Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy 339

Evolving the Methodology for Detection of Primary DNA Damage

Development, adaptation and assessment of the single cell gel electrophoresis (comet) assay

ERIK BIVEHED

ACTA UNIVERSITATIS
UPSALIENSIS
2023

ISSN 1651-6192
ISBN 978-91-513-1889-9
urn:nbn:se:uu:diva-510750

UPPSALA
UNIVERSITET

Dissertation presented at Uppsala University to be publicly examined in B42, Uppsala Biomedical Centre, Husargatan 3, Uppsala, Friday, 20 October 2023 at 09:15 for the degree of Doctor of Philosophy (Faculty of Pharmacy). The examination will be conducted in English. Faculty examiner: Associate professor Hanna Karlsson (Karolinska Institutet, Institute of Environmental Medicine).

Abstract

Bivehed, E. 2023. Evolving the Methodology for Detection of Primary DNA Damage. Development, adaptation and assessment of the single cell gel electrophoresis (comet) assay. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy* 339. 72 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1889-9.

Deoxyribonucleic acid (DNA) is one of the most important molecules in nature. It is the fundamental carrier of evolutionary information and constitutes the genetic blueprint of all living organisms. Being the sole source of information, it is vital for the cell to transmit the correct genetic information from generation to generation. DNA damage is a critical precursor to cancer development, highlighting the need for tests to predict genotoxicity and mutagenicity of various agents, including pharmaceuticals and environmental factors. This thesis focuses on enhancing the single cell gel electrophoresis (comet assay) for assessing primary DNA damage.

The work was concentrated around several fundamental aspects of the methodology where a novel statistical approach, Uppsala Comet Data Analysis Strategy (UCDAS), was developed for data evaluation. A proportional odds model tailored to continuous outcomes was used, accommodating the experimental design's hierarchy, large zero values, and avoiding data transformation. A revisit of the formulation of the electrophoresis medium led to the introduction of a low conductive lithium hydroxide-based solution, enabling higher field strengths, significantly reducing runtimes and increasing sensitivity compared to the conventional comet assay.

A lot of work was done on the investigation of the pH's impact on DNA integrity, revealing elevated background DNA damage at higher pH levels. Extended unwinding at pH >13, typical of the most commonly used versions of alkaline comet assays, jeopardizes the integrity of DNA, resulting in greater background DNA damage than at lower pH values. The study underscores pH's significance for DNA stability, highlighting risks associated with extremely alkaline conditions.

A new method was developed, the Polymerase Assisted DNA Damage Assay (PADDA), to label and quantify single- and double-strand DNA breaks selectively in comet heads and tails after exposure to established DNA-damaging agents. This approach also allowed detection of DNA damage inside comet heads, an ability lacking in traditional comet assays.

In conclusion, this research enhances DNA damage assessment methodologies, introducing new statistical innovations, novel electrophoresis mediums, and a novel technique for the selective detection and quantification of single- and double-strand breaks. These advancements deepen our understanding of DNA damage's complexities and underscore the crucial role of pH in influencing DNA stability and its implications for genotoxicity assessment.

Keywords: Comet assay, DNA damage, DNA strand breaks, Flash-comet, Genotoxicity, In vitro, Single cell gel electrophoresis, Polymerase assisted DNA damage assay (PADDA), TK6 cells, UCDAS, U937 cells

Erik Bivehed, Department of Pharmaceutical Biosciences, Box 591, Uppsala University, SE-75124 Uppsala, Sweden.

© Erik Bivehed 2023

ISSN 1651-6192
ISBN 978-91-513-1889-9
URN urn:nbn:se:uu:diva-510750 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-510750)

The most effective way to do it is to do it.

Amelia Mary Earhart
Aviation pioneer

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Bivehed, E.,** Gustafsson, A., Berglund, A., Hellman, B. (2019) Evaluation of potential DNA-damaging effects of Nitenpyram and Imidacloprid in human U937-cells using a new statistical approach to analyse comet data. *Exposure and Health*, 12:547-554

II **Bivehed, E.,** Söderberg, O., Hellman, B. (2020) Flash-comet: Significantly improved speed and sensitivity of the Comet assay through the introduction of lithium-based solutions and a more gentle lysis. *Mutation Research*, 858-860, 503240

III **Bivehed, E.,** Hellman, B., Söderberg, O., Heldin, J. (2023) Visualizing DNA single- and double-strand breaks in the Flash comet assay by DNA polymerase-assisted end-labelling. *Submitted manuscript.*

IV **Bivehed, E.,** Hellman, B., Fan, Y., Haglöf, J., Buratovic, S. (2023) DNA integrity under alkaline conditions: an investigation of factors affecting the comet assay. *Mutation Research*, 891: 503680

Reprints were made with permission from the respective publisher.

Contents

Introduction 11
  General 11
  Chemical properties of DNA and primary DNA damage 12
  Primary DNA damage 13
  Evaluation of primary DNA damage 14
  The single cell gel electrophoresis (comet) assay 15
    Overview of the experimental method 15
    Statistical evaluation of data 17
  Concerns 18

Aims of the present study 19

Experimental design and methodologies 20
  Preface and project overlapping methods 20
  General experimental procedures 20
    Cell cultures 20
    Precoating of slides 21
    Encapsulation of single cells in gels 21
    Cell lysis 21
    Alkaline unwinding and electrophoresis 21
    Neutralization and fixation 21
    Staining and comet scoring 21
  Experimental summaries of papers 22
    Paper I – Evaluation of potential DNA-damaging effects of nitenpyram and imidacloprid in human U937-cells using a new statistical approach to analyse comet data 22
    Paper II – Flash-comet: Significantly improved speed and sensitivity of the Comet assay through the introduction of lithium-based solutions and a more gentle lysis 23
    Paper III – Visualizing DNA single- and double-strand breaks in the Flash-comet assay by DNA polymerase-assisted end-labelling. 24
    Paper IV – Multifactorial investigation of the DNA integrity under alkaline conditions 27

Results and Discussion 29
  Development of a new statistical method for evaluation of comet data... 29
  Introduction of a low conductivity electrophoresis medium 35

Staining or labelling of the DNA? Development of the Polymerase Assisted DNA Damage Assay (PADDA) 38
The impact of altered pH on the DNA migration in the comet assay 44
Author remarks and future perspectives....55

Conclusions....57
Summary 57
Specific conclusions....57

Populärvetenskaplig sammanfattning 59

Acknowledgements....61

References....63

Abbreviations

8-oxoGua	8-oxo-7,8-dihydroguanine
A	Adenine
ALS	Alkali labile sites
ANOVA	Analysis of variance
AP-site	Apurinic or apyrimidinic site
B2M	Human β_2-microglobulin
C	Cytosine
CI	Confidence interval
CO_2	Carbon dioxide
DAPI	4′,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPI	Deoxyribonucleic acid polymerase I
ds	Double-stranded
DSBs	Double-strand breaks
EDTA	Ethylenediaminetetraacetic acid
Endo III	Endonuclease III
FAM	Fluorescein amidite
FBS	Fetal bovine serum
Fpg	Formamidopyrimidine DNA-glycosylase
G	Guanine
Gy	Gray
H_2O_2	Hydrogen peroxide
K	Potassium
KCl	Potassium chloride
LC-MS	Liquid chromatography-mass spectrometry
Li	Lithium
LiOH	Lithium hydroxide
LM	Low-melting
mA	Milliampere
$MgCl_2$	Magnesium chloride
MIRCA	Minimum information for reporting on the comet assay
mRNA	Messenger ribonucleic acid
Na	Sodium
Na_2EDTA	Disodium ethylenediaminetetraacetic acid
Na_2HPO_4	Disodium phosphate

NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
nt	Nucleotide
OECD	Organization for economic co-operation and development
PADDA	Polymerase assisted DNA damage assay
PBS	Phosphate-buffered saline
RADD	Repair assisted damage detection
RFU	Relative fluorescence units
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
RT-qPCR	Real-time quantitative polymerase chain reaction
S9	Post mitochondrial supernatant from human liver homogenate
SCGE	Single cell gel electrophoresis
SE	Standard error
SEM	Standard error of mean
ss	Single-stranded
SSBs	Single-strand breaks
T	Thymine
TBS	Tris-buffered saline
TDNA	Tail deoxyribonucleic acid intensity
TdT	Terminal deoxynucleotidyl transferase
T_m	Melting temperature
Tris	Tris(hydroxymethyl)aminomethane
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
U	Uridine
UCDAS	Uppsala comet data analysis strategy
UHPLC-UV	Ultra-high performance liquid chromatography-ultraviolet detection
UNG	Uracil-DNA glycosylase
V	Volt

Introduction

General

Deoxyribonucleic acid (DNA), is probably one of the most important molecules in nature. It is the fundamental carrier of evolutionary information and constitutes the genetic blueprint of all living organisms (1). It defines every operation of the cell, and the precise readout of the DNA sequence details the production of a messenger ribonucleic acid (mRNA), thereby defining the protein production and ultimately the function of the cell (2). Being the sole source of information, it is vital for the cell to carry the correct genetic information from generation to generation. During cell replication (mitosis), DNA has to be faultlessly replicated in order to produce genetically identical daughter cells. However, the DNA is constantly barraged by compounds that may inflict damages on the DNA. The body and the cell have several ways of defending the DNA from this constant attack, mainly by several well-preserved and high-fidelity repair mechanisms (3,4).

Even with fully functioning defenses the cell only has one set of DNA, and with this in mind, it is not surprising that even a small chemical modification of the DNA might result in devastating biological consequences.

The relentless bombardment of DNA damaging compounds is associated with mutagenic and carcinogenic events. That if they are left unchanged may lead to several adverse outcomes including cell death, inhibited mitosis and altered gene expression, ultimately leading to tumor development and carcinogenesis (5). Therefore, much efforts are now invested into the investigation and evaluation of the potential genotoxicity of chemicals in order to ensure safe pharmaceutical drugs as well as removing various harmful types of environmental, occupational and dietary substances from our everyday life.

Chemical properties of DNA and primary DNA damage

The chemical composition of the DNA is based on a phosphorylated deoxyribose sugar coupled to one of the four nucleobases, adenine (A), cytosine (C), guanine (G) and thymine (T). This unit of a sugar-phosphate and a nucleobase is known as a nucleotide (nt) and the genetic information is stored in the order of how the nucleotides are linked in the polymer chain. In eukaryotic cells, DNA is most prevalently found as a double-stranded helix where two separate DNA strands come together and bind in the opposite direction to each other. One goes from the 5' to 3' end, the other goes from the 3' to 5' end (Figure 1). The formation of the DNA helix is guided by the Watson and Crick base paring were the nucleobases either form two (A-T) or three (C-G) hydrogen bonds between each other. The base paired nucleotides are located in the center of the helix, and the phosphate groups form the outer surface.

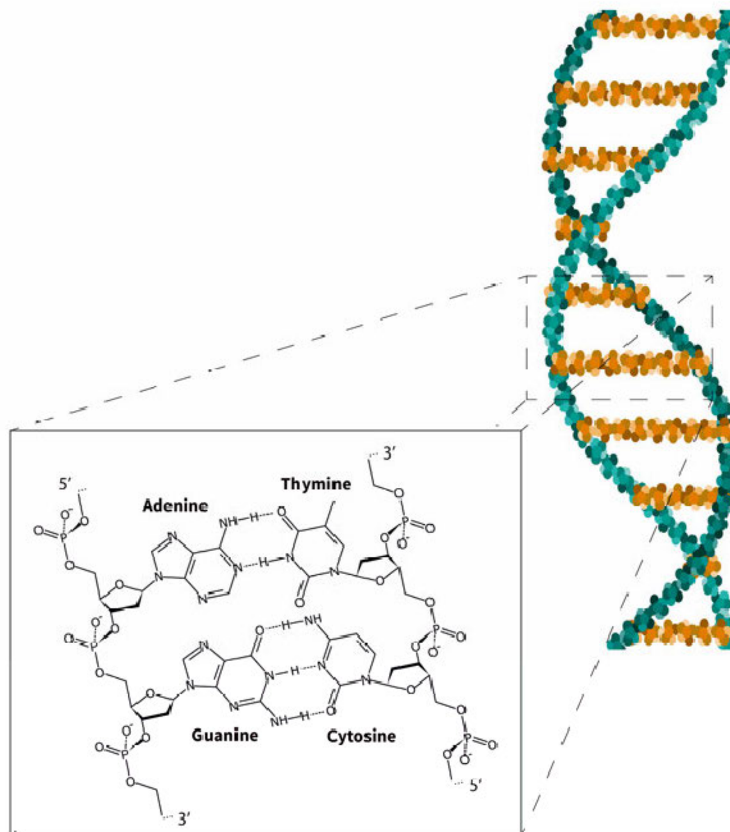

Figure 1. Molecular structure of DNA. Illustration by E. Bivehed

Due to the negative net charge of the phosphate groups, the DNA surface is negative which makes it quite hydrophilic and highly soluble in water (6). In contrast to the surface, the center of the helix is more hydrophobic with a high abundance of van der Waals interactions between the stacked nucleotides (6).

Primary DNA damage

Biological active chemical compounds such as drugs and genotoxins can cause base alterations in the DNA. The vast majority of these compounds mediate their effect through two major reaction categories: alkylation by electrophilic chemicals (e.g., nitrogen mustards such as chlorambucil and cyclophosphamide) or radical intermediates (e.g., reactive-oxygen species [ROS] generated both endogenously and from the reactions of chemicals like hydrogen peroxide) (7).

Alkylating agents are a diverse group of chemical compounds that can add alkyl groups to almost all heteroatoms in the DNA forming DNA adducts. The type and site of formed adduct is highly dependent on the structure (mono- or bifunctional, i.e., one or two electrophilic centers), reactivity (hard or soft reactivity) and steric elements of the agent in question (8-11). The nature of the various nucleophilic sites in DNA also affects the alkylation reaction and the most favored sites for alkylation in DNA are the ring nitrogens N7 and N3 of guanine and N3 of adenine (8,10). The direct effect of covalent binding of the alkylating agent to the DNA is destabilization of the nucleobase, resulting in ring-opening, deglycosylation and finally base loss. The loss of a base from the DNA leaves an apurinic or apyrimidinic site (AP-site), which is a possible site for mutation due to the incorrect incorporation of a new base to the DNA (12).

DNA damage caused by radical intermediates can be the result of exogenous chemical reactions with DNA-like copper-phenanthroline complexes and some chemotherapeutic agents (13-15). However, radical intermediates, like ROS, are constantly generated through endogenous sources in the cell as a result of respiration and metabolism. Oxidation of the DNA may directly result in DNA strand cleavage and oxidized nucleobases can, if left unrepaired, lead to mutations primarily through the formation of 8-hydroxyguanin, with subsequent G-T and C-A substitution (16-18).

Apart from the above-mentioned major reaction pathways, there are also other ways the DNA can be damaged. Bifunctional alkylating agents (e.g., chlorambucil) also have the possibility to covalently bind a second base thus crosslinking two strands, or to a protein. Further, hydrophobic heterocyclic ring structures (e.g., ethidium bromide), resembles the structure of the base pairs and can be inserted within double-stranded DNA (dsDNA) by noncovalent intercalation (19). An intercalating agent act as a steric hinder and cause

distortion of the DNA double helix thus interfering with enzymatic processes such as DNA replication, repair and transcription (20).

Another well-known DNA damaging phenomenon, that is unrelated to chemical reactions, is ionizing radiation. High energy electromagnetic waves (γ- and x-rays) and/or particles (α- and β-decays) are well-known DNA damaging agents and are frequently used for treatment of several types of cancers. When the radiation hits DNA it causes ionization within the molecular structure, resulting in disruption of the molecular bonds within and between the molecules. This disruption causes a diverse damage spectrum, including the generation of ROS and molecular bond breakage, which ultimately may lead to single-strand breaks (SSBs) with subsequent strand breaks in the DNA (21-24).

Primary DNA damage, regardless of type and reaction category, can be transformed into single- or double-strand breaks, either directly or by the cell's DNA repair systems. The origin of DNA strand breaks can also be caused by natural and frequent endogenous processes in the cell, such as replication, transcription, reparation and recombination (25,26). However, the biological consequences may not be that adverse for the cell. Single-strand breaks are usually easily and correctly repaired, because the analogous strand can be used as template. Double-strand breaks (DSBs) are much more problematic for the cell and often lead to adverse events, such as gene translocations, rearrangements and deletions that can spark tumor development (27). However, one prerequisite for tumor development is that the cell is actively dividing and not terminally differentiated.

Evaluation of primary DNA damage

Methods that efficiently detect DNA damage, such as single-stranded DNA (ssDNA) breaks, double-stranded DNA (dsDNA) breaks, or damaged bases, are necessary to determine the consequences of DNA-damaging agents, as well as the subsequent repair.

There are several methods available that efficiently can detect and pinpoint different DNA damages, such as single-strand breaks (SSBs), double-strand breaks (DSBs), or damaged bases, insertions, deletions and translocations. Several sequencing-based assays have been developed (28-30) providing detailed genetic information down to single nucleotide level and liquid chromatography coupled to mass spectrometry methods (31,32) can reveal molecular alterations in DNA down to atomic levels (33). Although powerful, these techniques come with significant drawbacks that restrict their use. From a structural/chemical point of view, they require extensive manipulation of the DNA through extraction like sonication, heating and denaturation and in case of liquid chromatography-mass spectrometry (LC-MS), labeling with isotopes, all of which may introduce additional DNA damage. Another limiting factor is

the requirement of expensive equipment, trained expert users and advanced data handling and processing.

There are however more accessible methods to detect DNA damage either based on antibodies, like the γH2AX assay (34) or enzyme based (TUNEL or RADD) (35,36) detection of specific DNA adducts or strand break. However, these come with other disadvantages, antibody and enzymatic strategies are restricted by their specificities to particular epitopes or templates and only detect and provide information regarding specific DNA damages. Since many of the acquired DNA damages are converted to strand breaks, either as a direct effect or as a consequence via the repair machinery, a technique like the single cell gel electrophoresis (i.e., the comet assay) makes it possible to get a gross estimate of strand breaks on a single cell level in a rather straightforward and simple way.

The single cell gel electrophoresis (comet) assay

Overview of the experimental method

In Uppsala during the late 1970s and early 80s, Rydberg, Östling and Johanson lay the foundation for what later would become the single cell gel electrophoresis assay, nowadays more commonly known as the comet assay. They encapsulated single cells in thin agarose gels and showed that the DNA of γ-irradiated cells started to diffuse out in the gel following lysis of the cells, while unirradiated DNA remained stationary in the nucleoid. Later they refined the method by submerging the gels in an electrophoresis solution and performed electrophoretic separation of the DNA (37-39). A few years later, Singh et al. and Olive et al. independently improved the method by introducing strong alkaline conditions (pH >13) (40,41). The alkaline unwinding and alkaline electrophoresis solution increased the sensitivity of the assay by further promoting unwinding and relaxation of the supercoiled DNA (40,42-44).

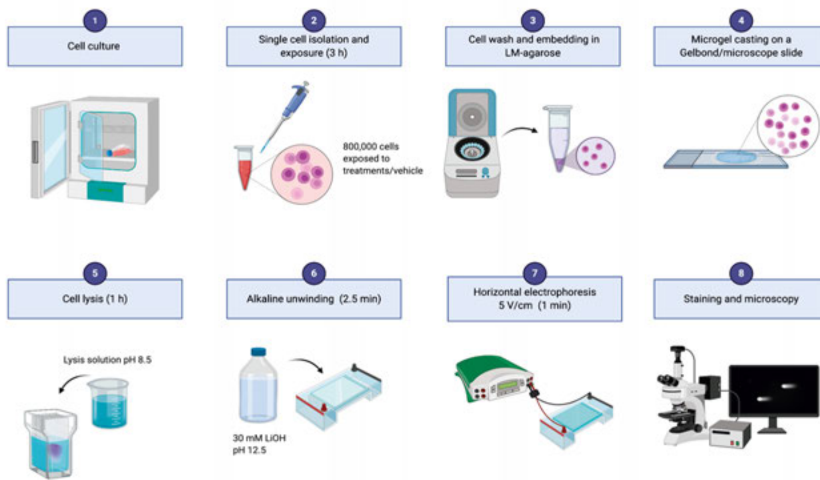

Figure 2. The eight experimental steps of a Flash-comet assay experiment. (1) Establishment of stable cell line culture or primary cell culture. (2) Single cell isolation and treatment of cells with xenobiotics and positive controls. (3) Treatment termination through cell wash with subsequent embedding of single cells in low-melting point (LM) agarose. (4) Casting of a thin agarose gel containing cells. (5) Lysis of cells using detergents and highly concentrated salt solution. (6) Alkaline unwinding allowing relaxation of the DNA structure, liberating DNA-loops and fragments. (7) Electrophoresis using an alkaline electrophoresis medium. (8) Staining and image analysis. Illustration by E. Bivehed.

The basic principle of the comet assay under alkaline conditions is to monitor the migration distance of negatively charged DNA loops moving through a sieving matrix when an electrical gradient is applied (45). The typical outline (Figure 2) of the comet assay includes exposure of cells, either when they are embedded in agarose, or when the cells are dispersed in medium or in culture. Next, a lysis step is performed with detergents and high molarity salt solution in order to obtain nucleoids. The gels are then subjected to alkaline unwinding that enhances the unwinding of DNA and convert apurinic/apyrimidinic sites (AP-sites) into strand breaks that further promotes DNA relaxation and allows more readily migration of the DNA out in the gel (40,45-47). Following the unwinding step, electrophoresis is conducted, through which an electric field is applied. Since DNA is predominately negatively charged, the unwinded and relaxed DNA will migrate towards the positively charged anode (48). The principle underlying the comet assay is that damaged DNA migrates towards the anode to a greater extent than undamaged DNA, thereby producing a comet-like appearance when the electrophoresed DNA is stained with a

fluorescent dye (Figure 3), hence the name comet assay. The level of DNA damage can be assessed by measuring several different parameters such as the tail length, tail moment or the tail intensity (45,46,49-53).

During the 90s, Collins et al. showed that by including an enzymatic digestion step with bacterial endonucleases the specificity of the method was highly improved. Incubation with endonuclease III (Endo III) recognizes oxidized pyrimidines including oxidized thymine and uracil (54), and by incubating with formamidopyrimidine DNA-glycosylase (Fpg) it is possible to detect and visualize oxidized purines, such as 8-oxo-7,8-dihydroguanine (8-oxoGua) (55,56).

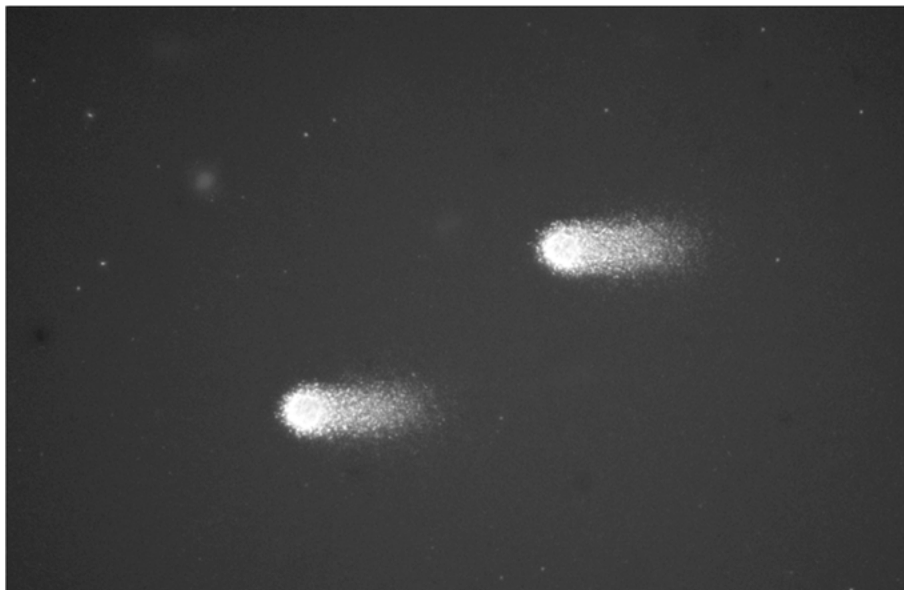

Figure 3. Visualization of DNA damage in TK6 cells stained with SYBR™ Gold, displaying the typical comet-like appearance following the electrophoresis.

Statistical evaluation of data

The statistical evaluation of comet data is quite challenging. The distribution of comet data is usually highly skewed and often contains many zero values (representing undamaged cells or comets without tails). Therefore, a typical comet data set does not conform to parametric distributions even after data transformation (57). Over the years there have been much work done to address these issues and several strategies have been put forward on how the statistical analysis of comet data should be conducted (58-62). As an example, in 2011 Bright et al. published a set of recommendations of how to do data analysis (58). They recommended that the best way to handle the skewness of the comet data was to log-transform and then aggregate the data on culture level before analyzing the data set using one-way ANOVA. However, this

suggestion assumes that the distribution after data transformation follows parametric distribution which is not always the case (63). Nor does the approach suggested by Bright et al. take the hierarchical nature of the data into account.

Concerns

The standard alkaline version of the comet assay that was introduced by Singh et al. is beyond any doubt the most widely cited protocol for performing the alkaline comet assay (40). The article has been cited over 8,600 times (Scopus, July 2023). Additionally, the in vivo version of the comet assay has been adopted into the Organization for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals (64). The method has been widely used for more than 35 years for genotoxicological assessments (65). However, it should be noted that there is still not a standardized protocol and the method is often highly criticized for its high inter-laboratory variation and lack of reliability (52). A fact that can be ascribed to the numerous modifications available of the protocol (42,48,50,66-89) affecting virtually all steps of the assay.

Some of the most common variations include: varying slide preparations using either low-melting point and/or normal-melting agarose, different concentrations of agarose (e.g., 0.5-1 %), different embedding of cells in single or double layer of agarose, different compositions of lysis solutions (e.g., varying concentrations or the inclusion or exclusion of components), different duration times of lysis, unwinding, and electrophoresis steps, different voltage gradients during electrophoresis, preservation of slides by air drying or fixation using ethanol, rehydration and staining, type of stains (ethidium bromide (40,90), acridine orange (38), propidium iodide (91), DAPI (90), SYBR™ Green (92), SYBR™ Gold (93), YOYO™-1 (90,94) or silver nitrate (95)). Different analysis strategies (type of image software and type of comet scoring) have also been used, as well as different parameters recorded (% tail DNA, tail moment, tail inertia, tail length or visual scoring), different ways of presenting data (mean or median values), different aggregation levels (experimental, slide or cell level) and type of statistical strategies for analyzing the result (T-test, one-way ANOVA, Kolmogorov–Smirnov, generalized linear model etc.). Moreover, to this day the comet assay is not able to discern what type of strand breaks that have occurred meaning that the method only provides a gross estimate of a mixture of single- and double-strand breaks.

Several publications have recently put forward consensus statements in order to recommend how to conduct, analyze and report assay conditions and results (50,52,81). Unfortunately, the problem of high inter-experimental variation or reliability of the assay remains, since the authors decline to provide a unified fundamental protocol and thus still allow numerous protocols to be used.

Aims of the present study

The present research project was focused on some of the fundamental aspects of the single cell gel electrophoresis methodology, hopefully contributing to further development and use of this technique for the assessment of primary DNA damage.

The specific aims of the included papers in my thesis were:

I To apply and implement a novel statistical methodology to analyze comet data.

II To evaluate and compare an alternative electrophoresis medium with a lower conductivity but preserved alkalinity.

III To develop a new method for the simultaneously detection of single- and double-strand breaks in the comet tail.

IV To evaluate three comet protocols in order to investigate which influence alkaline pH actually has on the integrity of DNA.

Experimental design and methodologies

Preface and project overlapping methods

The overall aim of this thesis was to develop and adapt the single cell gel electrophoresis technique using the general experimental outline for the comet assay (37,38,40,48,50) with some variations regarding the exposure and experimental conditions. In order to avoid unnecessary repetition and to be able to highlight specific modifications of the technique, a general experimental description of the comet assay under the most commonly used conditions (pH >13 during the unwinding and electrophoresis) will first be provided together with the cell culturing procedure used in all projects. New modifications of the single cell gel electrophoresis procedure suggested in our publications will be highlighted under respective project (paper), together with the additional materials and methods used.

General experimental procedures

Cell cultures

Two different cell lines have been used in this work; U937 (Paper I) and TK6 (Paper II-IV).

The human histiocytic lymphoma (U937) cells were cultivated in RPMI 1640 medium (Fisher Scientific, UK) complemented with 10 % fetal bovine serum (FBS; Biological Industries, Israel), 1 % Gibco™ Pen Strep (10,000 U penicillin/ml and 10,000 µg/ml streptomycin; Fisher Scientific, UK), and 1 % sodium pyruvate (Fisher Scientific, UK); hereafter referred to as complete U937 growth medium.

The human lymphoblastoid TK6 cells were cultivated in RPMI 1640 medium (Fisher Scientific, UK) supplemented with 10 % FBS (Biological Industries, Israel), 1 % Gibco™ Pen Strep (10,000 U penicillin/ml and 10,000 µg/ml streptomycin; Fisher Scientific, UK); hereafter referred to as complete TK6 growth medium.

In short, a working culture was established by carefully thawing an ampoule of frozen cells ($1x10^6$ cells) at 37 °C, and the cells were then washed with and resuspended in 20 ml complete growth medium (U937 or TK6). The cells were maintained in suspension in 75 cm^2 cultivation flask in a 37 °C

atmosphere with 5 % CO_2 until the experiments were performed on the cells on cultivation day 7.

Precoating of slides

Ordinary microscope slides were either precoated with 0.6 % (w/v) low-melting point agarose in ultra-pure water (100 µl) or with Gelbond™ film. The coated slides were allowed to dry at room temperature and protected from dust and other particles.

Encapsulation of single cells in gels

An aliquot of 30 µl of a freshly prepared single cell suspension was mixed with 210 µl melted 0.6 % (w/v) low-melting point agarose in PBS (pH 7.4). The cell/agarose mixture (60 µl) was layered on top of a pre-coated microscope slide and covered with a coverslip and allowed to solidify at 4 °C.

Cell lysis

After the agarose had set, the coverslip was gently removed and the slide carefully immersed in a freshly prepared lysis solution.

Alkaline unwinding and electrophoresis

The slides were then transferred to a horizontal electrophoresis tank filled with 1,700 ml of fresh alkaline electrophoresis solution completely submerging the slides. The slides were left in the alkaline solution for 40 minutes to allow for unwinding of the DNA. After the unwinding, the electrophoresis was conducted for 10-20 minutes.

Neutralization and fixation

The slides were then washed with neutralizing solution (0.4 M Tris; pH 7.5) for 15 minutes to reduce the alkalinity. After neutralization, the gels were desalted and DNA was fixated by sequential dehydration with ethanol (70:95:100 %; 2 minutes/step). The slides were allowed to dry at room temperature in a hood, and then stored in a sealed box until image analysis. All steps following lysis were conducted in a light protected environment to prevent additional DNA damage.

Staining and comet scoring

On the day of image analysis, the slides were rehydrated in neutralization solution (0.4 M Tris; pH 7.5) for approximately 10 seconds/slide. After

rehydration, the slides were stained with a fluorescent DNA binding dye (Propidium iodide, Hoechst, Acridine orange, or SYBR™ Gold). The slides were examined using a fluorescence microscope (Olympus BX60F-3, Olympus Optical, Japan). Digitized images (50 randomly selected nuclei per slide; employing three slides per treatment and culture) were scored using an ATV FireWire camera (Stingray; Allied Technologies, Germany) and the software Comet Assay IV (Perspective Instruments, UK).

Experimental summaries of papers

Paper I – Evaluation of potential DNA-damaging effects of nitenpyram and imidacloprid in human U937-cells using a new statistical approach to analyse comet data

This project was the first project during my thesis work and not designed nor intended to be a methodological investigation of the comet assay. It was primarily designed to investigate the potential genotoxicity of the neonicotinoids imidacloprid and nitenpyram, with or without the addition of a biotransformation system (S9-fraction). During this project it became evident that the alkaline version of the comet assay we used could have some drawbacks, and this insight prompted the following three projects presented in Paper II, III and IV.

In Paper I we used the standard protocol for the alkaline comet assay (as outlined above) that had been used in our laboratory for many years. What we additionally included was a metabolic activation system referred to as the S9-mixture (79). The S in S9 stands for supernatant fraction and the 9 is the fraction number after centrifugation a liver homogenate (Figure 4). The most commonly used version of S9 is the so-called "induced S9-fraction" obtained from rat or mouse that have been treated with strong cytochrome P450-inducing agents such as Aroclor 1254 or phenobarbital/5,6-benzoflavone (96). The reason for using an induced S9-fraction is to maximize the amount of metabolic enzymes. However, an issue with induced S9 is that it has poor metabolic resembling to the situation in the human liver, increasing the risk for obtaining false positive (or false negative) responses when identifying genotoxic (potential carcinogenic) agents. Therefore, we decided to use a pooled human S9 to keep the metabolic profile as close to the human situation as possible.

One issue we had during this project was how to handle the comet data. It is notoriously known to be highly skewed and rarely conform to a normal distribution. The whole method follows a hierarchical and nested structure, making the statistical evaluation a challenge. There have been many attempts to solve this problem and our laboratory has adopted several different statistical approaches (57-63,97). However, this time we had the opportunity to

collaborate with a statistician and together we developed a new statistical approach, which we have named Uppsala Comet Data Analysis Strategy (UCDAS), a proportional odds model tailored to continued outcomes.

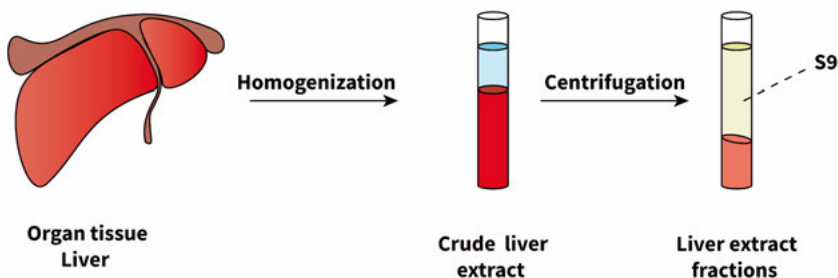

Figure 4. Preparation of metabolic activation system S9. Illustration by E. Bivehed.

Paper II – Flash-comet: Significantly improved speed and sensitivity of the Comet assay through the introduction of lithium-based solutions and a more gentle lysis

When we started to work on refining the comet assay it struck us that no one had ever looked into alternative electrophoresis solutions. Using a sodium hydroxide solution at sub-molar concentrations as electrophoresis medium is a rather crude way of conducting DNA separation. The major reason is that this separation is limited to relatively low field strengths which requires longer runtimes, increasing the risk for an uneven separation of DNA during the electrophoresis. Therefore, we decided to investigate a new alternative electrophoresis solution allowing for much higher field strengths. The aim was then to lower the runtimes for unwinding and electrophoresis and hopefully then also provide less assay-to-assay variations.

In the project we (*i*) formulated a new alkaline electrophoresis solution and (*ii*) performed an investigation of how pH impacts the stability of DNA. The new protocol, which we called the Flash-comet, has basically the same experimental steps as the standard protocol outlined above, but with some important specific modifications. After exposure, the cells were washed with a mild alkaline PBS solution (pH 8.5) at 4 °C. The cell lysis was modified by adjusting the pH to 8.5 together with a reduction of the concentration of DMSO from 10 to 5 %. The most prominent changes were that alkali metal ion was changed from sodium to lithium, and that Na_2-EDTA was omitted from the solution used for unwinding and electrophoresis. The new solution consisted of 30 mM lithium hydroxide (LiOH) with a pH of 12.5. The alkaline

unwinding was allowed for 2.5 minutes and the electrophoresis was conducted at a field strength of 5 V/cm for 1 minute.

Since we knew that our new electrophoresis solution was much more diluted and had a pH of 12.5 instead of >13 in the conventional sodium hydroxide (NaOH)/EDTA solution, we were unsure if it was able to promote for unwinding and relaxation of the DNA. Apart from comparing the two solutions on the same sample sets we also designed a molecular beacon experiment utilizing two probes, a self-quenched double-stranded DNA tagged with a FAM-fluorophore and a non-quenched single-stranded DNA probe that allowed us to investigate the unwinding capacity by monitoring the change in melting temperature of the probes when subjected to solutions with increasing pH (Figure 5).

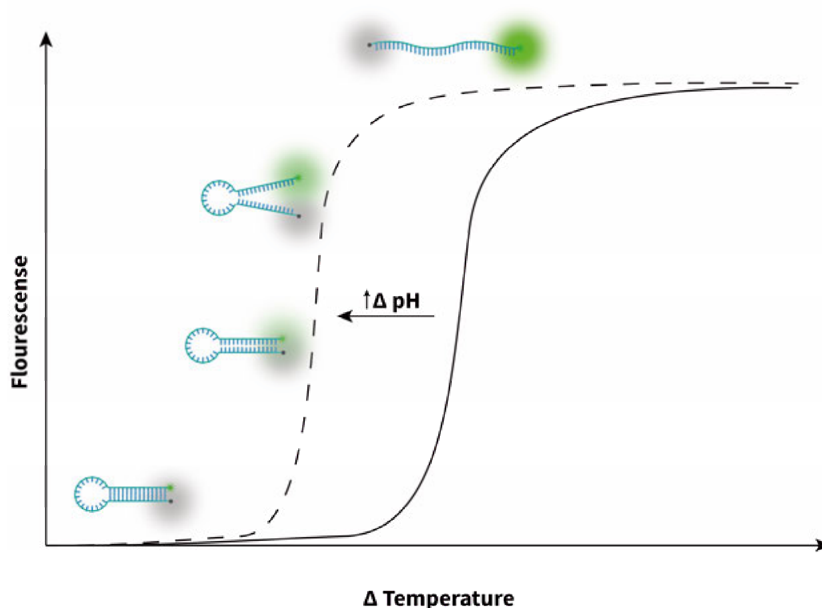

Figure 5. Outline of the molecular beacon experiment. The melting curve of a double-stranded DNA is monitored by increase in fluorescence. When the probe is subjected to increasing alkalinity a lowering of the melting temperature is seen, due to the destabilization of the Watson-Crick hydrogen bonds connecting the nucleobases. Illustration by E. Bivehed.

Paper III – Visualizing DNA single- and double-strand breaks in the Flash-comet assay by DNA polymerase-assisted end-labelling.

One of the major drawbacks of the conventional comet assay has been that it is only able to provide a gross estimate of strand breaks, meaning that it is unable to accurately distinguish between the less detrimental single-strand

breaks (SSBs) and more toxic double-strand breaks (DSBs). This has previously been suggested to be accomplished by performing a 2D-comet assay or by using DNA stains that have different spectral properties. However, both suggestions are based on misguided ideas (98). The 2D-comet builds on the notion that only DSBs are revealed if it is conducted under neutral conditions. This notion has been disproven by others (43-45). Regardless of methodology, both SSBs and DSBs will be present in the comets. The staining approach is only providing information of how the topology of DNA looks like and this strategy only provides information about the structure of the DNA in the comet and no information about the breaks.

Instead, we designed and developed a new procedure for selective and simultaneous detection of single- and double-strand breaks using the comet assay. Our new approach, which we call the Polymerase Assisted DNA Damage Assay (PADDA), is based on labeling of the DNA rather than staining it. Utilizing the selective recognitions and labeling with fluorescently labeled nucleotides of single-strand breaks by DNA polymerase I (DPI) and double-strand break by Terminal deoxynucleotidyl transferase (TdT). The strategy for the PADDA is described in Figure 6.

The method was evaluated using three different types of exposures; (*i*) chemically-induced damages by hydrogen peroxide, (*ii*) physically-induced damages by ionizing radiation (X-rays) and (*iii*) biologically-induced damages by selective induction of strand breaks via cutting the DNA using a nickase (Nt.BsmAI).

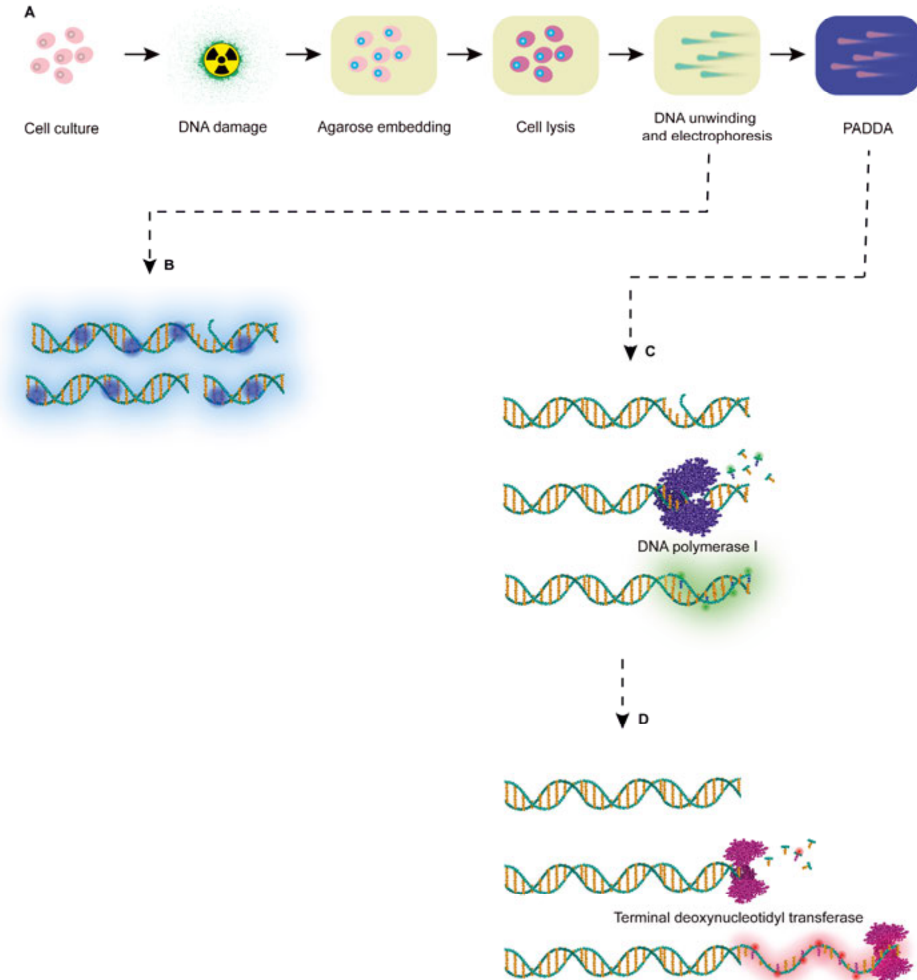

Figure 6. A: A general overview of how the Flash-comet protocol is combined with the PADDA-technique. B: When comets are stained in a conventional comet assay, fluorescent stains such as Hoechst 33342 or SYBR™ Gold are used for a global staining of DNA, not allowing any discrimination between DNA single strand breaks (SSBs) and double-strand breaks (DSBs). C and D: SSBs and DSBs are selectively labelled in two steps using the PADDA protocol. In the first step (C), SSBs are recognized by DNA polymerase I (DPI) which starts a template dependent polymerization of new fluorescently labelled nucleotides (fluorophores). This elongation will be terminated by a DSB. As a consequence of this, DPI will blunt DSBs and thus prime the DSBs for the second step in the PADDA (D). The latter step is mediated by the template independent elongation of free 3'OH-groups by terminal deoxynucleotidyl transferase (TdT), which incorporates another fluorophore than the one used in step one, during the elongation in the second step. Illustration by E. Bivehed.

Paper IV – Multifactorial investigation of the DNA integrity under alkaline conditions

During the development of the Flash-comet and the PADDA, we made several observations indicating that the alterations of the pH in the assay had a major effect on the outcome. These observations indicated that DNA damages were induced during the various steps of the comet protocol. Since the PADDA method now allowed us to selectively analyze the abundance of single- and double-strand breaks we became surprised to see that there was a relative high abundance of strand breaks in the non-treated control cells. This gave us the idea that the strong alkaline treatment during the comet assay actually induced more strand breaks than initially thought. Therefore, we designed a study to compare the pH effect on the DNA On the whole genome level by comparing the two most widely used protocols, the alkaline and neutral versions together with our Flash-comet using non-irradiated TK6 cells. On the gene level where we extracted the DNA from the same batch of TK6 cells that were used in the comet experiments. Pooled fractions of extracted DNA were treated with the alkaline electrophoresis solutions or buffers (pH 10 and 7) that were used in the different comet protocols. Following the alkaline treatment, the amplification efficiency of the human β_2-microglobulin (B2M) gene was evaluated by real-time quantitative polymerase chain reaction (RT-qPCR).

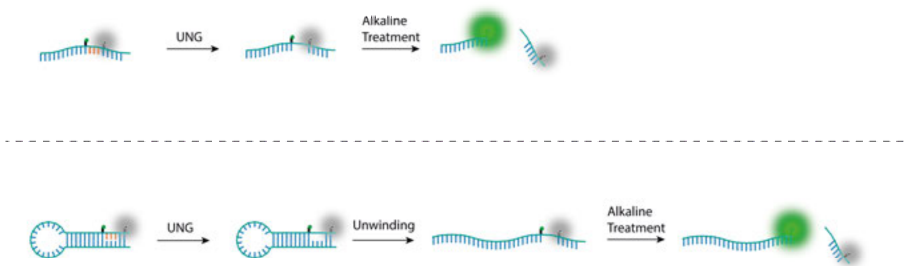

Figure 7. Outline of the molecular beacon experiment. The pH dependent conversion of a alkali labile site into a strand cleavage is monitored by increase in fluorescence as a consequence of the release of the quencher. Illustration by E. Bivehed.

One hypothesis was that the strong sodium hydroxide (NaOH) treatment during the conventional alkaline comet assay would not only cleave the alkali labile sites (ALS) but also disintegrate the DNA in an uncontrollable way. In an effort to investigate if this occurred, we set up an experiment at the DNA-molecular level, using a newly designed molecular beacon with a triplet of uracils in the sequence flanked by an internal FAM-fluorophore and a quencher at the 5´-end. By using the enzyme uracil-DNA glycosylase (UNG) we could then selectively remove the uracils to create an ALS-region within the beacon, As shown in Figure 7. The intact beacon and ALS-containing

beacon were then subjected to solutions with increasing pH, and the degree of strand cleavage was measured as a relative increase in fluorescence. It should be pointed out that this experiment was built on the assumption that the fragmentation of the strand resulted in a release of the quencher from the beacon. There could of course be other possible fragmentation patterns that would not result in the release of the quencher. Since DNA absorbs at 260 nm, we also monitored the reaction by using liquid chromatography with UV-detection (UHPLC-UV).

Results and Discussion

Development of a new statistical method for evaluation of comet data

When looking at the design and experimental outline of an in vitro comet assay experiment (Figure 8), it becomes evident that it resembles the Russian Matryoshka dolls. The hierarchical structure of the experiment is comprised of experimental units containing a chain of subunits, i.e., nuclei/comet within a gel, gels within slides, slides within treatments/exposures that are nested within cultures/samples, thus creating a highly clustered system.

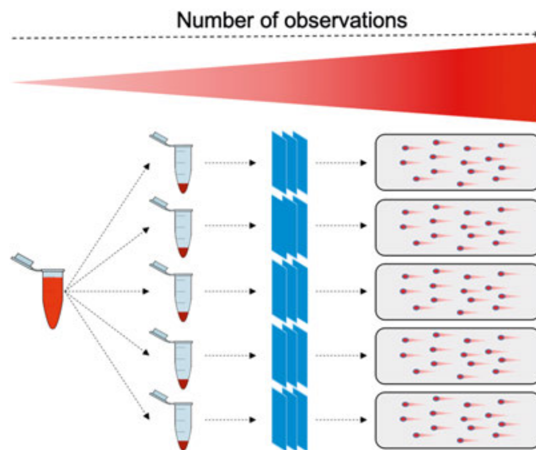

Figure 8. General outline and display of the hierarchical structure of a comet assay experiment. The experimental structure can be broken down into separate clusters with increasing number of observations. Culture (n=3), treatment cluster (n=4-7), slide cluster (n=9) and comet/cell cluster (n=450). Illustration by E. Bivehed.

Apart from the clustered design, the distribution of the experimental data is also frequently highly skewed and often include high levels of zero values (i.e., cells with low or no DNA damage resulting in comets without any tail) and also some extreme values, meaning cells with high DNA damage resulting in comets dominated by the tail (Figure 9).

Over the years there have been much work done to address these issues and several strategies for how to statistically analyze comet data have been

suggested (58-63,97). Still, the dominating statistical approach to analyze comet data is to use a one-way analysis of variance (ANOVA). However, this strategy has some crucial drawbacks: ANOVA is a parametric test requiring data with normal distribution, without extreme values and it cannot adjust for the hierarchical structure in a typical in vitro comet assay evaluating the potential genotoxicity of xenobiotics.

When comparing the requirements for the parametric statistic tests (ANOVA and T-test) and the design of the comet assay, it becomes obvious that such statistical approaches may not be the best choices when it comes to the evaluation of comet data. However, there are ways to improve the data to conform with parametric analyses, and one such trick is to log-transform the data, but this comes at a price. Since it is not possible to log-transform a zero value, you have to infer a small constant to the dataset, which consequently alter the original data as shown by Bright et al. 2011 (58). Moreover, the process of transformation still assumes that the data distribution post-transformation aligns with a normal distribution. Unfortunately this is not always the case (63).

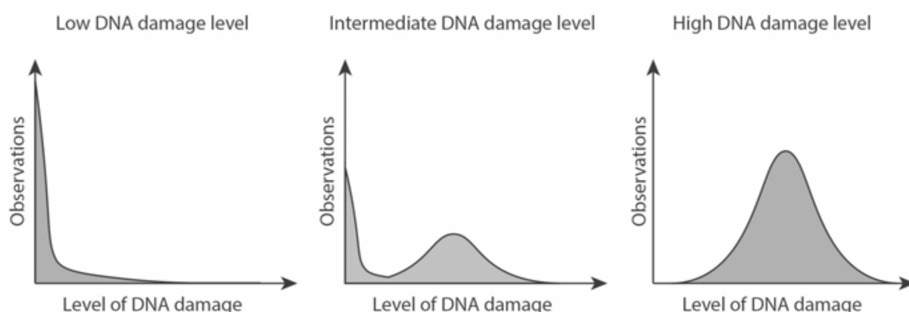

Figure 9. General representation of the data distribution and skewness of comet assay data. At low levels of DNA damage, the data follows a K-distribution with a lot of zero values. With increased DNA damage the distribution shifts towards a gaussian distribution. Illustration by E. Bivehed.

An alternative, and a seldomly used approach, is to use non-parametric statistic tests such as the Mann–Whitney U test (a non-parametric version of the T-test) or the Kruskal–Wallis H test (a non-parametric counterpart to ANOVA). The non-parametric strategies are distribution-free and insensitive to extreme and zero values and because of that, they seem to be better suited for analyzing comet data. Although being more robust than the parametric tests, the non-parametric tests also have some drawbacks, one being that the increased robustness results in decreased statistical power. They also share the same problem as the parametric tests as they cannot handle a highly clustered system.

In light of the statistical challenges associated with the comet data and that previously used (66,68,99-102) strategies were not optimal, we, together with

a statistician, aimed to find another statistical model that better explain our comet data better. We defined four requirements that the statistical model had to meet: (*i*) not requiring normal distributed data, (*ii*) being insensitive to extreme values, (*iii*) not requiring log-transformation and (*iv*) addresses the hierarchical structure of the data. The model that best suited our data and requirements was a proportional odds model tailored to continuous outcomes, using a strategy which we named Uppsala Comet Data Analysis Strategy (UCDAS).

When applied on the results obtained from the imidacloprid and nitenpyram experiments (Paper I), UCDAS seemed a little bit more conservative than the ANOVA combined with a Tukey post-hoc test, but, overall, the two different strategies for analyzing comet data basically gave similar results (Table 1 and

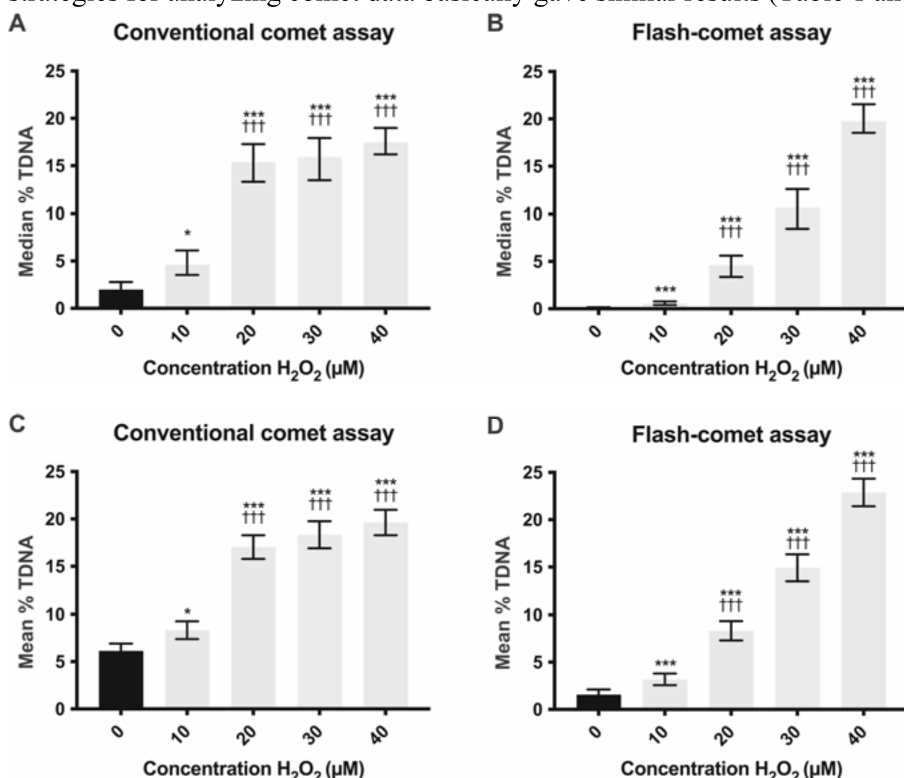

Figure 10. Comparison between the conventional alkaline comet assay (A and C) and the Flash-comet assay (B and D). TK6 cells were exposed to increasing concentrations of hydrogen peroxide (H_2O_2; 10-40 µM), or water as the negative control. The tail intensity was used as the marker of DNA-damage. The medians (± 95 % confidence interval; CI) in A and B and means (± 95 % CI) in C and D are presented after pooling the data from three independent experiments (n=450 cells). Statistical analysis was performed using our new proportional odds model tailored to continuous outcomes (UCDAS) and one-way ANOVA with Tukey post-hoc test.
* $P < 0.05$ (UCDAS) – in comparison to negative control
*** $P < 0.001$ (UCDAS) – in comparison to negative control
††† $P < 0.001$ (One-way ANOVA) – in comparison to negative control

2). None of the tested concentrations, of either Imidacloprid or Nitenpyram, did increase the level of DNA damage. When the cells were exposed for 3 hours in the presence of human S9, imidacloprid increased the level of DNA damage both at 5 and 50 μM according to the ANOVA analysis, but only at 5 μM according to the UCDAS analysis. However, when using the same statistical approaches on an increasing concentration range of hydrogen peroxide (H_2O_2) on TK6 cells (Paper II), it became evident that UCDAS has superior statistical power and is better in discriminating between treatment groups (Figure 10).

Table 1. DNA-migration in U937-cells after 3 hours of exposure of two different neonicotinoids without metabolic activation.

Exposure	**Concentration** (μM)	**Tail intensity** (% TDNA)		**Viability** (%)	**Number of Cultures/Slides/Cells**
		Median ± SE	Mean ± SEM		
Growth medium	0	0.5 ± 0.1	2.6 ± 0.2	95	6/18/900
Chlorambucil	3×10^3	14.8 ± 0.2***	15.2 ± 0.4†††	85	6/18/900
Imidacloprid	0.5	0.4 ± 0.3	2.6 ± 0.3	95	3/9/450
	5	0.3 ± 0.2	2.3 ± 0.2	95	3/9/450
	20	0.5 ± 0.2	2.5 ± 0.2	95	3/9/450
	35	0.4 ± 0.2	2.3 ± 0.2	95	3/9/450
	50	0.4 ± 0.1	2.3 ± 0.2	95	3/9/450
Nitenpyram	0.5	0.6 ± 0.2	2.9 ± 0.2	95	3/9/450
	5	0.4 ± 0.1	2.5 ± 0.2	95	3/9/450
	20	0.5 ± 0.2	2.9 ± 0.2	95	3/9/450
	35	0.7 ± 0.2	2.7 ± 0.2	95	3/9/450
	50	1.1 ± 0.2	3.4 ± 0.2	95	3/9/450

The tail intensity was used as the indicator of DNA damage. The medians (± standard error; SE) and means (± standard error of mean; SEM) presented in the table were calculated after pooling the data from three or six independent experiments (n=450 or 900 cells). Two different methods were used for the statistical analysis (*i*) our new proportional odds model tailored to continuous outcomes (UCDAS) and (*ii*) a traditional one-way ANOVA with Tukey post-hoc test.

*** $P < 0.001$ (UCDAS) – in comparison to vehicle control

††† $P < 0.001$ (ANOVA) – in comparison to vehicle control

Table 2. DNA-migration in U937-cells after 3 hours of exposure of two different neonicotinoids with metabolic activation using human S9-mix.

Exposure	**Concentration** (μM)	**Tail intensity** (% TDNA)		**Viability** (%)	**Number of Cultures/Slides/Cells**
		Median ± SE	Mean ± SEM		
Growth medium	0	1.4 ± 0.3	4.8 ± 0.1	90	3/9/450
Cyclophosphamide	100	3.8 ± 0.5***	8.6 ± 0.2†††	90	3/9/450
Imidacloprid	5	2.8 ± 0.5***	7.3 ± 0.1†††	90	3/9/450
	50	2.2 ± 0.4	6.8 ± 0.1†††	90	3/9/450
Nitenpyram	5	1.4 ± 0.4	6.2 ± 0.1	90	3/9/450
	50	1.2 ± 0.4	6.0 ± 0.1	90	3/9/450

The tail intensity was used as the indicator of DNA damage. The medians (± standard error; SE) and means (± standard error of mean; SEM) presented in the table were calculated after pooling the data from three independent experiments (n=450 cells). Two different methods were used for the statistical analysis (*i*) our new proportional odds model tailored to continuous outcomes (UCDAS) and (*ii*) one-way ANOVA with Tukey post-hoc test.

*** $P < 0.001$ (UCDAS) – in comparison to vehicle control

††† $P < 0.001$ (ANOVA) – in comparison to vehicle control

Introduction of a low conductivity electrophoresis medium

Since the single cell gel electrophoresis (SCGE-method) was introduced, a lot of work and much effort has been laid on evaluating if and how the different parameters in the comet assay protocol may affect the assay results. The main goal of these investigations has been to improve the robustness of the comet assay and reduce the inter- and intra assay variability (103). In-depth studies have evaluated virtually every step of the assay. It has, for example, been shown that increased agarose concentrations reduced the tail migration (104,105). Lysis solutions composition and lysis conditions have also been thoroughly investigated. However, these investigations show some conflicting results, in one study (106) the duration of the lysis in one study showed a time dependent increase in tail DNA, while another study (107) did not see such a time dependent increase in tail DNA. Furthermore, a study showing that the unwinding period, where the cells are incubated in a strong alkaline solution (pH >13) is enough to achieve lysis (41). Even if it seems possible to completely omit the lysis step (41), this approach has seldom been used. The effect the unwinding period (i.e., the time the DNA is incubated in strong alkaline solution to achieve DNA relaxation and conversion of ALS into strand breaks) has on the assay results is probably one of the most studied parameters (104,105,108,109). Most of these studies show a correlation between time and increased tail DNA. As indicated below, the latter observation was also confirmed by us during our unwinding experiment (Figure 11) in Paper II.

The basic electrochemical principal behind the comet assay is the same as for any other electrophoresis technique, namely that charged particles (DNA) are separated by their ability to move through a sieving matrix when an electrical gradient is applied over the gel. Consequently, the duration and voltage of the electrophoresis are also key variables when it comes to the degree of DNA migration in a comet assay. Higher voltage (increased speed) and/or longer duration (runtimes) will lead to an increase in tail DNA migration.

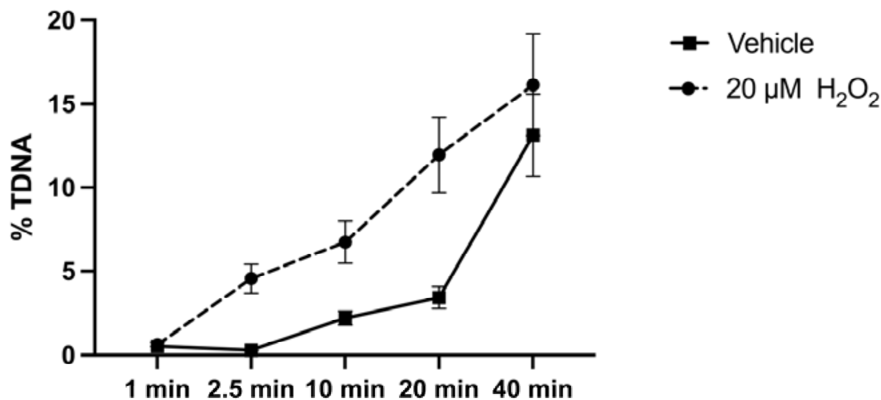

Figure 11. The effect of prolonged unwinding times (1-40 minutes) was investigated using duplicate set of TK6 cells treated with 20 μM hydrogen peroxide (H_2O_2) or vehicle (RPMI-1640). The cells were incubated in 30 mM lithium hydroxide (LiOH) at pH 12.5. The tail intensity was used as the indicator of DNA damage. The data are presented as mean (± 95 % confidence interval; CI) after pooling the data from two slides (n=100).

One major issue when dealing with electrophoresis systems is that they are restricted by heat generation and electrolyte exhaustion. The process of heat generation can, in part, be explained by Ohm's law. If you increase the voltage you increase the propulsion of the electrons passing through the system. Heat is generated due to friction of the electrons, caused by an increased resistance of the system that may destroy the samples and/or the sieving matrix. Furthermore, heat generation is also influenced by the temperature and conductivity of the electrophoresis solution. The conductivity of the solution depends on the concentration of the electrolyte and its molar conductivity, which in turn depends on the ionization, charge and hydration of the partitioning ions.

Apart from the generation of heat, an additional problem during a prolonged electrophoresis, is electrolyte exhaustion caused by a relocation of the free ions in the electrophoresis tank either to the anode or cathode. This will ultimately lead to an increase of the current (if the voltage is kept constant) in the system and additional heat generation. However, there are some ways to reduce the problems with heat generation and electrolyte exhaustion. Many protocols recommend running the electrophoresis with cold solutions and equipment (4 °C), which will delay the heat generation, allowing reasonable runtimes and DNA migration. To counteract the ion exhaustion, it is recommended to re-circulate the electrophoresis medium in the tank, thereby replenishing free ions to the system and at the same time allowing sufficient cooling of the electrophoresis medium.

However, even if cold solutions and re-circulation are used, one is still limited to perform the electrophoresis around 0.7 V/cm due to the high conductivity of the alkaline NaOH-based electrophoresis solution. We were actually

surprised when we realized that the composition of the electrophoresis medium has been overlooked and never investigated since the SCGE-assay was introduced three decades ago (at least we did not find any information about that in the literature). We therefore decided to evaluate an alternative electrophoresis medium to be used in the comet assay. We used the limitations and problems associated with the traditional alkaline NaOH-based solution as the foundation for the requirements of the new alternative electrophoresis medium. A major requirement was to lower the conductivity of the electrophoresis solutions, still preserving strong alkaline conditions in order to maintain the unwinding and DNA relaxation capacity. Apart from counteracting heat generation, a reduced conductivity would also allow a much higher voltage, vastly decreasing the runtimes, but still maintaining the DNA migration through the gel. Using a similar methodology as Brody and Kern (110), who presented a low-molarity conductive solution based on dilute solutions of alkali metals, we achieved to reduce the conductivity of the electrophoresis solution more than 10 times by replacing the sodium ion with the much smaller lithium ion.

The molar conductivity of an ion is dependent on the charge as well as how freely it can move through a solvent. Alkali metal ions, such as potassium (K^+), sodium (Na^+) and lithium (Li^+), do not exist free in a solution as they are always accompanied by an envelope of solvent molecules or sometimes by other ions (111). In aqueous solutions this is referred to the hydration of the ion, which means that several water molecules are bound to each alkali metal ion through by ion–dipole forces. The amount of water molecules that are bound to each ion is denoted as their coordination number (K:6, Na:5 and Li:4) (112). How strongly the water is bound to the ions are determined by their charge densities. The Na^+ and K^+ ions are large ions with low charge density, which means that their electrostatic interactions with the water molecules are weak and only form a single layered envelope of water molecules (111). Lithium ions, on the other hand, have a higher charge density due to the smaller size and therefore exhibits greater electrostatic interaction with the water molecules and forms a stronger water envelope that consists of several layers. Consequently, even if the larger ions, K^+ and Na^+, attract more water molecules, the interactions are much weaker compared to the much smaller Li^+ ion. This means that Li^+ is more “restrained” by the water, compared to K^+ and Na^+. Therefore it is more difficult for Li^+ to move through the water, i.e., having a reduced electromobility in the solvent, which lowers its molar conductivity (110). In Paper II we studied this effect using a low conductive alkaline electrophoresis solution based on LiOH and compared it with the conventional NaOH-based solution. Due to the lowered conductivity of the LiOH solution, we were able to run the electrophoresis at considerably higher field strength, compared to the conventional solution: from 0.7 V/cm (25 V) to 5 V/cm (150 V). As described previously, the voltage defines the speed of the

electrophoresis, so when we increased the voltage by a factor of approximately 10, we could also reduce the electrophoresis time from 10 minutes to 1 minute.

In Paper II, TK6 cells were exposed to an increasing concentration of hydrogen peroxide (from 10 to 40 µM) (Figure 10) or increasing levels of ionizing radiation (from 2.5 to 10 Gy) (Figure 12). The level of DNA damage was evaluated using either the conventional alkaline comet assay or our new low conductive alkaline electrophoresis solution based on LiOH, the Flash-comet. As shown in Figures 10 and 12, both protocols showed a significant increase of DNA damage at all concentrations tested. However, what was striking was that the Flash-comet had a much higher sensitivity and was able to more clearly discriminate between the different exposure concentrations/radiation levels.

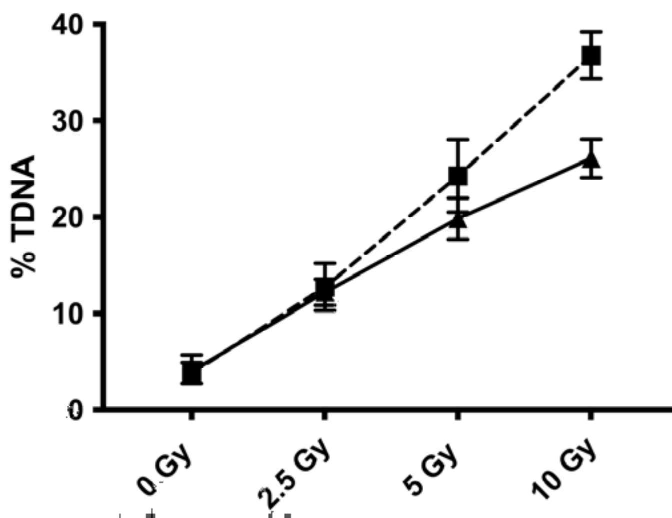

Figure 12. Dose-response relationship of DNA damage in TK6 cells corresponding to an increased level of irradiation of X-rays corresponding to 0, 2.5, 5 and 10 Gy. Flash-comet (squares) shows a steeper incline when compared to the conventional alkaline comet assay (triangles). The data is presented as means (± 95 % confidence interval; CI) after pooling the data from three slides (n=150 cells for each exposure).

Staining or labelling of the DNA? Development of the Polymerase Assisted DNA Damage Assay (PADDA)

One of the major concerns with the conventional comet assay is that it is unable to accurately distinguish between the less detrimental SSBs and more toxic DSBs. This has previously been suggested to be possible to achieve by running the comet assay under near neutral conditions assuming that this protocol is selective for DSBs, and then the alkaline version of the assay where SSBs,

DSBs and ALS are visualized. However, this concept is a misguided suggestion because both versions of the comet assay detect both DSBs and SSBs. The reason for this is that they are driven by the same fundamental principle. Undamaged intact DNA with its large size and supercoiled structure does not easily move through the gel during the electrophoresis. However, depending on the degree of strand breaks present in the DNA, whether single or double, leads to a relaxation of the supercoiled structure. The relaxation allows the DNA to move more easily through the matrix. During the alkaline version of the assay, the relaxation and unwinding process is enhanced by increasing the pH to above 13, which disrupts the Watson-Crick hydrogen bonds in the DNA, converting it into a single-stranded form and simultaneously cleaving the DNA backbone at the alkali-labile sites (discussed further down). Consequently, one could resemble the alkaline treatment as a lubricant of the DNA allowing it to move more freely than under neutral conditions, thereby increasing visualization of the damages (44,46,47,113). However, the migration of the DNA does not indicate whether the relaxation of the DNA is caused by SSBs or DSBs. During the staining process, the DNA will be stained with a fluorescent dye binding to the DNA resulting in a signal that will be identical regardless of the type of DNA strand breaks.

The most commonly used stains in the comet assay are either small DNA-binding molecules called intercalators, or so-called minor groove binders (114). Both types bind non-covalently to the DNA, but whereas intercalators such as propidium iodide, acridine orange or SYBR™ Gold, are stacked between DNA base pairs, minor groove binders such as Hoechst 33528 and DAPI, are governed by van der Waals interactions in AT-rich regions (115-117). Nowadays, SYBR™ Gold seems to be the golden standard in the comet assay, but there is no clear consensus about which stain that should be used for visualization of DNA in the assay (50,114). However, depending on the mode of binding, the DNA structure and the overall staining conditions, it may be worth noting that the staining process may influence the outcome of the comet assay (52,53). Some have suggested that certain stains have different emission spectra if they bind to single- or double-stranded DNA (118). However, this does not provide information about the type of DNA breaks that have occurred, but rather about the topology of the DNA in the comet tail (119).

Since a major drawback of the current protocols of the comet assay is that it is not able to discriminate between SSBs and DSBs, we thought that this could be done by changing the entire approach of visualizing the comets. Instead of using a stain, we utilized the inherent selectivity of DNA polymerases to recognize single- or double-strand breaks, and then use them as templates for new synthesis of DNA. By adding different fluorescently tagged nucleotides we could specifically label single- and double-strand breaks, not only in the comet tails but also in the comet heads (so far not evaluated in typical comet assay experiments).

The PADDA method was evaluated using three well-established ways of inducing DNA damage; chemically by hydrogen peroxide, physically by ionizing radiation and biologically using DNA cutting enzymes. Hydrogen peroxide was used to induce a mixture of both SSBs and DSBs. As expected, a high dose of 50 μM hydrogen peroxide induced massive DNA damage, producing clear and, as most of us doing the comet assay expect, typical comets with heads and tails (Figure 13A). During the radiation experiments we used excessive doses of X-rays in a misguided attempt to differentiate between SSBs and DSBs. The rational for using these extreme doses was to induce an overweight of DSBs. In the radiation experiments, the PADDA was indeed able to accurately label the comets, but to our surprise, our results clearly indicated that the SSBs were not transformed into DSBs, not even at an extremely high radiation dose (100 Gy). Although slightly misguided we did see that the PADDA actually was able to provide novel information regarding the nature of migration as the DSBs, but not the SSBs, seemed to have been clustered both in the comet heads (somewhat expected) but also in the comet tails, especially at the highest doses. This indicates that the larger structures of DNA migrate together, possibly reflecting crosslinked DNA or clustering of large chromatin structures containing multiple DSBs sites (Figure 14).

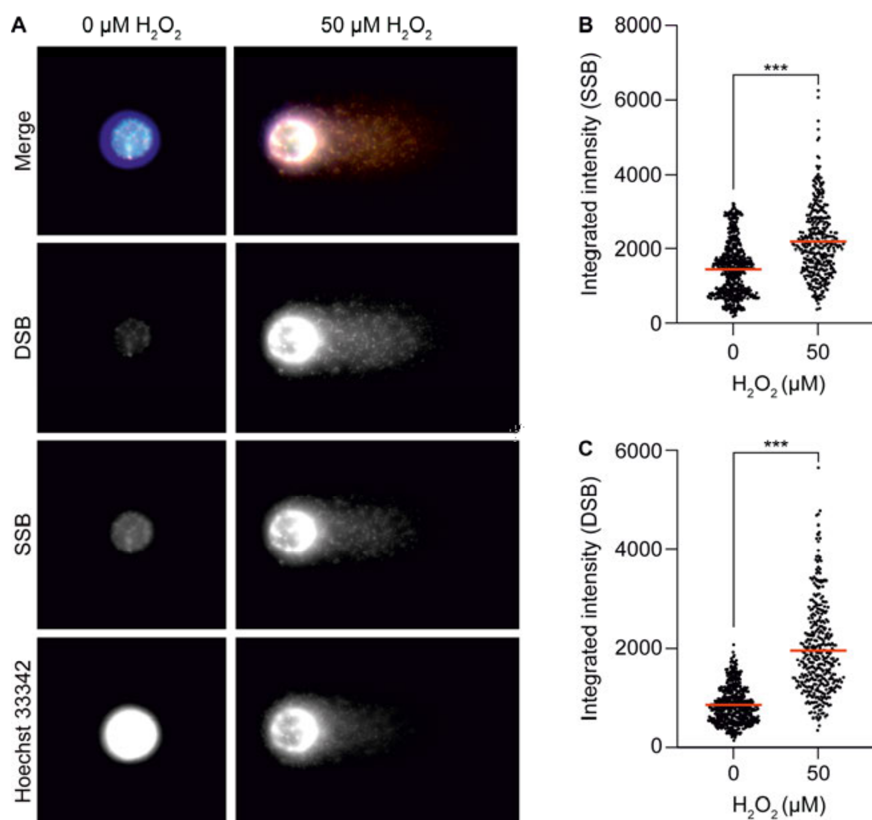

Figure 13. A shows the labelling of DNA single-strand breaks (SSBs, in green) and double-strand breaks (DSBs, in red) in comets from TK6 cells that have been exposed to vehicle alone or hydrogen peroxide (H_2O_2) for 15 minutes. After exposure, the cells were subjected to single cell gel electrophoresis using the Flash-comet protocol. After the electrophoresis, SSBs and DSBs were labelled using the PADDA technique where the fluorophore AF555 was used to label SSBs and AF647 to label DSBs. Hoechst 33342 (blue) was used for the global staining of DNA. The intensities of the labelled SSBs (B) and DSBs (C) were quantified using a modified comet assay pipeline for CellProfiler. Mean values for the intensities of SSBs and DSBs are marked with a red line (B, C). Statistical significance was evaluated using the Mann-Whitney test.

***$P < 0.001$

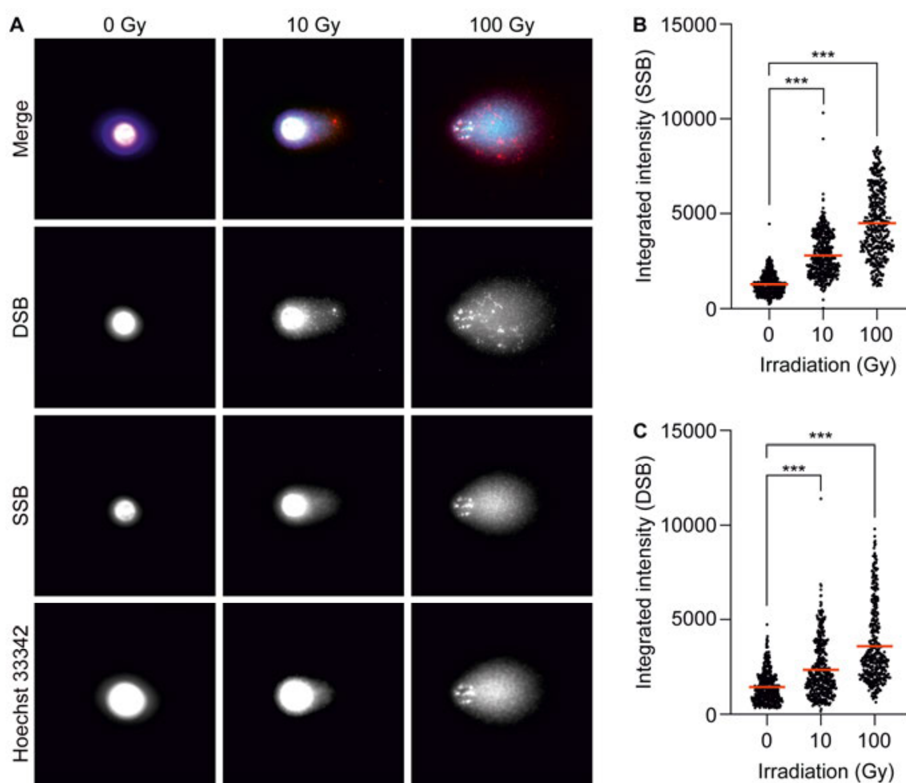

Figure 14. (A) shows the labelling of DNA single-strand breaks (SSBs, in green) and double-strand breaks (DSBs, in red) in comets from TK6 cells that had been exposed to vehicle or ionizing radiation from X-rays (0, 10 or 100 Gy). After exposure, the cells were subjected to single cell gel electrophoresis using the Flash-comet protocol. After the electrophoresis, SSBs and DSBs were labelled using the PADDA technique where the fluorophore AF488 was used to label SSBs and AF555 to label DSBs. Hoechst 33342 (blue) was used for the global staining of DNA. The intensities of the labelled SSBs (B) and DSBs (C) were quantified using a modified comet assay pipeline for CellProfiler. Mean values for the intensities of SSBs and DSBs are marked with a red line (B, C). Statistical significance was evaluated using the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

***$P < 0.001$

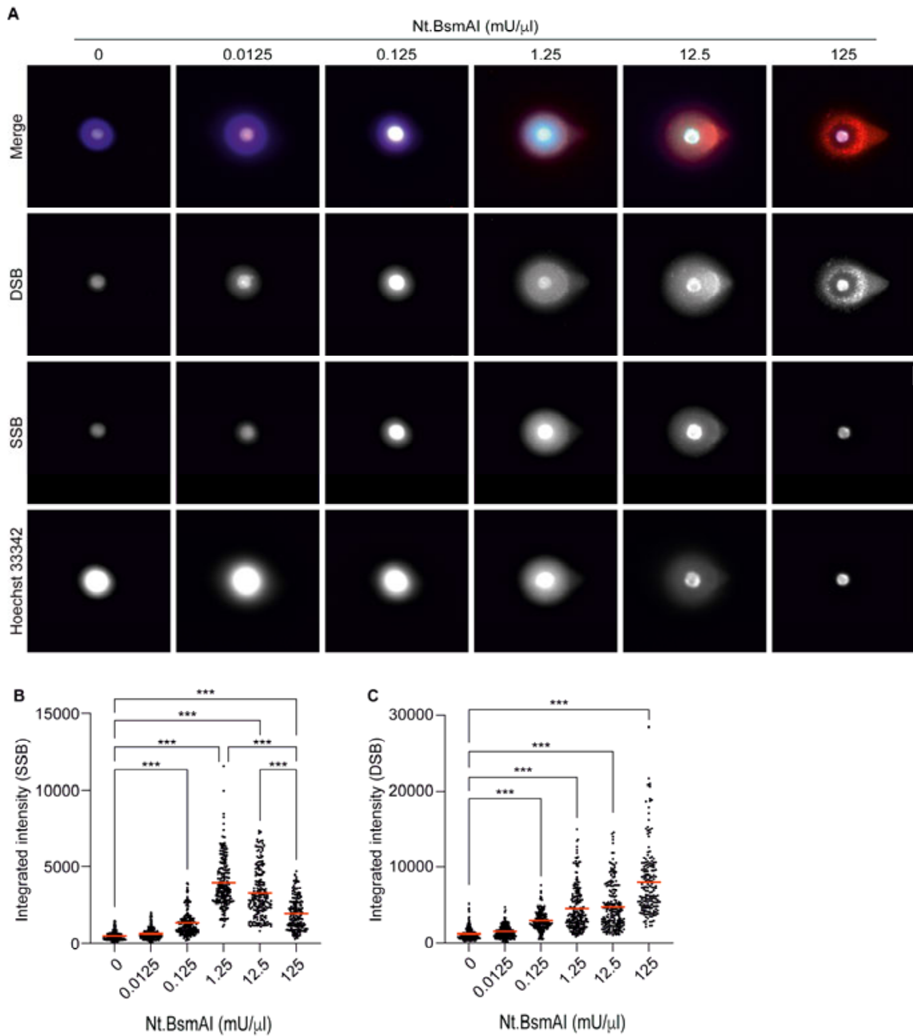

Figure 15. (A) shows the labelling of DNA single-strand breaks (SSBs, in green) and double-strand breaks (DSBs, in red) in comets from TK6 cells that had been exposed to vehicle or increasing concentrations of the nickase Nt.BsmAI. After lysis and enzyme reaction, the cells were subjected to single cell gel electrophoresis using the Flash-comet protocol. After the electrophoresis, SSBs and DSBs were labelled using the PADDA technique where the fluorophore AF488 was used to label SSBs and AF555 to label DSBs. Hoechst 33342 (blue) was used for the global staining of DNA. The intensities of the labelled SSBs (B) and DSBs (C) were quantified using a modified comet assay pipeline for CellProfiler. Mean values for the intensities of SSBs and DSBs are marked with a red line (B, C). Statistical significance was evaluated using the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

***P < 0.001

As both the chemical and physical experiments showed that the PADDA was able to label the DNA, we had still been unsuccessful proving the discriminatory capability. Therefore, we designed the third exposure experiment where we used a nicking endonuclease (Nt.BsmAI) that cleaves double-stranded DNA on only one strand leaving a single-strand break in the DNA. The rationale behind this experiment was that at really low concentrations, the amount of enzyme would not be able to produce DSBs in the DNA but when increasing the amount of enzyme, we would eventually reach a point where the enzyme started to cut overlapping, i.e., start to convert SSBs into DSBs. This experiment clearly demonstrated that the PADDA-technique is able to detect and discriminate SSBs and DSBs from each other (Figure 15A), especially at the higher concentrations of enzyme, were the signals from the labelled DSBs increased (Figure 15C), when, at the same time, the corresponding signals from SSBs decreased (Figure 15B).

During the development of the PADDA we always included a counter stain in order to find and evaluate the labeling of our comets. The fluorophores restricted us to use stains that did not have overlapping spectral properties, and after testing several stains, we selected the minor groove binder Hoechst 33528 since it had the most separated spectral properties from our fluorophores. This resulted in the following unintentional, but important finding: in our nicking enzyme experiment we were able to demonstrate the that the intensity of Hoechst stain clearly decreased with an increasing amount of DNA damage (Figure 15A). This was most likely a result of the loss of the helical structure of the DNA, deforming or destroying the minor groove. This finding showed, first hand, what major impact the choice of stain can have.

Another startling finding was that the levels of strand breaks in the non-treated control cells were surprisingly high in all experiments, indicating a relatively high abundance of DNA strand breaks also inside the comet heads (Figure 13-15). This type of strand breaks in the nucleus are missed in a conventional comet assay analysis. This clearly suggests that the comet-PADDA allows the detection of SSBs and DSBs also in DNA fragments that are too large to be pulled out from the nucleoids during the electrophoresis. These are previously undetected damages that may be of value to consider when it comes to the evaluation of the potential genotoxicity of various compounds in the comet assay.

The impact of altered pH on the DNA migration in the comet assay

One of the major questions throughout the projects presented in Paper II-IV is what effect the pH has on DNA stability. The first time it was considered was during the development of the Flash-comet. Since lithium hydroxide (LiOH)

is roughly 10 times less soluble in water than sodium hydroxide (NaOH), we were concerned that our low molar LiOH solution would not be strong enough to get an efficient alkaline unwinding of the DNA. The reduced solubility lowers the amount of available free hydroxide ions in the solution, restraining the pH range to a maximum of 12.8 in a saturated solution. Therefore, we designed the molecular beacon experiment (Paper II) to test and compare the DNA unwinding effect of the conventional electrophoresis solution and the lithium-based electrophoresis solution. We monitored the lowering of the melting temperatures (T_m) of a self-quenched double-stranded DNA (dsDNA) and non-quenched single-stranded DNA (ssDNA) molecular beacon when the beacons were dissolved in either the conventional buffer (pH >13), lithium-based (pH 12.5) or two PBS-buffers at pH 7 and 10. It was evident that the two electrophoresis solutions, conventional and LiOH-based, were equally capable in unwinding the probe (Figure 16) and that the dsDNA probe was fully dissociated over the entire temperature range in both solutions. In a follow-up experiment (Figure 17; unpublished), it was confirmed that it indeed was a clear pH-dependent lowering of the melting temperatures correlating with increased pH. We could also confirm that a complete dissociation of the dsDNA probe was fully achieved when the probe was dissolved in solutions with pH above 11.

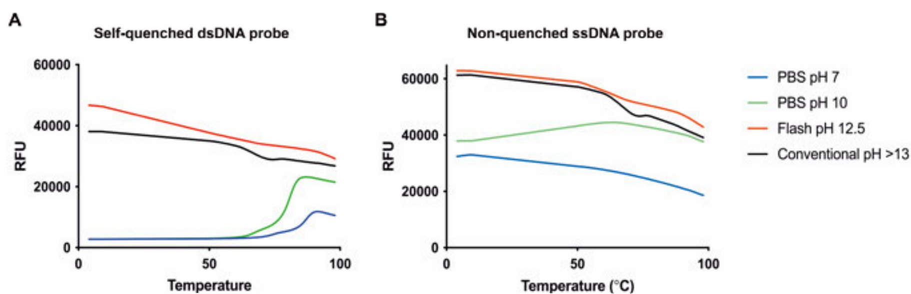

Figure 16. (A) The pH-dependent lowering of the melting temperature of a self-quenched double-stranded DNA (dsDNA). (B) A non-quenched single-stranded DNA (ssDNA) probe was used as control. The probes where incubated in either traditional electrophoresis solution pH >13 (black), the new low conductive electrophoresis solution pH 12.5 (red), saline solution (150 mM NaCl, 15 mM Tris,) adjusted to either pH 7 (blue) or 10 (green).

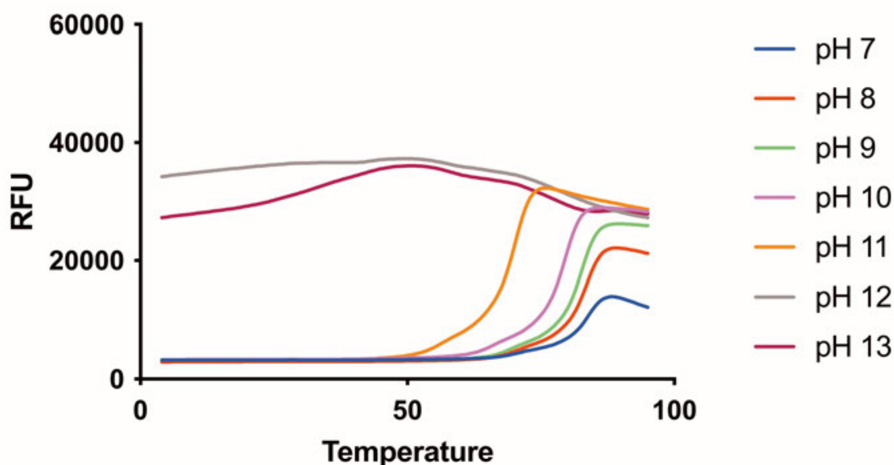

Figure 17. (A) The observed dissociation of a self-quenched double-stranded DNA (dsDNA) probe showing that with elevated pH 7-13 results in a lowered melting temperature.

One finding from these experiments, was that the influence of pH on the stability of the DNA structure was rather significant already at relative mild alkaline pH levels. Especially interesting was the observation of a quite dramatic decrease of the stability at pH 10. This will probably have an impact on the results obtained in a comet assay using extremely high pH, as it is well established that prolonged alkaline treatment leads to increased tail DNA migration. The majority of the protocols for the comet assay recommend a lysis solution that is adjusted to pH 10, but our data clearly suggest that the unwinding starts already at this pH level.

During lysis of the cell most of the proteins and histones are detached from the nuclei, leaving a supercoiled protein depleted DNA (47). One could then postulate that the driving forces, for keeping the DNA double helical structure intact after lysis, are (*i*) the electrical dipole moment caused by the negatively charged phosphate groups on the surface of the DNA (*ii*) the hydrogen bonds between the positively charged nucleotides and (*iii*) the high abundance of van der Waal interactions between the stacked nucleotides (120).

One of the major contributing forces that stabilize the dsDNA structure is the hydrogen bond between the nucleotides and that these bonds stay stable up until their respective pK_a-values. At the pK_a, half of the nucleotide is deprotonated, resulting in destabilization of its structure. At pH beyond the pK_a for a given nucleotide, the hydrogen bonds in the nucleotide will be disrupted. When looking at the pK_a-values of each nucleotide (121,122), it becomes apparent that whereas the C-G bonds are partially broken or possibly disrupted at pH 10-10.5, the A-T bonds are disrupted or broken at pH 11.

The deprotonation of hydrogen bonds probably explains our observations in the molecular beacon experiments (Paper II). It can be argued that the DNA of a lysed cell resembles our dsDNA probe, in that most of the proteins and histones are detached from the nuclei, leaving a supercoiled protein depleted DNA. Even if our probe is much smaller than genomic DNA, it could still follow similar pH-dependent destabilization processes. To confirm if we could observe the same effect on cellular DNA, we conducted a proof-of-concept experiment where TK6 cells (H_2O_2-treated and vehicle controls) were subjected to lysis with either an alkaline (pH 10) or a mildly alkaline (pH 8.5) lysis solution (Figure 18). Our hypothesis was confirmed: when a lysis solution at pH 10 was used, an increased level of DNA damage was observed both in the vehicle control and in the H_2O_2-exposed cells in comparison to when the milder alkaline lysis solution (pH 8.5) was used. Based on these observations, we decided that the pH during the lysis and in the wash solutions should be 8.5 in the Flash protocol, instead of pH 10 as in the most commonly used original protocol for the comet assay under alkaline conditions. We believe this to provide true DNA-preserving conditions, being in alignment with the reported pKa-values for the four different nucleotides.

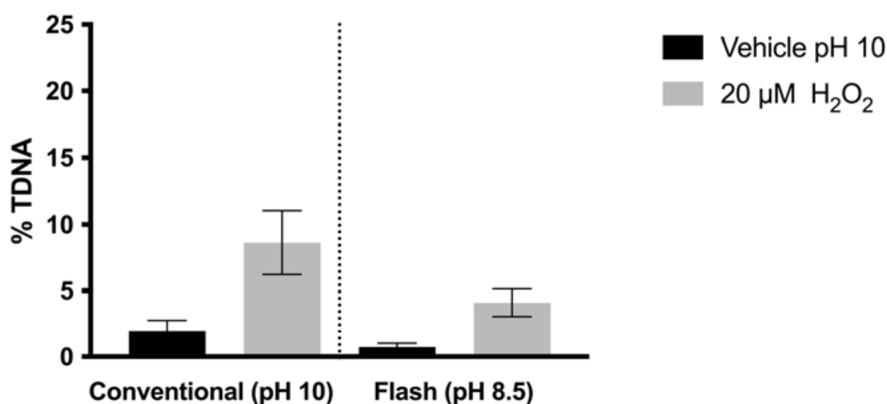

Figure 18. The effect on tail intensity depending on the pH of the lysis solutions. Duplicate sets of TK6 cells treated with 20 µM H_2O_2 or vehicle (RPMI-1640) were either incubated for 1 hour at 4 °C in conventional comet assay lysis buffer with the pH 10 (2.5 mM NaCl, 100 mM Na_2-EDTA, 10 mM Tris, adjusted with NaOH, 1 % Triton X-100 and 10 % DMSO) or a milder lysis buffer (2.5 mM NaCl, 100 mM Na_2-EDTA, 10 mM Tris, 1 % Triton X-100, 5 % DMSO) with the pH 8.5 using NaOH. The data are presented as mean (± 95 % confidence interval; CI) after pooling the data from two slides (n=100 cells).

After the publication of the Flash-comet, we received several comments regarding the short time for unwinding and whether or not it was able to convert

alkali labile site (ALS) into strand cleavage. These questions made us revisit the question regarding pH and DNA stability. As indicated previously, during the development of the PADDA we made observations that the control cells had surprisingly high amounts of signals (Figures 13-15). What made the signals so interesting is that they would never be identified in a normal staining process, indicating that a lot of strand breaks are present or generated in the comet head where it is generally believed to be less damaged DNA. The number of signals observed throughout the PADDA project were at levels that would be unlikely to be explained as naturally occurring. Hence, we started to wonder if these damages actually were caused during the assay itself.

Because, we already had started to question the use of extremely strong alkaline solutions (pH >13) during the development of the Flash, in combination with the fact that we had observed that pH has a major impact on the stability of DNA, we decided to design a three-levelled approach in order to investigate, in more detail, what effect pH has on the DNA stability. Firstly, the comet assay was employed on a whole genome level, using three different protocols: neutral (no alkaline unwinding), the Flash (pH 12.5 with 2.5 minutes of unwinding), and the alkaline (pH >13 with 40 minutes of unwinding). Secondly, real-time quantitative polymerase chain reaction (RT-qPCR) was used to study isolated DNA from irradiated and non-irradiated cells, looking at the effect on gene amplification of B2M gene exposing the extracted DNA to increasing pH levels. Thirdly, we designed new molecular beacons to examine the DNA stability on a molecular level, both with and without ALS insertions, which were then exposed to different pH levels.

We observed a substantial effect when elevating the pH (Paper IV) and this was also seen in Paper II. All three comet protocols showed background levels higher than we normally observe, with the most pronounced effect seen in the alkaline protocol using a pH >13 (Figure 19). This general increase in background damage was probably due to the fact that we used cryopreserved cells in this project, and we believe that the higher background levels of DNA damage was a consequence of the cryopreservation and thawing processes. The Flash protocol had the lowest increase in background damage, but it was still considerably higher than our historical controls. One can argue that the background level of damage in the Flash-comet was “too low”, possibly indicating that this protocol is less sensitive than the most commonly used protocol for the comet assay under alkaline conditions. However, as shown in Paper IV, ALS will be detected also at pH 12.5 if the time for the exposure of DNA to this pH is increased (for example, from 2.5 minutes to 30-40 minutes). This will probably also increase the background level of DNA damage in the Flash-comet. It is crucial to remember when comparing the Flash-comet with the other two protocols employed in Paper IV, that the samples are only subjected to the solutions in a fraction of the time compared to the other two protocols. Therefore, one has to separate what is going on at the various stages in the different methods when doing the comparison:

1. The cell lysis: since you will start having denaturation of the DNA, and partial disruptions of the hydrogen bonds, at pH 10 and above, this step is highly dependent on the pH used.
2. The alkaline unwinding (if such step is included): allows the DNA to relax and cleave ALS into strand breaks. Here, two important assumptions need to be regarded, firstly, all ALS present are either endogenous and/or caused by the treatment, and secondly, the time allowed for unwinding will affect the diffusion of DNA.
3. Field strength of the electrophoresis: this can be regarded as the throttle of the system, because if relaxed DNA is present it will move more rapidly under a strong electric field.

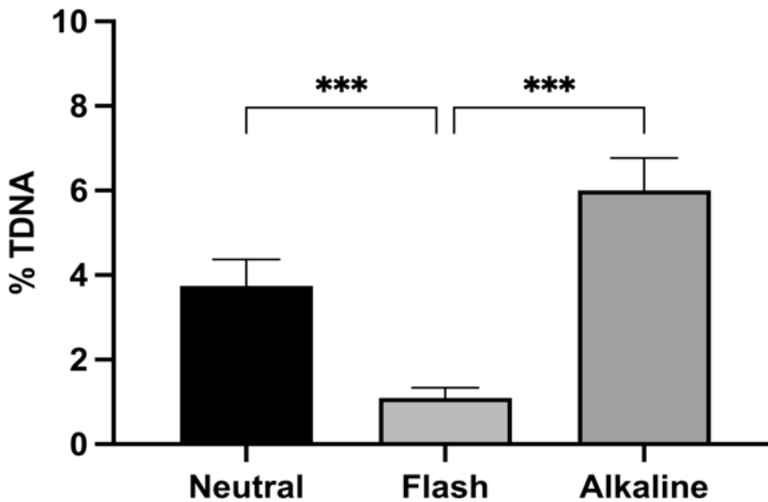

Figure 19. Using the percentage of DNA in the tail (% TDNA) as the indicator of DNA damage, the level of damage was measured in TK6 cells. Three different protocols for the comet assay were used: a neutral version (pH 10), the Flash version (pH 12.5) and an alkaline version at pH >13. The data are presented as means (± SEM) after pooling the data from three independent gels (n=150 analyzed comets per protocol). Statistical significance was evaluated using the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

*** $p<0.001$

Two out of the three points outlined above are directly linked to the pH used. Here, I must pause and provide a crucial clarification about the Flash-comet. As indicated above, it has been suggested that the Flash-comet protocol is not sensitive enough to accurately reveal the entire plethora of damages that can be visualized as in the conventional protocol using a pH >13. However, this is solely based on an unfortunate misinterpretation that the unwinding must be 2.5 minutes when conducting the Flash-protocol. However, this is not the case and something we also demonstrated during the unwinding time

experiments in Paper II (Figure 11). These results clearly showed that by increasing the unwinding times, you dramatically increase signals of DNA damage, most likely due to unwinding, relaxation and ALS conversion. However, what we also clearly showed in Paper II, using 2.5 minutes for the unwinding and 1 minute for the electrophoresis, was that it is no problem to reveal DNA relaxation (DNA strand breaks) also with these extremely short runtimes.

The different findings from Paper II, III and IV made us ask ourselves about the actual meaning of using the harsh alkaline treatments used in the conventional comet assay. Is it really necessary to use a pH as high as >13 during the unwinding and electrophoresis? Such a strong alkaline treatment has a striking similarity with the alkaline lysis method used for plasmid preparation (123) used in other genetic and biomolecular methodologies. In the alkaline lysis method, a nearly equally strong alkaline solution is used for the purpose of removing linear dsDNA from the plasmids. The chemical principle behind this is that plasmids can survive in an alkaline environment due to its circular conformation that provides increased stability. However, at the same time it has also been shown that the alkaline treatment actually damages the DNA, resulting in reduced recovery of the plasmids (124). This finding, in combination with our own observations, led us to investigate the impact of alkali treatment on linear DNA integrity by monitoring how the amplification performance of the B2M gene using RT-qPCR was affected after 60 minutes exposure to different pH levels. The result from this experiment showed a significant reduction of amplification quality which was directly correlated with the increase in alkaline treatment. The strongest effect was seen at pH >13 (Figure 20). Beacuse our result demonstrated a clear correlation between decreasing amplification efficiency and increasing pH, indicating that treatment with an extremely high pH by itself induces DNA degradation. Our interpretation of this finding was that alkaline treatment leads to degradation of the gene (or primer regions), lowering the quality and/or the amount of starting material accessible for amplification. This observation showed, once again, that strong alkaline treatment had deleterious effects on DNA. One question that still remained was whether the observed degradation of the DNA was caused by the conversion of ALS into strand breaks, or not.

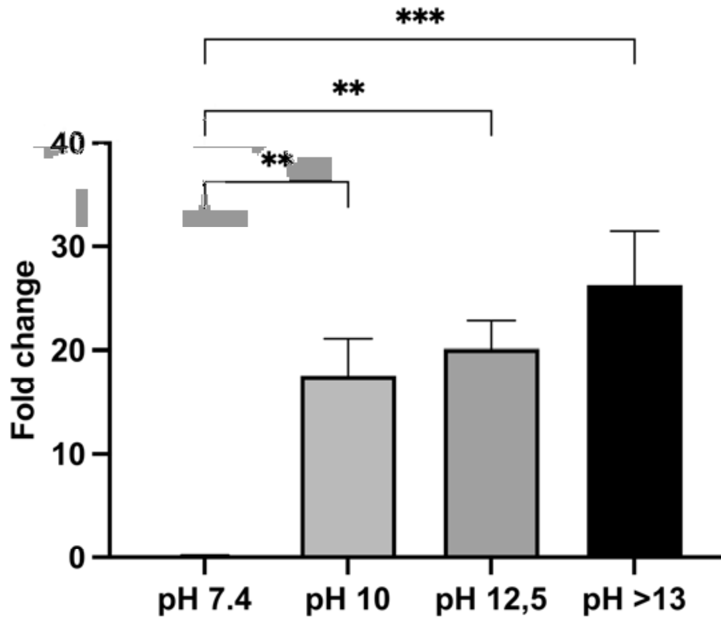

Figure 20. Transcript levels of the reporter gene β_2-microglobuline (B2M*)* in TK6 cells at pH 7.4 (used as controls), and under different alkaline conditions: pH 10, 12.5 or >13. The cells were exposed to the solutions with different pH during 60 minutes. Data are presented as means fold change (± SEM) relative to the controls. The number of observations was 9 for each treatment. Statistical significance was evaluated with a one-way ANOVA followed by Bonferroni's post hoc test.

** $p<0.01$; *** $p<0.001$

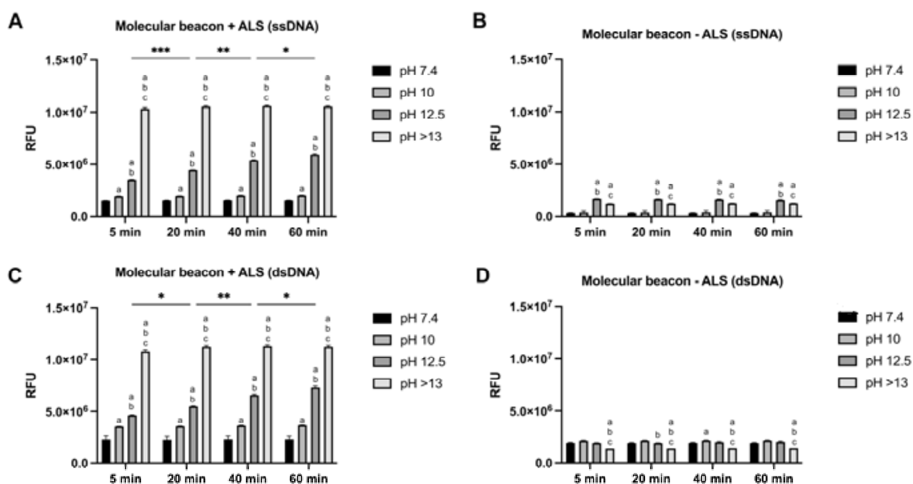

Figure 21. The effect on the stabilities of self-quenched DNA molecular beacons subjected to increasing strengths of alkaline treatment was measured over time. (A) single-stranded DNA (ssDNA) containing ALS, (B) non-ALS containing ssDNA, (C) double-stranded DNA (dsDNA) containing ALS, and (D) non-ALS containing dsDNA. The probes where incubated in the following solutions PBS at pH 7.4, TBS at pH 10, LiOH at pH 12.5 or NaOH pH >13. The data are presented as means (± SEM). Statistical significance was evaluated using a two-way repeated measures ANOVA followed by Tukey post hoc test.

Different from preceding time point: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Different from: a) pH 7.4 ($p<0.01$); b) pH 10 ($p<0.01$); c) pH 12.5 ($p<0.01$).

Therefore, we set up a new molecular beacon experiment to investigate to what extent, and how rapid, an ALS is converted into strand breaks at different pH conditions. Two different molecular beacons were used: one single-stranded DNA (ssDNA) and one double-stranded DNA (dsDNA). Both were constructed with a three-nucleotide uridine insertion with an internal fluorophore a the 3'-end of the uridine triplet and a quencher at the 5'-end of the beacon. ALS regions were then generated by treating the beacons with Uracil-DNA glycosylase (UNG). If a strand break would occur, this would lead to a release of the quencher, allowing the fluorophore to emit light. As seen in Figure 21A and C, at pH 12.5 there was a rapid increase in fluorescence already after 5 minutes with a gradual increase over time. At pH >13, the signal plateaued at maximum intensity already at 5 minutes, indicating a near instantaneous conversion of the ALS into a strand break. However, interestingly, when there were no ALS present in the probes, there was also no increase in fluorescence over time indicating that no fragmentation occurred.

The way this experiment was designed, it could only answer the question whether the treatment would cause a release of either the fluorophore or quencher from the DNA. The assumption was of course that this would occur

at the point of the ALS. However, we had no way to check if fragmentations occurred at any other place in the DNA-probe, leaving the fluorophore and quencher in proximity. Therefore, we also analyzed the single-stranded molecular beacons with ALS by liquid chromatography coupled to UV detection (UHPLC-UV). Since DNA absorbs at the wavelength of 260 nm we could independently, from the fluorophore/quencher, monitor what was going on with the DNA. What we observed was that no conversion of the beacon occurred at pH 7.4 nor at pH 10, and that the chromatograms at these pH levels only contained one single peak (the parent peak). However, when the pH was increased to 12.5 and >13, we lost the parent peak. Instead, daughter fragments emerged in the chromatograms indicating conversion of the ALS into strand breaks (Figure 22). This observation supported our theory that the ALS was converted to strand breaks. Since the beacon carried a three-uridine long insertion it would generate three possible sites for strand cleavage, thus generating three different fragments (shown as peaks in the chromatogram). Hence, our data suggest that the alkaline treatment seems to be rather selective towards AP-sites.

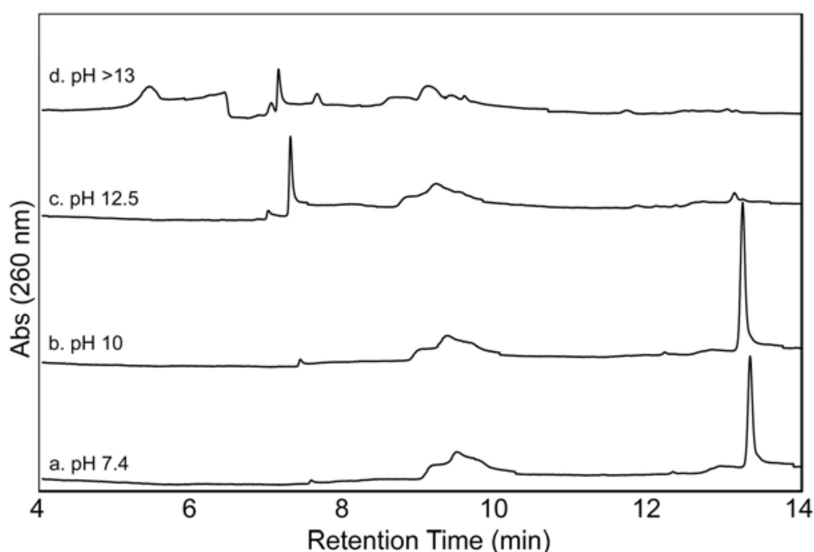

Figure 22. UHPLC-UV analysis of the products generated by strand cleavage of an alkaline-labile site (ALS) in a single stranded molecular beacon containing ALS after 60 minutes exposure time to the following solutions: (a) PBS at pH 7.4; (b), TBS at pH 10; (c), LiOH at pH 12.5, or (d) NaOH at pH >13. The peak at a retention time between 12 and 14 minutes represent the intact molecular beacon. The number of observations for each treatment was three.

The rate of ALS conversion was also investigated by repeated injections of the single-stranded molecular beacon using the same chromatographic system. As seen in Figure 23A, the conversion of the ALS was clearly time dependent

at pH 12.5, with almost no conversion after 2-30 minutes but fully fragmented into three daughter fragments after 60 minutes. However, a similar pattern of fragments could not be seen when the probe was incubated at pH >13. The latter pH only left trace levels of the parent probe after 30 minutes (Figure 23B). This left us in a conundrum, since we see full destruction of the beacon when subjected to the strongest alkaline treatment, but only if it contained an ALS region. When the same beacon without ALS was incubated at pH >13, no fragmentation occurred even after 60 minutes of exposure (Figure 24). This is noteworthy since the beacons used in the present study contained several modifications including mismatched non-DNA specific bases (uracil), covalently bound fluorophores and quenchers. All of which can be argued to be examples of DNA adducts, i.e., different types of primary DNA damages that could cause changes in the structural stability of the DNA. However, neither of the modifications, except for the ALS, resulted in fragmentation of the probes (Figure 24).

So where do we land in the question regarding the effects of alkaline pH on DNA stability? Based on the findings from our investigations it becomes clear that alkaline pH does have a major impact on the structural stability. We have several times observed, and also demonstrated, dramatic and deleterious effects on the integrity of the DNA when exposing the DNA to excessive alkalinity.

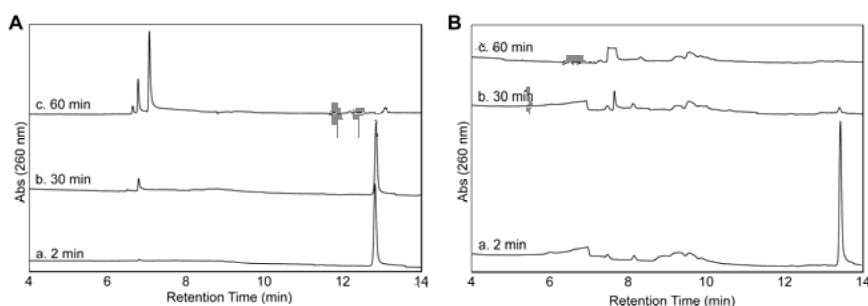

Figure 23. UHPLC-UV analysis of the products generated by strand cleavage in a single-stranded molecular beacon containing an alkali-labile site (ALS) after exposure during 2-60 minutes to either (A) 30 mM LiOH at pH 12.5 or (B) 0.3 M NaOH at pH >13. The peak at a retention time between 12 and 14 minutes represent the intact molecular beacon.

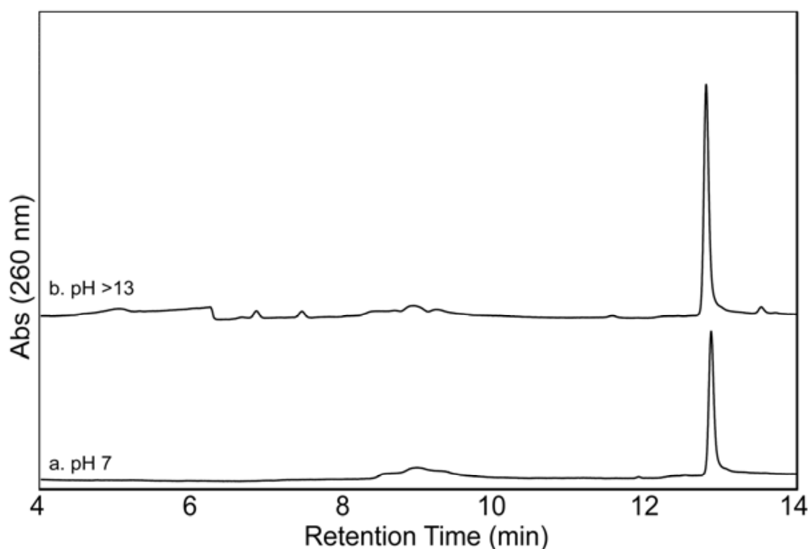

Figure 24. UHPLC-UV analysis of the products generated by strand cleavage in a single stranded molecular beacon without an alkali-labile site (ALS) after 60 minutes of exposure to either (a) PBS at pH 7.4, or (b) NaOH at pH >13. The peak at a retention time between 12 and 14 minutes represent the intact molecular beacon.

Author remarks and future perspectives

I have now spent more than five years exclusively working with, investigating and developing the comet assay. During this time, I have conducted hundreds, if not even close to thousands, of comet experiments and investigating almost all aspects of the technique. It has become evident to me that even if the technique is regarded as a well-established method for evaluating primary DNA damage, a lot of work still remains before it can be considered perfect. In this final part of my thesis, I would like to put forward my own personal thoughts regarding the comet assay, highlighting some key issues that have not gained enough spotlight previously, but that I believe are important.

In the beginning of this thesis I put forward several methodological concerns regarding the inter- and intra assay variability of the method. I have during my work tried to overcome some of these issues with the introduction of UCDAS, Flash, PADDA a focus on DNA integrity and different staining techniques. There are however also a few observation I made regarding the comet assay community that I consider to be of great concern.

One of the biggest issues that I have encountered during my work, is the general lack of transparency when it comes to report comet assay data. The comet assay community basically rely on two different scoring systems, “live-

capture" using commercial software or visual scoring using a grading system (grade I-IV). Neither of these techniques require capturing of actual images that can be evaluated by image analysis, and the scoring has always been regarded as a relatively "subjective process" as to what is regarded as a comet or not. This means that we put an enormous trust in the people generating the data to be consistent and error and bias free, since we have no way of evaluating the analysis without us replicating their experiments.

To me this is the fundamental reason as to why the comet assay suffers from such high variability, since we rely on humans, the interpretation will never be consistent or error free. However, this is something that can be completely avoided by using proper image analysis platforms, as we did during the PADDA development. I believe it would be much more beneficial for all users conducting comet assay to be able to see how comets are scored, what inclusion/exclusion criteria are used. This is the only way of ensuring objective and consistent analysis and I urge everyone to use image analysis over traditional scoring.

Here is also where I think the future lies, by starting the transition to image analysis you open up for developing autonomous analysis platforms based on artificial intelligence that can cope with much larger and more complex datasets and finding intricate details and patterns that are missed today, thus revealing more information from each experiment. This will also be possible to do at the fraction of the cost and time of what is required today.

Conclusions

Summary

The overall aim of this research project was to focus on some fundamental aspects of the single cell gel electrophoresis methodology, the comet assay, thereby hopefully contributing to further development and use of the method for assessment of primary DNA damage. The specific aims were: To apply and implement a novel statistical methodology to analyze comet data (Paper I); To evaluate and compare an alternative electrophoresis medium with lowered conductivity and preserved alkalinity (Paper II); To develop a new method for the simultaneously detection of single- and double-strand breaks in the comet tail (Paper III); To evaluate three comet protocols investigating the influence of alkaline pH on the integrity of DNA (Paper IV).

Specific conclusions

Paper I: No obvious genotoxic effect could be detected when U-937 cells were exposed to two different neonicotinoid substances Imidacloprid and Nitenpyram. A new statistical approach to evaluate comet assay data was developed, termed Uppsala Comet Data Analysis Strategy (UCDAS). A methodology that is based on a proportional odds model tailored to continuous outcomes, which addresses the hierarchical structure of the experimental outline, handles large zero values and does not require data transformation.

Paper II: A new low conductive electrophoresis medium was introduced and evaluated for the use in the comet assay. The new medium is based on lithium hydroxide, which provide a lowered conductivity, allowing six-time higher field strengths to be used, leading to dramatically lowered runtimes and provide an increased sensitivity compared to the conventional comet assay.

Paper III: A novel method was developed where it is possible to label and selectively quantify single- and double-strand breaks in the comet heads and tails in cells that had been subjected to well established chemically, physically or biologically DNA damaging exposures. The method also provides the ability to monitor DNA damages in the comet heads, which cannot be done by conventional comet assays.

Paper IV: The findings indicate that pH poses a substantial risk to DNA integrity, leading to significantly higher background levels of DNA damage compared to less alkaline conditions. It was shown that the Flash protocol of the comet assay detects DNA single- and double-strand breaks after 2.5 minutes unwinding at pH 12.5, but also ALS, if the time for unwinding is increased to 60 minutes. Unwinding of DNA at pH >13, using a relatively long unwinding time, i.e., conditions most commonly used in the alkaline version of the comet assay, will jeopardize the integrity of DNA, inducing higher background levels of DNA damage than under less alkaline conditions. Our study demonstrates the importance of understanding the influence of pH on DNA stability and provides insights into risks associated with alkaline environments, especially at pH >13.

Populärvetenskaplig sammanfattning

Deoxiribonukleinsyra eller DNA är den mest centrala organiska molekylen i naturen och den återfinns i alla former av liv vi känner till. DNA är livets bärare av information och brukar ibland liknas vid en ritning eller recept över hur cellen, organet och slutligen hur hela organismen ska fungera och vara uppbyggd. Eftersom det bara finns en uppsättning av DNA i varje cell är det av yttersta vikt för cellen att bevara det intakt eftersom annars går informationen förlorad, ungefär som en hårddisk som kraschar och där det inte finns en backup. Om DNA går sönder, oftast i form av ett enkelsträngs- (mindre allvarlig skada) eller dubbelsträngsbrott (väldigt allvarlig skada), går informationen förlorad och det kan få flera allvarliga konsekvenser såsom att cellen dör eller får förändrade funktioner/egenskaper på grund av att DNA muterats. Mutationer kan i värsta fall leda till en utveckling av cancer. Det finns kemikalier eller gifter som specifikt kan skada DNA på olika sätt och eftersom det teoretiskt räcker med en enda skada för att starta utvecklingen av cancer är det oerhört viktigt att kunna hitta och identifiera dessa kemikalier innan de når och kan skada arvsmassan.

Vår forskargrupp fokuserar just på dessa kemikalier och det kallas på fackspråk för genetisk toxikologi och för att studera skador på arvsmassan finns det en uppsjö av metoder. Vår forskning har under de senaste åren framförallt handlat om att vidareutveckla en metod som heter kometsvanstestet (på engelska comet assay), men även ta fram nya metoder för att på ett bättre sätt förstå hur dessa kemikalier orsakar brott på DNA-strängen.

Kometsvanstestet är en metod som först utvecklades i Uppsala på 1980-talet av Östling och Johanson och den kan användas för att mäta graden av strängbrott i individuella celler, vilket var helt nytt på den tiden. Det görs genom att bädda in cellerna i en extremt tunn gel av agaros på ett mikroskopglas. Efter att gelen har stelnat löser man upp (lyserar) cellerna med hjälp av diskmedel och salt och det enda som finns kvar efter behandlingen är ett nystan av DNA. För att kunna undersöka om det finns strängbrott i nystanet måste man få DNA-molekylen att röra på sig. Det görs genom att sänka ner gelerna i ett bad av stark natriumhydroxidlösning (propplösare, lut eller kaustiksoda) och koppla på en elektrisk spänning i lutbadet. Vad som händer när man utsätter DNA, som är negativt laddat, för en elektrisk spänning är att det börjar röra på sig mot pluspolen (anoden). Beroende på om det finns strängbrott i DNA-nystanet kommer det kunna röra sig olika lätt genom gelen, ju mer skador

desto lättare kan DNA röra sig. Om man låter strömmen vara på tillräckligt länge och det finns skador i arvsmassan så bildar DNA-nystanet till slut något som väldigt mycket efterliknar en komet, därav namnet kometsvanstestet. Vi kan sen färga in DNA-molekylen med en fluorescerande DNA-färg och tillsammans med ett mikroskop kan vi mäta och räkna ut hur mycket av DNA som befinner sig i huvudet av kometen (oskadat DNA) eller i svansen (skadat DNA), men det går tyvärr inte att särskilja mellan dubbel- och enkelsträngsbrott i kometen.

Den här metoden är en mycket effektiv och billig metod att använda för att undersöka skador på arvsmassan, men som alla metoder har den sina begränsningar och tillkortakommanden. Det som tyvärr oftast kritiseras är att resultaten varierar beroende på vem som utför experimentet eller var det utförs och det finns flera orsaker till detta. Mitt forskningsprojekt har i huvudsak fokuserat på att försöka komma runt och lösa några av dessa orsaker för att göra metoden mer effektiv och informativ. Jag har i huvudsak fokuserat på de grundläggande delarna av metoden, förändrat en del av de lösningar som används och utformat ett nytt sätt att räkna på resultaten samt undersökt på molekylär nivå vad som händer med DNA när man låter det ligga i natriumhydroxid under en längre tid. Vi kom även på att om man ersätter natriumhydroxiden med litiumhydroxid kunde man få DNA-molekylen att röra sig mycket snabbare genom gelen. På så sätt kan vi nu få fram tydligare resultat på en bråkdel av den tid som tidigare krävdes.

Tillsammans med en annan forskargrupp vid institutionen, har vi utvecklat en helt ny metod som för första gången möjliggör särskiljning mellan enkel- och dubbelsträngsbrott. Istället för att använda en färg som färgar allt DNA på samma sätt, använder vi enzymer – cellens små byggmästare – som upptäcker och reparerar skadade områden i DNA. Dessa enzymer använder byggstenar som är färgade med olika nyanser av fluorescerande färg beroende på vilken typ av skada som repareras.

Utöver möjligheten att särskilja olika typer av skador kan vi nu även upptäcka helt nya och okända skador som tidigare inte upptäcktes med den konventionella metoden. Förhoppningsvis kommer vårt arbete med kometsvanstestet att reducera variationen i resultaten, ge mer informativa resultat och öka vår förståelse för hur kemikalier kan skada arvsmassan.

Acknowledgements

The work presented in this thesis was carried out at the Division of Drug Safety and Toxicology, Department of Pharmaceutical Biosciences, Faculty of Pharmacy, Uppsala University.

This is undeniably one of the most difficult sections to compose, and I have given it my utmost effort. Although I may not individually mention everyone who contributed and supported me, your involvement has made an enduring impact on me and my work.

To my supervisor and friend *Professor Björn Hellman*. I am deeply grateful for the opportunity of being your final PhD student. When reflecting on our relationship, I would describe it as a dynamic alliance characterized by a delicate equilibrium between freedom, encouragement, and responsibility. Your unwavering trust, guidance, and support have allowed me to fully immerse myself in my own projects and find immense joy in the pursuit of my ideas, ultimately leading to the shared triumph when finally reaching the point for publication of the final results. I will always be grateful for your endless patience and guidance when I have suggested new project ideas and it has left a truly lasting impact on me. As our mentor-student relationship now comes to a close, I take a moment to reflect on the phrase "Comet revolution" that you so enthusiastically introduced some years ago. Initially, I held a negative perspective towards it. However, as I conclude this text, I am delighted to admit that I have come to fully embrace it. Together, we have initiated a path of innovation and impact, even if on a modest scale. We have ventured beyond the confines of the established Comet community, forging an independent path that challenges old beliefs establishing a fresh trajectory for future exploration - a genuine revolution, indeed.

To my co-supervisors *Ola Söderberg* and *Johan Heldin* and together with the entire pharmaceutical cell biology research group. To have your support has been invaluable to me. I am deeply grateful for the fruitful discussions, cooperation and for allowing me to be included in a bigger research group.

To my co-supervisor *Malin Andersson, w*ho, despite unforeseen circumstances leading to a prolonged absence always provided enthusiasm, guidance and never-failing willingness to discuss theoretical aspects of my work.

Sonja Buratovic, you are a wonderful friend and you have become like an extra sister to me. Thank you for all your endless support, all good laughs and

fun discussions regarding everything and nothing! I wish our paths would have crossed many years ago.

Stina Lundberg, thank you for always being there for me, even at times I didn't even know I needed help! I'm grateful for all the fun we have had during the years and that you have pushed and dragged me along to everything from dinners to becoming a blood donor.

To all my co-authors for their incredible support and contributions throughout my work. Your collaboration has been a driving force, and I'm truly grateful for our shared journey.

Thanks to all present and former colleagues, friends and students at the department of Pharmaceutical Biosciences. Especially thanks to the Drug safety and toxicology group: *Faranak, Sebastian, Julia, Lisa, Emelie, Rein, Oskar, Eva, Paula, Daiane, Lennart, Michael, Birger, Mats, Raili, Carin, Lena* and *Magnus*.

I would like to thank my extended family and relatives for all your assistance and empowerment. With a special heartfelt gratitude to *Siv* and *Bo-Ragnar* for your help caring for Astrid and for allowing us to live with you during the end of Hanna's pregnancy. It gave us the possibility to give Astrid a wonderful summer, Hanna the time to rest and me with possibility to finish this text.

To my parents *Lena* and *Håkan* and my sister *Charlotte.* Your constant belief in me and your unwavering encouragement have played an essential role in shaping the person I am today. Your support, both personally and professionally, has been a cornerstone of my accomplishments, and I am truly grateful for the warmth you've brought into my life. An additional thanks to my father – my toughest critic, my greatest support. Your unwavering assistance, time, and guidance through chemical puzzles, both simple and complex, mean the world to me.

Even if I regard the completion of this journey as one of my greatest achievements. It accounts for nothing when I think of you two, *Astrid* and *Nils*. You are, and forever will be, my greatest achievements in life and nothing is more precious to me than you two!

Finally, to my *Hanna*, you are the love of my life and my best friend. Besides being the greatest mother to our children, your support and love is the true source of my energy. Without you this would never have be possible. Thank you for being you and remember that I love you with all my heart!

References

1. Dunham, I., Kundaje, A., Aldred, S.F., Collins, P.J., Davis, C., Doyle, F., Epstein, C.B., Frietze, S., Harrow, J., Kaul, R. *et al.* (2012) An integrated encyclopedia of DNA elements in the human genome. *NATURE*, **489**, 57-74.
2. Crick, F. (1970) CENTRAL DOGMA OF MOLECULAR BIOLOGY. *NATURE*, **227**, 561-&.
3. Hoeijmakers, J.H.J. (2001) Genome maintenance mechanisms for preventing cancer. *NATURE*, **411**, 366-374.
4. Lindahl, T. (1993) INSTABILITY AND DECAY OF THE PRIMARY STRUCTURE OF DNA. *NATURE*, **362**, 709-715.
5. Hanahan, D. and Weinberg, R.A. (2011) Hallmarks of Cancer: The Next Generation. *CELL*, **144**, 646-674.
6. Thaplyal, P. and Bevilacqua, P.C. (2014) Experimental approaches for measuring pKa's in RNA and DNA. *Methods Enzymol*, **549**, 189-219.
7. Gates, K.S. (2009) An overview of chemical processes that damage cellular DNA: spontaneous hydrolysis, alkylation, and reactions with radicals. *Chem Res Toxicol*, **22**, 1747-1760.
8. Lawley, P.D. (1974) Some chemical aspects of dose-response relationships in alkylation mutagenesis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **23**, 283-295.
9. Ho, T.-L. (1975) Hard soft acids bases (HSAB) principle and organic chemistry. *Chemical Reviews*, **75**, 1-20.
10. Beranek, D.T. (1990) Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutation research*, **231**, 11-30.
11. Singer, B. and Grunberger, D. (2012) *Molecular biology of mutagens and carcinogens*. Springer Science & Business Media.
12. Laval, J., Boiteux, S. and O'Connor, T.R. (1990) Physiological properties and repair of apurinic/apyrimidinic sites and imidazole ring-opened guanines in DNA. *Mutation research*, **233**, 73-79.
13. Greenberg, M.M. (2007) Elucidating DNA damage and repair processes by independently generating reactive and metastable intermediates. *Org Biomol Chem*, **5**, 18-30.

14. Ueda, J., Takai, M., Shimazu, Y. and Ozawa, T. (1998) Reactive oxygen species generated from the reaction of copper(II) complexes with biological reductants cause DNA strand scission. *Arch Biochem Biophys*, **357**, 231-239.
15. Bales, B.C., Kodama, T., Weledji, Y.N., Pitié, M., Meunier, B. and Greenberg, M.M. (2005) Mechanistic studies on DNA damage by minor groove binding copper-phenanthroline conjugates. *Nucleic Acids Res*, **33**, 5371-5379.
16. Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S. and Loeb, L.A. (1992) 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G----T and A----C substitutions. *The Journal of biological chemistry*, **267**, 166-172.
17. Kasai, H. and Nishimura, S. (1984) Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. *Nucleic acids research*, **12**, 2137-2145.
18. Shibutani, S., Takeshita, M. and Grollman, A.P. (1991) Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature*, **349**, 431-434.
19. Waring, M.J. (1981) DNA modification and cancer. *Annu Rev Biochem*, **50**, 159-192.
20. Long, E.C. and Barton, J.K. (1990) ON DEMONSTRATING DNA INTERCALATION. *Accounts Chem. Res.*, **23**, 271-273.
21. Mavragani, I.V., Nikitaki, Z., Kalospyros, S.A. and Georgakilas, A.G. (2019) Ionizing Radiation and Complex DNA Damage: From Prediction to Detection Challenges and Biological Significance. *Cancers (Basel)*, **11**, 1789.
22. Brunborg, G., Eide, D.M., Graupner, A., Gutzkow, K., Shaposhnikov, S., Kruszewski, M., Sirota, N., Jones, G.D.D., Koppen, G., Vanhavere, F. *et al.* (2023) Calibration of the comet assay using ionising radiation. *Mutat Res Genet Toxicol Environ Mutagen*, **885**, 503560.
23. Harper, J.V., Anderson, J.A. and O'Neill, P. (2010) Radiation induced DNA DSBs: Contribution from stalled replication forks? *DNA Repair (Amst)*, **9**, 907-913.
24. Nickoloff, J.A., Sharma, N. and Taylor, L. (2020) Clustered DNA Double-Strand Breaks: Biological Effects and Relevance to Cancer Radiotherapy. *Genes (Basel)*, **11**.
25. Loeb, L.A., Springgate, C.F. and Battula, N. (1974) Errors In Dna Replication As A Basis Of Malignant Changes 1. *Cancer research*, **34**, 2311-2321.
26. Blokzijl, F., de Ligt, J., Jager, M., Sasselli, V., Roerink, S., Sasaki, N., Huch, M., Boymans, S., Kuijk, E., Prins, P. *et al.* (2016) Tissue-specific mutation accumulation in human adult stem cells during life. *Nature*, **538**, 260-264.

27. Neijenhuis, S., Verwijs-Janssen, M., Kasten-Pisula, U., Rumping, G., Borgmann, K., Dikomey, E., Begg, A.C. and Vens, C. (2009) Mechanism of cell killing after ionizing radiation by a dominant negative DNA polymerase beta. *DNA repair*, **8**, 336-346.
28. Hu, J., Lieb, J.D., Sancar, A. and Adar, S. (2016) Cisplatin DNA damage and repair maps of the human genome at single-nucleotide resolution. *Proc Natl Acad Sci U S A*, **113**, 11507-11512.
29. Okabe, S. (1986) [On apprehension of student nurses in giving injections: analysis of the mental state by a Japanese version of the State-Trait Anxiety Inventory and reliability and validity of results]. *Kango Kyoiku*, **27**, 181-185.
30. Elacqua, J.J., Ranu, N., DiIorio, S.E. and Blainey, P.C. (2021) DENT-seq for genome-wide strand-specific identification of DNA single-strand break sites with single-nucleotide resolution. *Genome Res*, **31**, 75-87.
31. Balbo, S., Turesky, R.J. and Villalta, P.W. (2014) DNA adductomics. *Chem Res Toxicol*, **27**, 356-366.
32. Krug, K., Jaehnig, E.J., Satpathy, S., Blumenberg, L., Karpova, A., Anurag, M., Miles, G., Mertins, P., Geffen, Y., Tang, L.C. *et al.* (2020) Proteogenomic Landscape of Breast Cancer Tumorigenesis and Targeted Therapy. *Cell*, **183**, 1436-1456 e1431.
33. Kanaly, R.A., Hanaoka, T., Sugimura, H., Toda, H., Matsui, S. and Matsuda, T. (2006) Development of the adductome approach to detect DNA damage in humans. *Antioxid Redox Signal*, **8**, 993-1001.
34. Kinner, A., Wu, W., Staudt, C. and Iliakis, G. (2008) Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic Acids Res*, **36**, 5678-5694.
35. Kyrylkova, K., Kyryachenko, S., Leid, M. and Kioussi, C. (2012) Detection of apoptosis by TUNEL assay. *Methods Mol Biol*, **887**, 41-47.
36. Holton, N.W., Ebenstein, Y. and Gassman, N.R. (2018) Broad spectrum detection of DNA damage by Repair Assisted Damage Detection (RADD). *DNA Repair (Amst)*, **66-67**, 42-49.
37. Ostling, O. and Johanson, K.J. (1987) Bleomycin, in contrast to gamma irradiation, induces extreme variation of DNA strand breakage from cell to cell. *Int J Radiat Biol Relat Stud Phys Chem Med*, **52**, 683-691.
38. Ostling, O. and Johanson, K.J. (1984) Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Commun*, **123**, 291-298.
39. Rydberg, B. and Johanson, K.J. (1978) In Hanawalt, P. C., Friedberg, E. C. and Fox, C. F. (eds.), *DNA Repair Mechanisms*. Academic Press, pp. 465-468.

40. Singh, N.P., McCoy, M.T., Tice, R.R. and Schneider, E.L. (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res*, **175**, 184-191.
41. Olive, P.L., Banáth, 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. *Radiation Research*, **122**, 86-94.
42. Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C. and Sasaki, Y.F. (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen*, **35**, 206-221.
43. Collins, A.R. (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol*, **26**, 249-261.
44. Collins, A.R., Dobson, V.L., Dusinska, M., Kennedy, G. and Stetina, R. (1997) The comet assay: what can it really tell us? *Mutat Res*, **375**, 183-193.
45. Shaposhnikov, S.A., Salenko, V.B., Brunborg, G., Nygren, J. and Collins, A.R. (2008) Single-cell gel electrophoresis (the comet assay): loops or fragments? *Electrophoresis*, **29**, 3005-3012.
46. Azqueta, A. and Collins, A.R. (2013) The essential comet assay: a comprehensive guide to measuring DNA damage and repair. *Arch Toxicol*, **87**, 949-968.
47. Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivao, I., Giovannelli, L., Kruszewski, M., Smith, C.C. and Stetina, R. (2008) The comet assay: topical issues. *Mutagenesis*, **23**, 143-151.
48. Olive, P.L. and Banath, J.P. (2006) The comet assay: a method to measure DNA damage in individual cells. *Nat Protoc*, **1**, 23-29.
49. Hellman, B., Vaghef, H. and Bostrom, B. (1995) The concepts of tail moment and tail inertia in the single cell gel electrophoresis assay. *Mutation research*, **336**, 123-131.
50. Collins, A., Moller, P., Gajski, G., Vodenkova, S., Abdulwahed, A., Anderson, D., Bankoglu, E.E., Bonassi, S., Boutet-Robinet, E., Brunborg, G. *et al.* (2023) Measuring DNA modifications with the comet assay: a compendium of protocols. *Nat Protoc*, **18**, 929-989.
51. Kumaravel, T.S., Vilhar, B., Faux, S.P. and Jha, A.N. (2009) Comet Assay measurements: a perspective. *Cell Biol Toxicol*, **25**, 53-64.
52. Moller, P., Azqueta, A., Collia, M., Bakuradze, T., Richling, E., Bankoglu, E.E., Stopper, H., Bastos, V.C., Langie, S.A.S., Jensen, A. *et al.* (2023) Inter-laboratory variation in measurement of DNA damage by the alkaline comet assay in the hCOMET ring trial. *Mutagenesis*.
53. Moller, P., Azqueta, A., Sanz-Serrano, J., Bakuradze, T., Richling, E., Bankoglu, E.E., Stopper, H., Bastos, V.C., Langie, S.A.S., Jensen, A. *et al.* (2023) Visual comet scoring revisited: a guide to scoring comet assay slides and obtaining reliable results. *Mutagenesis*.

54. Collins, A.R., Duthie, S.J. and Dobson, V.L. (1993) Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis*, **14**, 1733-1735.
55. Collins, A.R., Dusinská, M., Gedik, C.M. and Stětina, R. (1996) Oxidative damage to DNA: do we have a reliable biomarker? *Environmental health perspectives*, **104 Suppl 3**, 465-469.
56. Dušinská, M. and Collins, A. (1996) Detection of Oxidised Purines and UV-induced Photoproducts in DNA of Single Cells, by Inclusion of Lesion-specific Enzymes in the Comet Assay. *Alternatives to Laboratory Animals*, **24**, 405-411.
57. Verde, P. and Rottmann, A. (2015) Statistical Models to Analyze Genotoxicological Experiments with the Comet Assay Data. *Ann Biom Biostat*, **2**, 1025.
58. Bright, J., Aylott, M., Bate, S., Geys, H., Jarvis, P., Saul, J. and Vonk, R. (2011) Recommendations on the statistical analysis of the Comet assay. *Pharm Stat*, **10**, 485-493.
59. Heberger, K., Kolarevic, S., Kracun-Kolarevic, M., Sunjog, K., Gacic, Z., Kljajic, Z., Mitric, M. and Vukovic-Gacic, B. (2014) Evaluation of single-cell gel electrophoresis data: combination of variance analysis with sum of ranking differences. *Mutation research. Genetic toxicology and environmental mutagenesis*, **771**, 15-22.
60. Lovell, D.P. and Omori, T. (2008) Statistical issues in the use of the comet assay. *Mutagenesis*, **23**, 171-182.
61. Moller, P. and Loft, S. (2014) Statistical analysis of comet assay results. *Frontiers in genetics*, **5**, 292.
62. Wiklund, S.J. and Agurell, E. (2003) Aspects of design and statistical analysis in the Comet assay. *Mutagenesis*, **18**, 167-175.
63. Lovell, D.P., Thomas, G. and Dubow, R. (1999) Issues related to the experimental design and subsequent statistical analysis of in vivo and in vitro comet studies. *Teratogenesis, carcinogenesis, and mutagenesis*, **19**, 109-119.
64. OECD. (2016) *Test No. 489: In Vivo Mammalian Alkaline Comet Assay*.
65. Moller, P. (2018) The comet assay: ready for 30 more years. *Mutagenesis*, **33**, 1-7.
66. Demma, J., Engidawork, E. and Hellman, B. (2009) Potential genotoxicity of plant extracts used in Ethiopian traditional medicine. *Journal of ethnopharmacology*, **122**, 136-142.
67. Vaghef, H., Wisen, A.C. and Hellman, B. (1996) Demonstration of benzo(a)pyrene-induced DNA damage in mice by alkaline single cell gel electrophoresis: evidence for strand breaks in liver but not in lymphocytes and bone marrow. *Pharmacology & toxicology*, **78**, 37-43.
68. Andersson, M., Agurell, E., Vaghef, H., Bolcsfoldi, G. and Hellman, B. (2003) Extended-term cultures of human T-lymphocytes and the

comet assay: a useful combination when testing for genotoxicity in vitro? *Mutat Res*, **540**, 43-55.

69. Speit, G. and Hartmann, A. (2006) In Henderson, D. S. (ed.), *DNA Repair Protocols: Mammalian Systems*. Humana Press, Totowa, NJ, pp. 275-286.
70. Collins, A.R. and Azqueta, A. (2012) In Conn, P. M. (ed.), *Methods in Cell Biology*. Academic Press, Vol. 112, pp. 69-92.
71. Burlinson, B. (2012) In Parry, J. M. and Parry, E. M. (eds.), *Genetic Toxicology: Principles and Methods*. Springer New York, New York, NY, pp. 143-163.
72. Vasquez, M. and Frötschl, R. (2016), pp. 345-382.
73. Fairbairn, D.W., Olive, P.L. and O'Neill, K.L. (1995) The comet assay: a comprehensive review. *Mutation Research/Reviews in Genetic Toxicology*, **339**, 37-59.
74. Mamur, S., Yuzbasioglu, D., Unal, F. and Yilmaz, S. (2010) Does potassium sorbate induce genotoxic or mutagenic effects in lymphocytes? *Toxicology in vitro : an international journal published in association with BIBRA*, **24**, 790-794.
75. Wu, R.A., Ding, Q., Lu, H., Tan, H., Sun, N., Wang, K., He, R., Luo, L., Ma, H. and Li, Z. (2020) Caspase 3-mediated cytotoxicity of mealworm larvae (Tenebrio molitor) oil extract against human hepatocellular carcinoma and colorectal adenocarcinoma. *Journal of ethnopharmacology*, **250**, 112438.
76. Jurica, K., Benkovic, V., Sikiric, S., Kopjar, N. and Brcic Karaconji, I. (2020) Liver function and DNA integrity in hepatocytes of rats evaluated after treatments with strawberry tree (Arbutus unedo L.) water leaf extract and arbutin. *Drug Chem Toxicol*, **43**, 127-137.
77. Kowalczyk, K., Roszak, J., Brodecki, M., Domeradzka Gajda, K., Szparaga, M., Sobanska, Z. and Stepnik, M. (2020) The effect of inhibitors of phosphatidylinositol 3-kinase-related kinases on dibenzo[def,p]chrysene genotoxicity measured by gammaH2AX levels and neutral comet assay in HepG2 human hepatocellular cancer cells. *Toxicology in vitro : an international journal published in association with BIBRA*, **63**, 104749.
78. Ventura, C., Pereira, J.F.S., Matos, P., Marques, B., Jordan, P., Sousa-Uva, A. and Silva, M.J. (2020) Cytotoxicity and genotoxicity of MWCNT-7 and crocidolite: assessment in alveolar epithelial cells versus their coculture with monocyte-derived macrophages. *Nanotoxicology*, 1-25.
79. Sasaki, Y.F., Nakamura, T. and Kawaguchi, S. (2007) What is better experimental design for in vitro comet assay to detect chemical genotoxicity? *Alternatives to Animal Testing and Experimentation*, **14**, 499-504.
80. Azqueta, A., Muruzabal, D., Boutet-Robinet, E., Milic, M., Dusinska, M., Brunborg, G., Moller, P. and Collins, A.R. (2019) Technical

recommendations to perform the alkaline standard and enzyme-modified comet assay in human biomonitoring studies. *Mutation research*, **843**, 24-32.
81. Møller, P., Azqueta, A., Boutet-Robinet, E., Koppen, G., Bonassi, S., Milić, M., Gajski, G., Costa, S., Teixeira, J.P., Costa Pereira, C. *et al.* (2020) Minimum Information for Reporting on the Comet Assay (MIRCA): recommendations for describing comet assay procedures and results. *Nature protocols*.
82. Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C. and Sasaki, Y.F. (2000) Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and molecular mutagenesis*, **35**, 206-221.
83. Olive, P.L. (2002) In Didenko, V. V. (ed.), *In Situ Detection of DNA Damage: Methods and Protocols*. Humana Press, Totowa, NJ, pp. 179-194.
84. Collins, A.R. and Dusinská, M. (2002) Oxidation of cellular DNA measured with the comet assay. *Methods Mol Biol*, **186**, 147-159.
85. Collins, A.R. (2011) In Gautier, J.-C. (ed.), *Drug Safety Evaluation: Methods and Protocols*. Humana Press, Totowa, NJ, pp. 137-147.
86. Speit, G. and Rothfuss, A. (2012) In Bjergbæk, L. (ed.), *DNA Repair Protocols*. Humana Press, Totowa, NJ, pp. 79-90.
87. Bajpayee, M., Kumar, A. and Dhawan, A. (2013) In Dhawan, A. and Bajpayee, M. (eds.), *Genotoxicity Assessment: Methods and Protocols*. Humana Press, Totowa, NJ, pp. 325-345.
88. Anderson, D., Dhawan, A. and Laubenthal, J. (2013) In Dhawan, A. and Bajpayee, M. (eds.), *Genotoxicity Assessment: Methods and Protocols*. Humana Press, Totowa, NJ, pp. 347-362.
89. Catalán, J., Suhonen, S., Huk, A. and Dusinska, M. (2014) In Sierra, L. M. and Gaivão, I. (eds.), *Genotoxicity and DNA Repair: A Practical Approach*. Springer New York, New York, NY, pp. 241-268.
90. Gichner, T., Mukherjee, A. and Veleminsky, J. (2006) DNA staining with the fluorochromes EtBr, DAPI and YOYO-1 in the comet assay with tobacco plants after treatment with ethyl methanesulphonate, hyperthermia and DNase-I. *Mutat Res*, **605**, 17-21.
91. Olive, P.L., Banáth, J.P. and Fjell, C.D. (1994) DNA strand breakage and DNA structure influence staining with propidium iodide using the alkaline comet assay. *Cytometry*, **16**, 305-312.
92. Ward, T.H. and Marples, B. (2000) Technical report: SYBR Green I and the improved sensitivity of the single-cell electrophoresis assay. *Int J Radiat Biol*, **76**, 61-65.
93. McNamee, J.P., McLean, J.R., Ferrarotto, C.L. and Bellier, P.V. (2000) Comet assay: rapid processing of multiple samples. *Mutat Res*, **466**, 63-69.

94. Malyapa, R.S., Bi, C., Ahern, E.W. and Roti Roti, J.L. (1998) Detection of DNA damage by the alkaline comet assay after exposure to low-dose gamma radiation. *Radiat Res*, **149**, 396-400.
95. Nadin, S.B., Vargas-Roig, L.M. and Ciocca, D.R. (2001) A silver staining method for single-cell gel assay. *J Histochem Cytochem*, **49**, 1183-1186.
96. Obach, R.S. and Dobo, K.L. (2008) Comparison of metabolite profiles generated in Aroclor-induced rat liver and human liver subcellular fractions: considerations for in vitro genotoxicity hazard assessment. *Environ Mol Mutagen*, **49**, 631-641.
97. Tug, T., Ickstadt, K., Kunz, M., Sutter, A. and Igl, B.W. (2020) Statistical analysis of in vivo alkaline comet assay data - Comparison of median and geometric mean as centrality measures. *Regul Toxicol Pharmacol*, 104808.
98. Cortes-Gutierrez, E.I., Fernandez, J.L., Davila-Rodriguez, M.I., Lopez-Fernandez, C. and Gosalvez, J. (2017) Two-Tailed Comet Assay (2T-Comet): Simultaneous Detection of DNA Single and Double Strand Breaks. *Methods Mol Biol*, **1560**, 285-293.
99. Boldbaatar, D., El-Seedi, H.R., Findakly, M., Jabri, S., Javzan, B., Choidash, B., Goransson, U. and Hellman, B. (2014) Antigenotoxic and antioxidant effects of the Mongolian medicinal plant Leptopyrum fumarioides (L): an in vitro study. *Journal of ethnopharmacology*, **155**, 599-606.
100. Durling, L.J., Busk, L. and Hellman, B.E. (2009) Evaluation of the DNA damaging effect of the heat-induced food toxicant 5-hydroxymethylfurfural (HMF) in various cell lines with different activities of sulfotransferases. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, **47**, 880-884.
101. Andersson, M.A., Petersson Grawe, K.V., Karlsson, O.M., Abramsson-Zetterberg, L.A. and Hellman, B.E. (2007) Evaluation of the potential genotoxicity of chromium picolinate in mammalian cells in vivo and in vitro. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, **45**, 1097-1106.
102. Bergqvist, M., Brattstrom, D., Stalberg, M., Vaghef, H., Brodin, O. and Hellman, B. (1998) Evaluation of radiation-induced DNA damage and DNA repair in human lung cancer cell lines with different radiosensitivity using alkaline and neutral single cell gel electrophoresis. *Cancer letters*, **133**, 9-18.
103. Collins, A.R., El Yamani, N., Lorenzo, Y., Shaposhnikov, S., Brunborg, G. and Azqueta, A. (2014) Controlling variation in the comet assay. *Frontiers in genetics*, **5**, 359.
104. Azqueta, A., Gutzkow, K.B., Brunborg, G. and Collins, A.R. (2011) Towards a more reliable comet assay: optimising agarose

concentration, unwinding time and electrophoresis conditions. *Mutat Res*, **724**, 41-45.

105. Ersson, C. and Moller, L. (2011) The effects on DNA migration of altering parameters in the comet assay protocol such as agarose density, electrophoresis conditions and durations of the enzyme or the alkaline treatments. *Mutagenesis*, **26**, 689-695.
106. Enciso, J.M., Sanchez, O., Lopez de Cerain, A. and Azqueta, A. (2015) Does the duration of lysis affect the sensitivity of the in vitro alkaline comet assay? *Mutagenesis*, **30**, 21-28.
107. Enciso, J.M., Gutzkow, K.B., Brunborg, G., Olsen, A.K., López de Cerain, A. and Azqueta, A. (2018) Standardisation of the in vitro comet assay: influence of lysis time and lysis solution composition on the detection of DNA damage induced by X-rays. *Mutagenesis*, **33**, 25-30.
108. Yendle, J.E., Tinwell, H., Elliott, B.M. and Ashby, J. (1997) The genetic toxicity of time: importance of DNA-unwinding time to the outcome of single-cell gel electrophoresis assays. *Mutation research*, **375**, 125-136.
109. Speit, G., Trenz, K., Schutz, P., Rothfuss, A. and Merk, O. (1999) The influence of temperature during alkaline treatment and electrophoresis on results obtained with the comet assay. *Toxicology letters*, **110**, 73-78.
110. Brody, J.R., Calhoun, E.S., Gallmeier, E., Creavalle, T.D. and Kern, S.E. (2004) Ultra-fast high-resolution agarose electrophoresis of DNA and RNA using low-molarity conductive media. *Biotechniques*, **37**, 598, 600, 602.
111. Varma, S. and Rempe, S.B. (2006) Coordination numbers of alkali metal ions in aqueous solutions. *Biophys Chem*, **124**, 192-199.
112. Mahler, J. and Persson, I. (2012) A study of the hydration of the alkali metal ions in aqueous solution. *Inorg Chem*, **51**, 425-438.
113. Koppen, G., Azqueta, A., Pourrut, B., Brunborg, G., Collins, A.R. and Langie, S.A.S. (2017) The next three decades of the comet assay: a report of the 11th International Comet Assay Workshop. *Mutagenesis*, **32**, 397-408.
114. Azqueta, A., Muruzabal, D., Boutet-Robinet, E., Milic, M., Dusinska, M., Brunborg, G., Moller, P. and Collins, A.R. (2019) Technical recommendations to perform the alkaline standard and enzyme-modified comet assay in human biomonitoring studies. *Mutat Res Genet Toxicol Environ Mutagen*, **843**, 24-32.
115. Sischka, A., Toensing, K., Eckel, R., Wilking, S.D., Sewald, N., Ros, R. and Anselmetti, D. (2005) Molecular mechanisms and kinetics between DNA and DNA binding ligands. *Biophys J*, **88**, 404-411.
116. Zhang, X.X., Brantley, S.L., Corcelli, S.A. and Tokmakoff, A. (2020) DNA minor-groove binder Hoechst 33258 destabilizes base-pairing adjacent to its binding site. *Commun Biol*, **3**, 525.

117. Kolbeck, P.J., Vanderlinden, W., Gemmecker, G., Gebhardt, C., Lehmann, M., Lak, A., Nicolaus, T., Cordes, T. and Lipfert, J. (2021) Molecular structure, DNA binding mode, photophysical properties and recommendations for use of SYBR Gold. *Nucleic Acids Res*, **49**, 5143-5158.
118. McMaster, G.K. and Carmichael, G.G. (1977) Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc Natl Acad Sci U S A*, **74**, 4835-4838.
119. Cosa, G., Focsaneanu, K.S., McLean, J.R., McNamee, J.P. and Scaiano, J.C. (2001) Photophysical properties of fluorescent DNA-dyes bound to single- and double-stranded DNA in aqueous buffered solution. *Photochem Photobiol*, **73**, 585-599.
120. Feng, B., Sosa, R.P., Martensson, A.K.F., Jiang, K., Tong, A., Dorfman, K.D., Takahashi, M., Lincoln, P., Bustamante, C.J., Westerlund, F. *et al.* (2019) Hydrophobic catalysis and a potential biological role of DNA unstacking induced by environment effects. *Proceedings of the National Academy of Sciences of the United States of America*, **116**, 17169-17174.
121. Stewart, R. and Harris, M.G. (1977) Amino group acidity in nucleotide bases. *Canadian Journal of Chemistry*, **55**, 3807-3814.
122. Verdolino, V., Cammi, R., Munk, B.H. and Schlegel, H.B. (2008) Calculation of pKa values of nucleobases and the guanine oxidation products guanidinohydantoin and spiroiminodihydantoin using density functional theory and a polarizable continuum model. *J Phys Chem B*, **112**, 16860-16873.
123. Feliciello, I. and Chinali, G. (1993) A modified alkaline lysis method for the preparation of highly purified plasmid DNA from Escherichia coli. *Anal Biochem*, **212**, 394-401.
124. Stadler, J., Lemmens, R. and Nyhammar, T. (2004) Plasmid DNA purification. *J Gene Med*, **6 Suppl 1**, S54-66.

// Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy 339

Editor: The Dean of the Faculty of Pharmacy

A doctoral dissertation from the Faculty of Pharmacy, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy".)

ACTA UNIVERSITATIS
UPSALIENSIS
2023

Distribution: publications.uu.se
urn:nbn:se:uu:diva-510750