should have clearly separate excitation/emission spectra from probe fluorophore</li> <li>• Slides can also be stored overnight in the dark at -20 °C. However, viewing immediately after staining is preferable as fluorescent signals may fade.</li> <li>• To aid visualisation of hybridisation signals, 60× magnification or more is preferable.</li> </ul>
Imaging	View images using appropriate filter set and microscope.	<ul style="list-style-type: none"> <li>• Care should be taken to select suitable fluorophores for probes/counterstain, ensuring a minimum of overlap in the excitation/emission spectra.</li> <li>• Likewise, the microscope and filter settings should be calibrated to minimise bleed-through of signals</li> <li>• Triplicate experiments are recommended.</li> </ul>
Analysing	Count 25 or 50 cells per slide using standard comet analysis. Record number and location of hybridisation signals	<ul style="list-style-type: none"> <li>• Several published studies have analysed between 25 and 50 cells per slide. However, it is recommended that not less than 50 cells are counted without statistical verification to justify using fewer numbers</li> </ul>

Fluorescent probes are hybridised to the agarose-embedded cells using standard *in-situ* hybridisation techniques. Typically, the probe is mixed with a hybridisation buffer and denatured by heating, before being added to the slides. The DNA of cells contained on the slides is also denatured, either by alkaline treatment prior to the hybridisation<sup>15</sup> or by heat.<sup>8</sup> If heat denaturation is preferred, it is usual to carry out codenaturation of the probe and target cellular DNA simultaneously on a hotplate. The slides are then placed in a humidified box overnight at the appropriate temperature (typically 37–42 °C) to allow hybridisation to occur. Following this, posthybridisation washing steps are carried out, whereby the slides are moved through a series of wash buffers to remove any unbound probe and minimise nonspecific binding. Finally, the overall cellular DNA is counterstained with a suitable fluorescent dye. During microscopic examination, specific hybridisation signals need to be clearly visible against the counterstained total genomic DNA, therefore care should be taken to ensure there is minimum overlap in the excitation and emission spectra for the different fluorophores used. Likewise, it is of utmost importance that the microscope and filter settings being used for analysis are calibrated correctly to allow clear visualisation of cells and minimise bleed-through of fluorescent signals, which can otherwise interfere with analysis of cells.

Analysis of cells involves examining both the distribution of total genomic DNA in the comet, together with the number of hybridisation signals, and the location of each signal. Overall DNA distribution is measured using standard Comet assay analysis software, whereby the amount of DNA in the head and tail of each selected cell is assessed using a number of parameters, including % tail DNA and Olive tail moment, the two preferred measurements of DNA damage in Comet assay experiments. Then, in the same selected cells, the number of signals and the position of each hybridisation signal in the head or tail of the comet is recorded, thereby giving an indication of whether it lies in, or close to, a region of damaged DNA. The appearance of hybridisation signals in the comet tail generally indicates that the region of DNA within, or around, the probe contains strand breakage. Information about where exactly the DNA breakage occurs in relation to the probed region is obtained by counting the frequency distribution of signals in each comet. Increase in signal number would suggest the probed region itself contains strand breakage, since the probe will bind to each broken DNA fragment from the target region. Of course, control cells must always be included to give an indication of baseline damage for both overall DNA and hybridisation signals. Figure 6.1 shows representative examples of images from Comet-FISH experiments, demonstrating the differences between an unirradiated, control cell (Figure 6.1(a)) and an irradiated, damaged cell (Figure 6.1(b)). Similar images can be obtained using a variety of different DNA-damaging agents to generate comets that are visualised using selected fluorescent stains/probes.

Both cells have been hybridised with Spectrum Orange-labelled TP53 probe, which fluoresces with a pink/orange colour and total genomic DNA counterstained with 4',6-diamidino-2-phenylindole (DAPI), which fluoresces blue. Visualisation was performed using an epifluorescence microscope (Nikon



**Figure 6.1** Representative images of cells processed using the Comet-FISH protocol.

Eclipse E400) fitted with a Nikon X60 fluor lens (Plan Apo 60:1 N.A. 1.4) and equipped with a Hamamatsu Orca digital CCD camera. A double bandpass filter set (Chroma HiQ) tuned for DAPI (excitation 370 nm, emission 450 nm) and spectrum orange (excitation 560 nm, emission 590 nm) was utilised, allowing simultaneous detection of DAPI and Spectrum Orange labels.

- (a) Unirradiated control cell showing little DNA damage as evidenced by the absence of a comet tail. Two hybridisation signals (pink) are clearly visible in the intact head.
- (b) Immediately after exposure to  $\gamma$ -radiation a large comet tail is visible, reflecting the extent of DNA damage. Several p53 hybridisation signals

are located in the comet tail, indicating that strand breakage has occurred near and/or within the probed region.

Using this novel approach, several interesting papers have been published over the past decade in which the Comet-FISH assay has been utilised to investigate the cellular response following DNA damage.

## 6.3 Applications of the Comet-FISH Assay

### 6.3.1 Discovery of the Comet-FISH Assay

Two independent laboratories were the first to report the Comet-FISH assay as a new technique. In 1997, Santos *et al.*<sup>17</sup> used the technique to detect centromeres and telomeres of chromosomes, as well as regions of the O<sup>6</sup>-methylguanine DNA methyltransferase (MGMT) gene, in human lymphocytes and concluded that the method offered potential for studying chromatin ultrastructure and spatial organisation in the nucleus. At the same time, another study, by McKelvey-Martin *et al.*,<sup>8</sup> successfully hybridised a locus-specific identifier (LSI) probe for TP53 to both human leukocytes and bladder cancer cells and proposed the Comet-FISH assay as a powerful tool for investigating damage and repair in specific DNA sequences in individual cells. Together, these two studies provided a springboard for many other studies to investigate DNA damage and repair and nuclear chromatin arrangement. These are discussed below and details of each experiment presented in Table 6.2.

### 6.3.2 Using Comet-FISH to Measure DNA Damage

Comet-FISH studies using whole chromosome paint (WCP) probes have generated interesting results about the susceptibility of different chromosome regions to DNA damage. Differences in DNA damage have been detected between chromosomes in human lymphocytes exposed to UV-A, with telomeres shown to be more sensitive than centromeres.<sup>18</sup> In a similar experiment on human lymphocytes, an inverse correlation was shown between density of active genes and sensitivity to UV-A damage, which the authors proposed was due to the higher repair enzyme activity located at active gene loci.<sup>19</sup> In a further experiment, exposure of healthy mucosal cells taken from patients with oropharyngeal carcinoma to benzo(a)pyrene-diolepoxide (BPDE) resulted in higher strand breakage in chromosomes 3, 5 and 8 compared to chromosome 1.<sup>20</sup> Since chromosomes 3, 5 and 8 are known to show alterations in carcinomas of the upper aerodigestive tract, this led to the suggestion that the Comet-FISH assay was a method that could allow the detection of gross chromosomal aberrations and selected genetic alterations in cells.<sup>20</sup> Hence, it may prove useful for investigating other chromosomes and large DNA regions for susceptibility to DNA damage in response to a wide variety of DNA-damaging agents. Furthermore, it offers potential as a predictive test for detecting specific

**Table 6.2** Summary of Comet-FISH studies.<sup>a</sup> This summarises the various Comet-FISH experiments published to date. Where known, details of probe label and size are given, as well as treatment conditions for cells and chemical concentrations used. All experiments utilised the alkaline version of the Comet assay, unless otherwise indicated in the Treatment column

<i>Cell type</i>	<i>Treatment</i>	<i>Probe</i>	<i>Results</i>	<i>Ref.</i>
Human leukocytes	Cells treated <i>in vitro</i> with ferric-nitrosylacetate. (Fe-NTA) (1–500 $\mu$ M) [Alkaline Comet assay using Endo III; Fpg]	Texas Red-labelled probe to TP53 (10.6 Mb)	Fe-NTA enhanced migration of TP53 signals into comet tail	36
Human lymphoblastoid cell line (TK6)	Cells treated <i>in vitro</i> with melphalan, etoposide or hydroquinone (HQ) (final concentration for all compounds = 0.25%)	Chromosome band probes 5q31 (200 kB) and 11q23 (350 kB)	All 3 compounds induced DNA breaks at both probed regions. HQ caused more DNA damage at 5q31 than 11q23	25
Primary human colon cells and colon adenoma cells (LT97)	Cells treated <i>in vitro</i> with hydrogen peroxide ( $H_2O_2$ ) (0–150 $\mu$ M), trans-2-hexenal (0–1600 $\mu$ M) & 4-hydroxy-2-nonenal (HNE)(0–250 $\mu$ M)	Digoxigenin- or Texas Red-labelled probes to APC, KRAS & TP53	All 3 regions showed increased dose-dependent migration of signals into tails for all 3 compounds. TP53 more sensitive to damage than KRAS, APC and overall DNA in $H_2O_2$ -treated primary colon cells and in all cells treated with HNE- & trans-2-hexenal.	29
Primary human colon cells and colon adenoma cells (LT97)	Cells treated <i>in vitro</i> with Fe-NTA(1–1000 $\mu$ M)	Texas Red-labelled probe to TP53 (10.6 Mb)	Fe-NTA enhanced migration of TP53 signals into comet tail in both cell types	33

Primary human colon cells and colon adenoma cells (LT97)	Cells treated <i>in vitro</i> with uranyl-nitriotriscetate. (UNTA)(0–1000 µM)	Texas Red-labelled probe to TP53 (10.6 Mb)	32
Mouse peripheral leukocytes	Mice irradiated with 0–4 Gy X-rays. Cells collected 30 min, 24 h and 30 days after exposure	Biotinylated probes to Abl (127 kB), TP53 (127 kB), Ret(150 kB)	37
Normal mammary epithelium (AG11134) and breast cancer cell line (MDA-MB468)	Cells irradiated <i>in vitro</i> with 2 and 10 Gy $\gamma$ -radiation OR exposed to H <sub>2</sub> O <sub>2</sub> (100 µM) [Neutral Comet assay]	TP53 probe (145 kB) ; HER-2 probe (190 kB) ; ZNF217 probe (320 kB) – all labelled with either Spectrum Orange and Spectrum Green	34
Human peripheral blood cells	Cells treated <i>in vitro</i> with bleomycin (0–100 IU/ml) and/or cisplatin	Telomere-specific peptide nucleic acid (PNA) probes	23
Chinese hamster ovary (CHO) cell line, human fibrosarcoma cell line (HT1080), acute lymphoblastic leukaemia cell line (CCRF-CEM)	Cells treated <i>in vitro</i> with bleomycin (0–100 IU/ml) or mitomycin C (MMC) (0–100 µg/ml)	Telomere-specific PNA probes	24
Healthy oropharyngeal mucosal cells biopsied during surgery of oropharyngeal carcinoma (10 patients)	Cells treated <i>in vitro</i> with benzo(a)pyrene-diolepoxide (BPDE) (9 µM)	Whole chromosome paint (WCP) probes to Chr 1, 3, 5 & 8	20
CHO cell line	Cells treated <i>in vitro</i> with UVC (1 J/m <sup>2</sup> ) OR with H <sub>2</sub> O <sub>2</sub> (0.2 mM) OR with photosensitiser Ro 19-8022 f/b irradiation at 0.33 m with 1000 W halogen lamp.	DHFR gene, Exon 1-biotinylated Exon 6-fluorescein; MGMT gene, bases 8-33 – biotinylated, bases 463-488 – fluorescein; TP53 gene, exon 2 – biotinylated, exon	35
		U-NTA enhanced migration of TP53 signals into comet tail in both cell types All 3 regions showed increased dose-dependent fragmentation after irradiation Both TP53 and HER-2 loci more susceptible than ZNF217 loci to damage by irradiation and H <sub>2</sub> O <sub>2</sub> Cisplatin reduces telomere signal migration more than total DNA, indicating crosslinking by cisplatin is preferentially telomeric Dose-dependent detection of telomeric signals in comet tail observed for CHO, CCRF-CEM, but less so for HT1080. Significantly higher damage in Chr 3, 5, & 8 compared to Chr 1 in healthy mucosa of patients with oropharyngeal carcinoma Signals from both TP53 probes located in tail of damaged cells. However, DHFR probe signals were rarely observed in comet tail and only one of MGMT	

Table 6.2 (Continued).

Cell type	Treatment	Probe	Results	Ref.
Human peripheral blood cells	Cells treated <i>in vitro</i> with bleomycin (0–100 IU/ml) or MMC (0–100 µg/ml)	11-fluorescein (all probes 26bp)	signals appeared consistently in tail. Preferential repair of strand breaks in TP53 gene and oxidised bases in MGMT gene observed.	22
Primary human colon cells	Cells treated <i>in vitro</i> with H <sub>2</sub> O <sub>2</sub> (0–150 µM) OR trans-2-hexenal (0–1600 µM)	Telomere-specific PNA probes	Breakage frequency for DNA in vicinity of telomeres found to be proportional to overall DNA	31
Primary human colon cells & human colon adenoma cell line (LT97)	Cells treated <i>in vitro</i> with H <sub>2</sub> O <sub>2</sub> (18.8–150 µM) OR HNE (100–250 µM)	Digoxygenin-labelled probe to TP53 (~80 kB)	TP53 gene region was more sensitive than global DNA to damage caused by H <sub>2</sub> O <sub>2</sub> and trans-2-hexenal	30
Bladder cancer cell lines (RT4 & RT112)	Cells treated <i>in vitro</i> with γ-radiation (0–10 Gy)	Digoxygenin-labelled probe to TP53 (~80 kB)	TP53 gene region was more sensitive than global DNA to damage caused by HNE in both primary cells and cell line	15
Bladder cancer cell line (RT4)	Cells treated <i>in vitro</i> with MMC (0–200 µg/ml)	Spectrum Orange-labelled probe to TP53 (~200 kB)	Repair of strand breaks in TP53 gene region was faster than overall DNA during first 15 min following 5 Gy irradiation	14
		Spectrum Orange-labelled probe to TP53 (~145 kB)	Repair of MMC-induced crosslinks in TP53 gene region was faster than overall DNA during first 4 h following treatment	38

Human peripheral blood leukocytes, double-strand-break repair deficient fibroblast cell line (MO59J) and parental fibroblast cell line (MO59 K)	Cells irradiated <i>in vitro</i> with X-rays (0–52 Gy) [Alkaline and neutral Comet assay both used]	Biotinylated whole human genome probe	DNA breakage detection (DBD)-FISH successfully distinguished between double- and single-strand breaks	27
<i>Vicia faba</i> plant cells	Cells treated <i>in vitro</i> with endonucleases DNase I, FokI or EcoRI [Alkaline & Neutral Comet assay both used]	Digoxigenin-labelled probes to FokI repeat (~59 bp), 25S-rDNA gene, intergenic space and telomere repeat.	Increased FokI signals in tails of cells treated with FokI. rDNA signals were randomly distributed for FokI and DNase treated cells. rDNA and telomere signals rarely found in tail in EcoRI treated cells.	19
Human lymphocytes	Cells irradiated <i>in vitro</i> with UV-A (500 kJ/m <sup>2</sup> )	WCP probes to Chr 1, 2, 3, 8, 9, 11, 14, 18, 19, 21, X & Y	Inverse correlation found between density of active genes and sensitivity to UV-A	18
Human lymphocytes	Cells irradiated <i>in vitro</i> with UV-A (500 kJ/m <sup>2</sup> )	Various digoxigenin-labelled probes to centromeres, $\alpha$ -satellites, telomeres & whole chromosomes, c-myc, TP53 and TP58	Telomeres more sensitive than centromeres to UV-A induced damage. c-myc locus more sensitive to chromosome breakage than TP53 and TP58. Chr X more sensitive than Chr 1 to UV-A	8
Human leukocytes & bladder cancer cell line (HT1376)	HT1376 cells treated <i>in vitro</i> with $\gamma$ -radiation (10 Gy)	Spectrum Orange-labelled TP53 probe (~200 kB)	Successful visualisation of TP53 hybridisation spots in head and tail of comets.	17
Human lymphocytes	No treatment [Neutral Comet assay]	biotinylated SO- $\alpha$ AllCen probes to all centromeres (~86 bp); biotinylated probes to all telomeres;	Centromeres demonstrate dispersed localisation along migrated DNA. Telomeres localized as concise nodules	

**Table 6.2** (Continued).

<i>Cell type</i>	<i>Treatment</i>	<i>Probe</i>	<i>Results</i>	<i>Ref.</i>
		biotinylated probe to Chr 7 centromere ; 3 biotinylated probes to segments of MGMT ; digoxigenin-labeled probe to Chr 3 long arm telomere	near nuclear membrane. MGMT signals detected in both comet head and tail.	

**Abbreviations.** Fe-NTA: ferric-nitriiotriacetate, Endo III: endonuclease III, Fpg: formamidopyrimidine-DNA glycosylase, TP53: tumour protein 53, HQ: hydroquinone, H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide, HNE: hydroxyl-2-nonenal, APC: adenomatous polyposis coli, U-NTA: uranyl-nitriiotriacetate, MMC: mitomycin C, PNA: peptide nucleic acid, BPDE: benzo(a)pyrene-diolepoxide, WCP: Whole chromosome paint, Chr: chromosome, DHFR: dihydrofolate reductase, MGMT: O<sup>6</sup>-methylguanine-DNA methyltransferase, CHO: Chinese hamster ovary, DBD: DNA breakage detection, SO-aAllCen: synthetic oligomer- $\alpha$  all centromeres.

endogenous risk factors or genetic biomarkers for disease, an extension of an idea already postulated for the Comet assay alone since it is a method that is reasonably fast, sensitive and requires only a few cells to generate results.<sup>21</sup>

The potential use of the Comet-FISH assay in understanding disease progression is further underlined by a number of studies that have used telomere-specific peptide nucleic acid (PNA) probes to investigate DNA breakage in, and adjacent to, telomeres. These studies have demonstrated that telomeric DNA damage can be detected in human blood leukocytes exposed to bleomycin, mitomycin C (MMC) and cisplatin.<sup>22–24</sup> Thus, an insight can be gained into telomere fragility, which is of great importance in aging and malignant transformation of cells. More recent experiments using chromosome band probes have also demonstrated breakage at 5q31 and 11q23 loci in human lymphoblastoid TK6 cells following *in-vitro* exposure to melphalan, etoposide or hydroquinone (HQ), with HQ causing increased damage at 5q31 in particular.<sup>25</sup> Since many malignant cells demonstrate large-scale chromosomal abnormalities, the Comet-FISH assay could prove to be a useful approach for detecting such damage. Moreover, these studies also lend credence to the suggestion that DNA damage is not random throughout the genome and is likely to be affected by nuclear architecture and ultrastructure.<sup>26</sup> Thus, the Comet-FISH assay also shows promise as a valuable technique for investigating the role that higher-order chromatin structure has in influencing the susceptibility of different DNA regions to damage.

Comet-FISH studies are not just restricted to mammalian cells. A study on *Vicia faba* plant cells used DNA probes to specific chromosomal domains, such as FokI element-containing heterochromatin, nucleolus-organising regions (NORs) and telomeres, following treatment of the cells with various endonucleases.<sup>27</sup> This study demonstrated that the distribution of FISH signals between comet head and tail reflected the distribution of restriction endonuclease cleavage sites within these domains and proposed that the technique would allow localisation of various DNA-damage endpoints in genotoxicity studies. Since the Comet assay is already widely used in genotoxicity testing, employing the Comet-FISH assay as well affords the extra opportunity to gain genetic information about DNA damage in specific regions of the genome.

With respect to specific gene loci, many studies have used the Comet-FISH assay to detect DNA damage and repair in response to different DNA-damaging agents. In particular, the TP53 gene has been investigated by several studies, since damage to this crucial gene plays a major role in the development of cancer. Although an early Comet-FISH study showed little damage in the TP53 gene in human lymphocytes exposed to UV-A,<sup>18</sup> subsequent studies have demonstrated that damage to this region occurs in human bladder cancer cells in response to both  $\gamma$ -radiation<sup>15,28</sup> and MMC.<sup>14</sup> Similarly, damage to this gene region has also been detected by the Comet-FISH assay in primary human colon cells and colon adenoma cells following treatment with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), trans-2-hexenal and 4-hydroxy-2-nonenal (HNE).<sup>29–31</sup>

This damage reflects the fact that these toxic compounds are produced *in vivo* via oxidative stress mechanisms, such as lipid peroxidation that produces the compounds trans-2-hexenal and HNE, and may contribute to cancer development by reacting with DNA bases. Moreover, Gleib *et al.*<sup>29</sup> also demonstrated significantly different sensitivities between TP53, APC and KRAS genes in colon cells. In their experiments, dose-dependent migration of hybridisation signals into comet tails was observed for all 3 loci in both primary colon cells and colon adenoma cells in response to H<sub>2</sub>O<sub>2</sub>, trans-2-hexenal and HNE. However, the TP53 region was more sensitive to damage than KRAS, APC and overall DNA in H<sub>2</sub>O<sub>2</sub>-treated primary colon cells and in all cells treated with HNE & trans-2-hexenal, particularly in HNE-treated adenoma cells. Since alterations in APC and KRAS are early events in colon carcinogenesis, they concluded that all three compounds tested could potentially initiate cancer, but only HNE was important for cancer progression.

The Comet-FISH assay has also detected damage to the TP53 gene region in primary human colon cells and colon adenoma cells following treatment with uranyl nitratotriacetate (U-NTA)<sup>32</sup> and ferric nitratotriacetate (Fe-NTA)<sup>33</sup> which emphasised the potentially genotoxic effects of iron and uranium *in vivo* through their interactions with DNA. Damage to both TP53 and HER-2/neu gene regions has been shown in  $\gamma$ -irradiated breast cancer cells,<sup>34</sup> whilst H<sub>2</sub>O<sub>2</sub>-treated human lymphocytes<sup>35</sup> and Fe-NTA-treated human leukocytes also demonstrate damage in TP53 gene.<sup>36</sup> In leukocytes from X-ray-irradiated mice, the Comet-FISH assay has been used to detect damage in TP53, Ret and Abl1,<sup>37</sup> whilst increased susceptibility of the c-myc gene was detected in human lymphocytes exposed to UV-A.<sup>18</sup>

Taken together, these studies demonstrate the ability of the Comet-FISH assay to measure the extent of DNA damage in specific genes and DNA regions, particularly those related to disease progression. It could therefore prove to be a valuable tool in understanding the cellular response to damage and its biological effects and may also prove to be useful in deriving appropriate and effective interventions in disease pathology.

### 6.3.3 Using Comet-FISH to Quantify DNA Repair

DNA repair of specific genes/gene regions can also be measured using the Comet-FISH assay. Fernandez *et al.* performed FISH using a whole genome probe following both neutral and alkaline versions of the Comet assay, calling their technique DNA-breakage detection-FISH (DBD-FISH).<sup>38</sup> They demonstrated that damage and repair of both double-strand breaks and single-strand breaks could be analysed simultaneously in irradiated leukocytes cells by analysis of fluorescence intensity and surface area of each comet and concluded that the use of different probes would allow similar analysis of repair in specific DNA sequences. Indeed, several studies have shown some intriguing results for the TP53 gene. In our laboratory we have demonstrated that the actively

transcribed TP53 gene region in bladder cancer cell lines is preferentially repaired in comparison to the overall genome following treatment with both  $\gamma$ -irradiation<sup>15,28</sup> and MMC.<sup>14</sup> These findings have been corroborated by other studies that have shown that damage in the TP53 gene is repaired more rapidly than total DNA in human lymphocytes treated with H<sub>2</sub>O<sub>2</sub><sup>35</sup> and  $\gamma$ -irradiated breast cancer cell lines.<sup>34</sup> Given these observations, it is tempting to speculate that this preferential repair is a reflection of the occurrence of transcription-coupled repair (TCR) in the cell. Preferential repair has been shown to occur in TP53<sup>39,40</sup> and other transcriptionally active genes<sup>41–44</sup> in comparison to the overall genome in mammalian cells following UV damage using other methods and it is likely a similar repair hierarchy exists in response to other DNA-damaging agents. Indeed, recent investigations in our laboratory have demonstrated that in  $\gamma$ -irradiated normal fibroblasts, the TP53 gene region was preferentially repaired compared to both the transcriptionally inactive hTERT gene region and the overall genome, whereas in TCR-deficient Cockayne syndrome (CS) fibroblasts, this preferential repair was not observed (personal communication).

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### 6.3.4 Summary of Studies

Taken together, the studies above have demonstrated that the Comet-FISH assay can detect DNA damage and repair in a number of genes, gene regions and loci. In theory, any gene could be detected if a suitable probe is available. Along with the assay's relative speed and sensitivity, this means that it has vast potential as a laboratory technique for studying the cellular response to damage, with the added advantage of being able to study specific genes and gene regions of interest, particularly those associated with disease. This also makes it an attractive candidate for use in a clinical setting whereby data can be quickly obtained from patient cell samples, thereby helping to inform the development of treatment that is tailored to the individual needs of the patient. This would prove particularly beneficial in cancer management, where increasing emphasis is being placed on personalised medicine.

However, a recent review of the potential of the Comet assay for use in the management of cancer raised concerns about reproducibility and validation of the assay, uncertainties that would also apply to the Comet-FISH assay.<sup>45</sup> Furthermore, it is worth remembering that the nature of the Comet assay precludes us from stating categorically that damage and repair is occurring within a given gene. Rather, examining the position of each hybridisation spot enables us to conclude whether or not the damage and repair is occurring in the vicinity of the gene of interest, whilst counting changes in spot number allows us to estimate if damage and repair is within the probed region. These are important subtleties to grasp in analysing Comet-FISH data and, as many authors of the studies above have acknowledged, the Comet-FISH assay has certain limitations and therefore caution must be taken in the interpretation of results to ensure the correct conclusions are arrived at.

## 6.4 Limitations of Comet-FISH Assay

### 6.4.1 Practical Difficulties

It is worth stating that the development and optimisation of the Comet-FISH assay has not been without its problems and investigators have encountered several practical difficulties with this technique. *In-situ* hybridisation to cells embedded in agarose is not ideal as the gel matrix can affect the ability of probes to hybridise effectively to their target, especially if hybridisation is reliant on temperatures above which the agarose melts. Although this can be overcome to some extent by dehydrating the agarose prior to hybridisation and by using alkaline denaturation instead, larger probe fragments can still become trapped in the gel matrix itself, resulting in inefficient and/or nonspecific binding. Researchers have also found that standard hybridisation washing steps may not be sufficient to remove all unhybridised probe, whilst excessive washing can result in dislodging the delicate gel from the slide, with the loss of all cells into the wash buffer. For the same reason, use of signal amplification steps is also undesirable as extra washing steps increase the likelihood of gel loss. Although several of the published studies listed in Table 6.2 have used probes that require secondary antibodies, many of them acknowledge the difficulties that this presents in obtaining slides with sufficiently low background for accurate analysis.

It is also worth remembering that many of the fluorescent probes described in the Comet-FISH studies above can only be purchased commercially and are often expensive to buy, which increases the pressure on researchers to generate good-quality comet slides each time for analysis. One alternative is to design specifically labelled probes against coding sequences in the gene under investigation, which has the added advantage of not being restricted to what is commercially available in terms of probe target or probe size. One study has successfully used this approach<sup>35</sup> and it may be a viable alternative for researchers working on a tight budget or where the analysis of large numbers of samples or time points are required.

### 6.4.2 Imaging Difficulties

Even if gels remain intact, microscopic examination can often reveal high background and nonspecific fluorescence that can mask true signals and make accurate analysis difficult. Unfortunately, despite collaborations and dialogue with several software companies, no research group has yet reported the successful use of a reliable software package for accurately counting hybridisation signals from comet slides, preferring instead to manually count signals. As well as being time consuming and laborious, this can also result in user subjectivity, leading to problems with data collection during image analysis. Hybridisation signals close to the head/tail boundary in a comet may be considered by one user to lie in the head, but in the tail by another user. Similarly, two signals close together may be recorded as one signal by some users, perhaps

due to poor eyesight or simple fatigue. Hence, the development of computer imaging technology that is capable of detecting and counting hybridisation spots accurately is a priority if this problem is to be resolved. Once a suitable technology becomes available, this will go a long way to helping to generate data that is free from user bias.

### 6.4.3 Interpretation of Results

In order to interpret Comet-FISH results, it is imperative that there is a clear understanding of what occurs to DNA during the Comet assay process. The essential basis of the Comet assay recalls the experiments of Cook *et al.*<sup>46</sup> who investigated nuclear structure by lysis of cells with nonionic detergent and high-molarity sodium chloride in order to remove the membrane and cytoplasm and disrupt the nucleosomes. This left a nucleoid structure in which negatively supercoiled DNA, RNA and proteins form a nuclear matrix. Cook *et al.* proposed that the DNA was attached at intervals to the matrix in a series of loops, thereby preventing free rotation of the DNA. However, using ionising radiation or the intercalating agent ethidium bromide was enough to damage the DNA and therefore relax the supercoiling in these loops.

The Comet assay works on a similar basis, with the issue of DNA loops and matrix attachment still fundamental to the understanding of the results. Strand break(s) in a loop of DNA will result in that region of DNA being pulled to one side by electrophoresis to form the comet tail. However, if the DNA loop is attached to the nuclear matrix it will reach a point whereby it can stretch no further, unless a further break in this region produces an unattached fragment of DNA. With regard to Comet-FISH experiments, this means that it is not possible to state conclusively that the position of the hybridisation signal in the head or tail shows damage in the probed region. Instead, we can only surmise that a break must have occurred in the vicinity of the probed region, unless the number of spots has increased. A signal may simply be detected in the tail due to electro-stretching effects as a strand of broken DNA migrates into the tail. Likewise, a signal in the head may actually be from damaged DNA but is restricted from migrating due to close attachment to the matrix. Furthermore, an extremely damaged region of DNA may generate fragments that migrate so far from the head that they are not considered part of that cell, or may be so small as to be completely undetectable. Therefore, counting the number of hybridisation signals is crucial as an increase in spot number in cells can only occur from breaks within the probed region. However, this counting must take into account the ploidy of the cells, particularly with regards to cancer cells, and if they are at various stages of the cell cycle, and clear baseline data with regard to hybridisation signal number is also essential.

It is also crucial to take into account the size of the probe used as the probability that a probed region of DNA will contain a strand break will depend to some extent upon how large the targeted area is. For example, if we accept the long-held assumption that 1 Gy of irradiation randomly introduces

0.31 breaks per  $10^9$  Da of cellular DNA,<sup>47</sup> it is clear that a large probe has more likelihood of targeting a region that happens to contain strand breakage than a smaller probe. Added to this, it is clear that there are distinct differences in the sensitivity of different DNA regions to damage, caused by factors such as chromatin structure,<sup>26</sup> DNA repair efficiency<sup>41</sup> and transcriptional activity,<sup>48</sup> therefore DNA damage and strand breakage throughout the genome is unlikely to be absolutely uniform. Furthermore, even if strand breakage does occur, the DNA may be prevented from migrating due to attachment to the nuclear matrix as discussed above, meaning hybridisation signals may remain in the comet head. Hence, it is important to have an appreciation of all these issues with regards to each individual study performed in order to draw the correct conclusions and subsequent implications from the data recorded.

## 6.5 Conclusion

Over the past decade, the Comet-FISH assay has proven a rapid and relatively simple procedure for measuring DNA damage and repair in both gene-specific loci and whole chromosomes in a variety of different cell types. The versatility of the assay means it offers great potential as a method for assessing DNA damage in specific gene regions, as well as the overall genome, in individual cells in response to many damaging agents, with clear implications for both basic science research and clinical application. However, the usefulness of this assay relies heavily on the correct analysis of results and on an accurate understanding of the dynamics of DNA movement under the conditions of the assay. Without due appreciation of these aspects, data generated from this assay can be misinterpreted and unreliable. Hence, there is a clear need for experiments that investigate fundamental issues relating to validation and standardisation of the Comet-FISH assay, ideally by comparison with other techniques used for measuring gene- and region-specific DNA damage and repair and nuclear architecture. Only by carrying out such experiments can the Comet-FISH assay gain widespread acceptance as a valuable and reliable method and thereby deliver on its potential for investigating DNA damage and repair in specific gene regions.

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