

# Investigation of genome instability in colon carcinoma cells caused by methotrexate

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Lina Liljenfeldt



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Abstract Methotrexate is a commonly used chemotherapy drug which acts by blocking the metabolism of folic acid, necessary to make DNA, in cells. In this project, it was investigated whether methotrexate causes damage to the genome of different colon carcinoma cancer cell lines. Two genome instability assays were used: the cytokinesis-block micronucleus assay and the comet assay. The conclusion from the obtained results was that methotrexate did not appear to harm the genome, but the effect of methotrexate on genome stability should be investigated further to corroborate these results.		
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Supervisors <b>Donna Albertson</b> <b>Comprehensive Cancer Center, University of California, San Francisco</b>		
Scientific reviewer <b>Anders Isaksson</b> <b>Department of Genetics and Pathology, Uppsala University</b>		
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<b>Biology Education Centre</b> Box 592 S-75124 Uppsala	<b>Biomedical Center</b> Tel +46 (0)18 4710000	<b>Husargatan 3 Uppsala</b> Fax +46 (0)18 555217

# **Investigation of genome instability in colon carcinoma cells caused by methotrexate**

**Lina Liljenfeldt**

## **Sammanfattning**

Methotrexate är en folsyra-antagonist och används ofta inom kemoterapi. Läkemedlet fungerar genom att blockera metabolismen av cellers folsyra, vilket har en betydande del i syntesen av DNA och RNA. Indirekt kan man alltså säga att methotrexate blockerar syntesen av DNA vilket gör den lämplig att använda som kemoterapeutiskt läkemedel.

Mitt projekt gick ut på att se om methotrexate skadar tarmcancer cellers genom. Min handledare använder ofta methotrexate i sin forskning och ville försäkra sig om det inte skadar genomet hos cancercellerna. Man har i vissa fall kunnat se att användandet av methotrexate orsakar DNA brott.

För att undersöka detta fick jag använda mig av två olika instabilitetsassayer. Den första är kometsvanstestet, även kallat Comet assay. Den andra är en assay som blockerar cytokinesen hos celler, kallas Cytokinesis-block micronucleus assay (CBMN).

Comet assay är en enkel metod att registrera DNA-strängsbrott med hos enskilda celler.

Metoden baserar sig på att denaturerade DNA fragment kan migrera ut ur cellen under påverkan av ett elektriskt fält, medan oskadat DNA stannar inuti cellkärnan. Detta resulterar i en kometsvans där storleken på svansen används till att mäta nivån av DNA skador.

CBMN assayen går ut på att mäta frekvensen av mikrokärnor i celler och därmed frekvensen av kromosombrott. Detta gör man genom att blockera cytokinesen hos cellerna vilket begränsar metoden till delande celler, vilket är de som producerar mikrokärnor.

Jag utförde dessa två instabilitetsassayer på fem olika tarmcancer cell-linjer och med fyra olika methotrexate koncentrationer, samt negativ och positiv kontroll. Tyvärr fick jag inga resultat från mitt CBMN försök på grund av trasig maskinvara. Däremot fick jag mycket data från mina Comet assay försök och de resultat jag fick fram pekar mot att methotrexate inte skadar genomet hos de tarmcancer cell-linjer jag använde mig av.

**Examensarbete 20p i Civilingenjörsprogrammet Molekylär bioteknik**

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# 1. Introduction

## 1.1 Cancer

Cancer is characterized by an uncontrolled cell division which leads to an aberrant tissue growth. The unregulated growth is caused by mutations or changes in expression of genes that encode for proteins controlling cell division and for survival factors. These alterations can occur due to external or hereditary factors.

The uncontrolled cell growth leads to the formation of either a benign tumour or a malignant tumour, cancer. Benign tumours are not cancer and they do not spread to other parts of the body. Malignant tumours on the other hand have the potential to spread to other parts of the body and invade other organs, which makes cancer fatal.

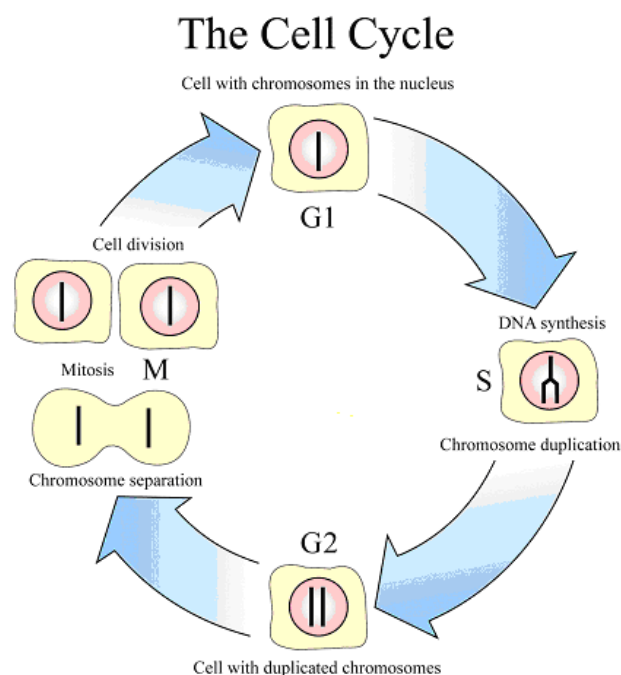
## 1.2 Genome aberrations

Development of tumours involves the acquisition of genetic and epigenetic alterations and corresponding changes in gene expression that modify normal growth control and survival pathways. These alterations can depend on several things, such as point mutations, epigenetic mechanisms and aneuploidy. [1]

There are two mechanisms involved in aneuploidy (a change in the number of chromosomes that can lead to a chromosomal disorder) in somatic cells: nondisjunction of chromosomes during anaphase and chromosome loss. In the first mechanism one daughter cell becomes trisomic and the other monosomic due to an abnormal segregation. In the second process the lagging chromosome may be lost, it can form a micronucleus or it can be randomly incorporated into either of the daughter nuclei. [13]

Studying DNA damage at the chromosome level is an important part of genetic toxicology since chromosomal mutation is an important event in carcinogenesis.

## 1.3 The Cell Cycle



**Figure 1.** Schematic overview of the cell cycle. Picture used with permission from Nobelprize.org

The G1 phase: while the cell carries on with its usual metabolic activities it is also preparing to duplicate itself. Preparations include increasing the amount of cytoplasm and mitochondria.

In G1 a diploid cell has a complement of 2N chromosomes, where N is the number of germ cell chromosomes.

The end of the G1 phase is a “point of no return” in which the cell is committed to dividing.

The S phase: DNA synthesis i.e. the cell duplicates its DNA.

The G2 phase: Now the cell has a complement of 4N chromosomes. The cell continues with metabolism and growth, preparing to undergo mitosis.

The M phase: The cell segregates its chromosomes so that the two daughter cells receive a complement of 2N. There are four stages of mitosis – prophase, metaphase, anaphase and telophase. Telophase is accompanied by cytokinesis, the division of the cytoplasm, and the two new daughter cells are in G1 phase again.

#### **1.4 The cytokinesis-block micronucleus assay**

The cytokinesis-block micronucleus (CBMN) assay is used to measure micronuclei, nucleoplasmic bridges (NPBs), nuclear buds, and cell death (necrosis or apoptosis). The cytokinesis-block micronucleus assay is based on the blocking of cytokinesis by cytochalasin-B so that scoring is restricted to dividing cells which are the cells that express micronuclei produced as a result of chromosome breakage. The use of cytochalasin-B eliminates the confusing effect of altered cell division on micronuclei expression.

The micronucleus index in human cells is today one of the standard tests for genetic toxicology testing. [2, 3, 11]

#### **1.5 The Comet Assay**

The Comet Assay or single-cell gel electrophoresis was developed and optimized in the mid-1980s to early 1990s to analyze DNA damage in individual cells. [10] This assay is widely used in research, genotoxicity investigations, and as a pre-screening assay in the pharmaceutical industry. [14] Before the Comet Assay it was only possible to get a measurement of the average response of a population. This is a big limitation since the response of a tumor to a cytotoxic treatment is often not dependent on the average amount of DNA damage in the cells but on the response of a small population of cells. [10]

The Comet Assay is based on the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of an electric field, while undamaged DNA migrates slower and therefore remains within the nucleus when a current is applied. This will result in a DNA ‘comet’ tail shape and migration pattern which allows for the measurement of DNA damage. This gives a good indication of whether all cells are responding similarly to a cytotoxic treatment or whether there is a wide heterogeneity among the individual cells. [6]

Analysis of the comets is carried out on a computer; in this degree project CometScore was used.

A limitation with the Comet Assay is the fact that it requires the use of a single-cell suspension which makes it impossible to get information on the location of a cell in a tumor. This limitation can be solved by combining the Comet Assay with cell sorting and selection methods [10]. This application will not be further discussed report.

## **2. Aim of this project**

Methotrexate (mtx) is an antimetabolite drug which means that it is capable of blocking the metabolism of folic acids in cells. Folic acid is needed for the *de novo* synthesis of the nucleoside thymidine, required for DNA synthesis. Methotrexate, therefore, inhibits the synthesis of DNA, RNA, thymidylates proteins. It is therefore widely used as a chemotherapeutic drug.

An effect of methotrexate administration is a cycling process of deoxyuracil monophosphate (dUMP) removal, reincorporation and removal. This can lead to a progressive accumulation of DNA strand breaks. [8]

The cytotoxic effect of methotrexate has earlier been investigated and the activation of the apoptotic pathway in response to methotrexate has been demonstrated by the presence of typical hallmarks such as chromatin condensation, nuclear fragmentation, internucleosomal DNA cleavage and cell cycle perturbations. [8]

In this degree project, my task was to investigate how much damage methotrexate causes to the genome of different colon carcinoma cell lines by exposing them to different concentrations of methotrexate. The levels of damage on these treated cells were investigated by performing the instability assays Cytokinesis-block micronucleus assay and Comet assay.

### 3. Materials and methods

#### 3.1 Cell lines

Here follows a brief description of the used cell lines in this study.

##### 3.1.1 HCT116 + chr3

HCT116 is a human colon carcinoma cell line which is mismatch repair deficient (MMR-deficient) due to mutation and silencing of *MLH1*. The HCT116+chr3 cell line on the other hand is MMR-proficient, because the hMLH1 expression was restored by a chromosome 3 transfer. [8, 9]

##### 3.1.2 KU86 +/-

KU86 is a cell line that arises from HCT116, deficient for the stability gene KU86. The protein KU binds to double stranded DNA ends, including DNA with blunt ends or single strand overhangs, stem-loop structures and telomeres. KU takes part in non homologous end-joining repair of DNA double strand breaks. It also has a role in the initiation of DNA replication and in telomere maintenance. [9]

##### 3.1.3 BLM-/- and BLM+/-

Also this is a cell line that arises from HCT116, deficient for the stability gene BLM. Mutations in BLM cause Bloom Syndrome, which is an autosomal recessive disorder. BLM is a member of a family of RecQ helicases, which unwind double stranded DNA and are necessary for DNA replication and repair. [9]

##### 3.1.4 266C3

266C3 is a cell line in which HCT116 is generated MMR proficient by transfection of a retrofitted BAC carrying *MLH1*. [9]

#### 3.2 Chemicals

##### 3.2.1 Methotrexate

The main target of methotrexate is dihydrofolate reductase (DHFR), an enzyme that plays a key role in nucleotide metabolism. Inhibition of DHFR leads to the impairment of new DNA synthesis. [8] Methotrexate is therefore said to inhibit the synthesis of DNA, RNA, proteins and thymidylates.

Methotrexate is cell-cycle S-phase selective and its effect is greater on rapidly dividing cells which are replicating their DNA and therefore inhibits the growth of these cells.

### 3.2.2 Cytochalasin-B

Cytochalasin-B is a cell-permeable mycotoxin which inhibits cytoplasmic division by blocking the formation of contractile microfilaments, i.e. blocks the cytokinesis. [2]

### 3.3 Cell culture and methotrexate treatment

The cells were cultured in Dulbecco's Modified Eagle Medium, D-MEM High Glucose (UCSF Cell Culture Facility) with 10% fetal bovine serum (FBS) and 1 x nonessential amino acids at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The media for the HCT116+chr3 and 266C3 was supplemented with 400 µg/ml geneticin disulfate, an aminoglycoside antibiotic which acts as a selective agent of transfected mammalian, yeast, plant and bacteria cells. Methotrexate was added to the media in four different concentrations 25nM, 50nM, 100nM and 200nM. The cells were, after a non determined culture time, resuspended in the media with methotrexate and exposed to it for 36 hours.

### 3.4 Cell counting

Cells were counted using a hemacytometer, to obtain  $1 \times 10^5$  cells/ml for each methotrexate concentration measurement and cell line for the Comet assay. Approximately 500-1000 cells were used per Cometslide.

Cells for the cytokinesis-block micronucleus assay were counted using the same method and a cell concentration of  $0.5 \times 10^6$  cells/ml was used for each methotrexate concentration and cell line.

### 3.5 Cytokinesis-block micronucleus assay

After the methotrexate treatment the cells were washed with Modified Eagle Medium, D-MEM High Glucose with 10% FBS and 1 x nonessential amino acids, and resuspended in media supplemented with 4,5 µg/ml cytochalasin-B on coverslips. After a culture time of 28 hours the cells were washed with PBS. Fixation and staining were carried out using Diff Quik, a dip stain purchased from VWR Scientific.

### 3.6 Comet assay

A brief summary of the assay is that cells are embedded in agarose and placed on a slide. The slide is then submersed in a lysis solution, an alkaline solution, rinsed to remove salt and exposed to a low voltage.

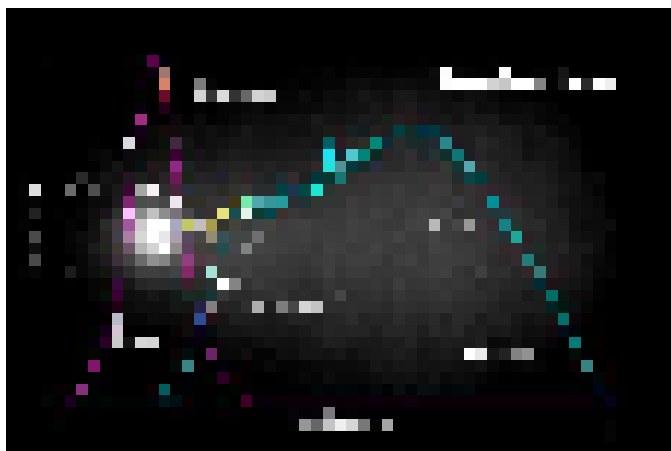
For each cell line, 6 samples were prepared – 1 negative control (no methotrexate), 4 samples treated with 25nM, 50nM, 100nM and 200nM methotrexate respectively, and 1 positive control. The positive control was carried out using 100µM hydrogen peroxide for 30 minutes on ice which gave good comets.

Cells were harvested and resuspended in 1X PBS, Ca<sup>++</sup> and Mg<sup>++</sup> free, that the PBS is calcium and magnesium free is important to inhibit endonuclease activities. Agarose was prepared according to the Comet assay instructions and then mixed with the cell samples and dropped on the comet slides. The slides were placed at -4°C in the dark for 30 min to allow the agarose to set before they were immersed in a lysis solution for 60 minutes. An alkaline solution was prepared and the slides were immersed in the solution for 60 minutes. The alkaline solution unwinds and denatures DNA and hydrolyzes the sites of damage.

The slides were then washed in TBE buffer twice, for 5 minutes. The slides were then placed in a horizontal electrophoresis apparatus and run for 10 minutes at 20V. Slides were placed in 70% alcohol for 5 minutes and then air dried. Slides were stained with SYBR 1 Green, diluted with TE buffer. An anti-fading solution was also applied before looking at the slides in the fluorescence microscope. [6]

### 3.7 CometScore

Image analysis methods vary but are fairly comparable; there are commercial and Web-based programs available. CometScore™ is used in this article and is a free comet scoring software that easily scores different metrics of the comet; such as tail length, tail area, % DNA in the tail and tail moment.[14]



**Figure 2.** A schematic figure over the different metrics that CometScore scores. The picture is published with permission from AutoComet.com

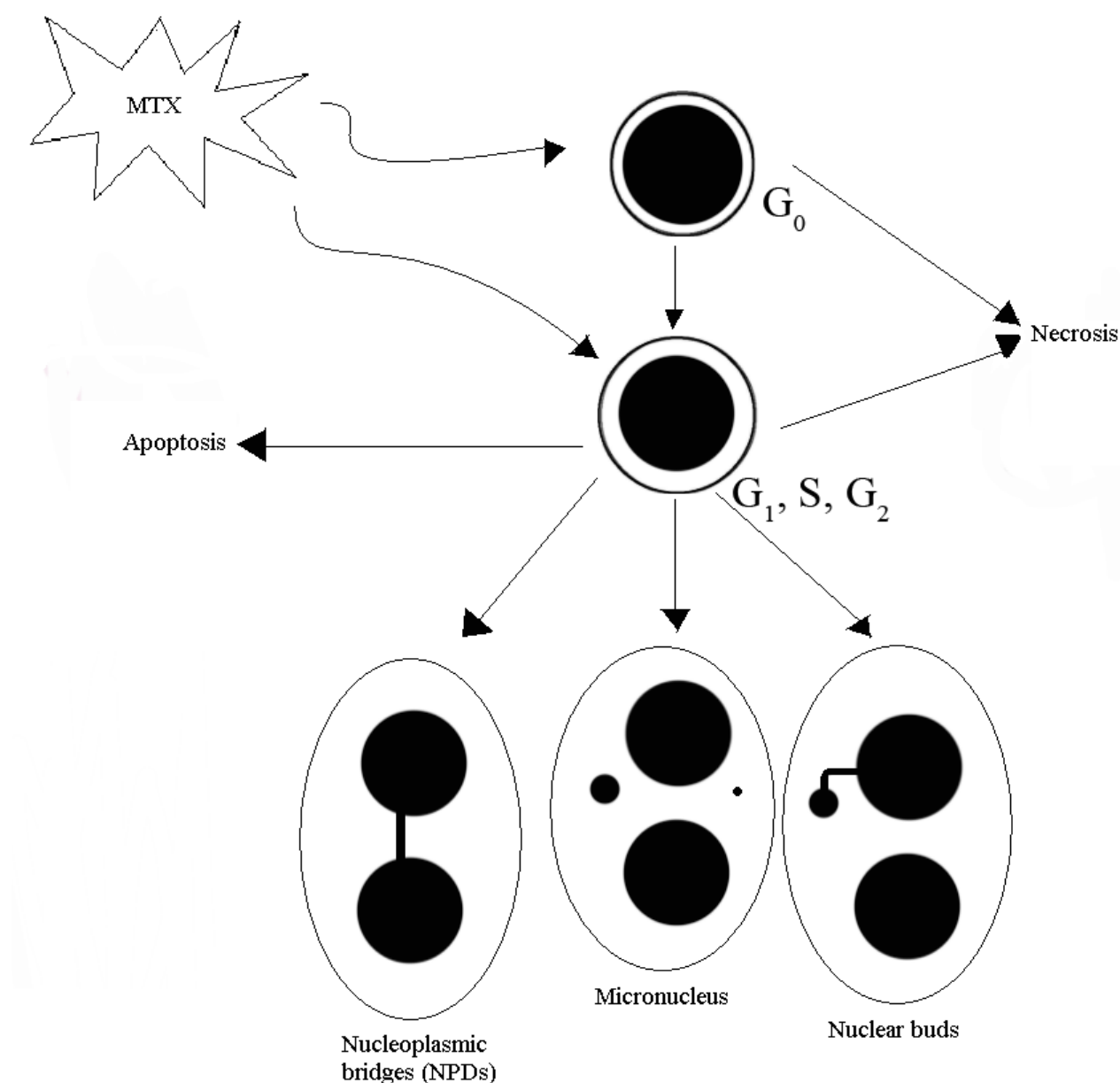
We have in this report concentrated on the comet length, comet area, % DNA in tail and tail length parameters since we found them the most interesting. % DNA in tail gives information about the distribution of DNA between the tail and the head of comet and can be used to evaluate the degree of DNA damage. [6]

CometScore was used to obtain the results that follow.

## 4. Results

### 4.1 Cytokinesis-block micronucleus assay

No results from the cytokinesis-block micronucleus assay could be obtained since the microscope needed broke down and no replacement microscope could be found. Here are some of the possible outcomes of the cytokinesis-block micronucleus assay.



**Figure 3.** The possible fates of cultured cytokinesis-blocked cells following exposure to methotrexate.

#### 4.1.1 Nucleoplasmic bridges (NPDs)

NPDs are a measure of chromosome rearrangement. NPDs are assumed to occur when the centromeres of dicentric chromosomes are pulled to opposite poles of the cell during anaphase. Normally it is almost impossible to observe these dicentric anaphase bridges before

the formation of the nuclear membrane, because cells go through the anaphase and telophase so rapidly, completing cytokinesis and breaking the NPD when the daughter cells separate. However, in the cytokinesis-block micronucleus assay cytokinesis is blocked which gives NPDs the opportunity to accumulate and the nuclear membrane can form around the chromosomes, allowing NPDs to be observed. [11]

#### **4.1.2 Micronucleus**

Micronucleus formation is a hallmark of genetic toxicity; as such, micronuclei are used as indicators of genotoxicity caused by drug candidates. Micronucleus form when chromosome fragments lag behind at anaphase during nuclear division. The cytokinesis-block micronucleus assay is the preferred method for measuring micronucleus since scoring is restricted to once-divided cells, which can be recognized by having two nucleuses (binucleated cells) after inhibition of cytokinesis by Cytochalasin-B. Restricting scoring of micronucleus to binucleated cells prevents confounding effects caused by suboptimal cell division kinetics which is a major variable in the cytokinesis-block micronucleus assay. [1, 2]

#### **4.1.3 Nuclear buds**

Nuclear buds are characterized by having the same morphology as a micronucleus with the exception that they are linked to the nucleus by a stalk of nucleoplasmic material. Nuclear budding occurs in the S-phase but the duration of the process and the extrusion of the micronucleus are unknown.

A hypothesis is that the nucleus has the ability to sense excess DNA that does not fit well within the nuclear matrix and that it can eliminate this excess DNA by forming it to a micronucleus and then exclude it from the cell by extrusion of the micronucleus. Nuclear budding is therefore a marker of gene amplification and/or altered gene dosage.

Irradiation is also believed to cause nuclear buds. [11]

#### **4.1.4 Apoptosis and necrosis**

The cytokinesis-block micronucleus assay can also measure cell death, both accidental cell death (necrosis) and programmed cell death (apoptosis). [11]

#### **4.1.5 Scoring Cytokinesis-block micronucleus assay**

The cytokinesis-blocked cells scored for micronucleus frequency have to satisfy the following criteria:

- (i) Cells should have two nuclei of approximately equal size
- (ii) Cells should not contain more than 6 MN
- (iii) The 2 nuclei may be attached by a fine nucleoplasmic bridge
- (iv) The 2 nuclei may overlap slightly or touch each other at the edges

Criteria for identifying micronuclei.

MN are morphologically identical to, but smaller than normal nuclei. They also have the following characteristics:

- (i) A diameter between 1/16 and 1/3 that of the main nuclei
- (ii) They are non-refractile
- (iii) They are not linked to the main nuclei via a nucleoplasmic bridge
- (iv) MN may sometimes overlap the boundaries of the main nuclei

[2]

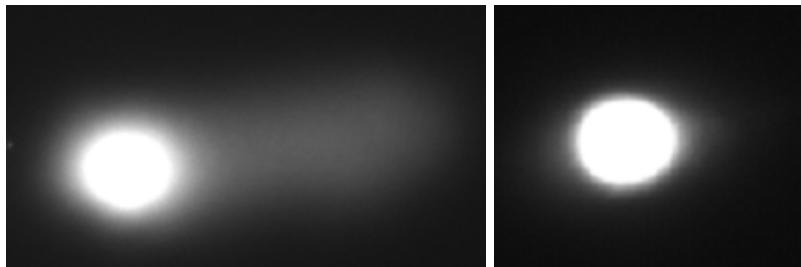
In this cytokinesis-block micronucleus assay experiment, Diff-Quik was used to stain the cells. The plan was then to take images of all the slides and score manually using these images. But unfortunately the equipment needed was not working. I did look at the cells though and could see binucleated cells and some micronucleus but that is nothing I can base

any conclusions from, neither could I see any differences between the different cell lines or methotrexate treatments.

Scoring micronucleus can also be done using an application that automatically identifies and quantifies micronuclei. An example of such application is the Micronucleus BioApplication from Cellomics, Inc.

## 4.2 Comet assay

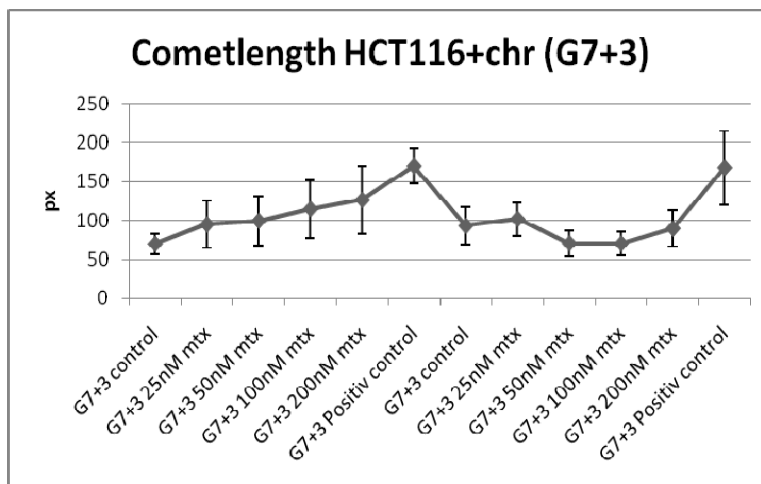
For every Cometslide several pictures were taken and at least 75 cells were measured for each Cometslide. Average values and standard deviations were calculated for cometlength, cometarea, % DNA in tail and tail length. A table of all these values for two experiments can be seen in appendix 1 & 2. In the appendices 3 to 10 figures over all the parameters and cell lines KU86, BLM+/-, BLM-/- and 266C3 can be found. The values to the left are from the first experiment and the values to the right are from the second experiment.



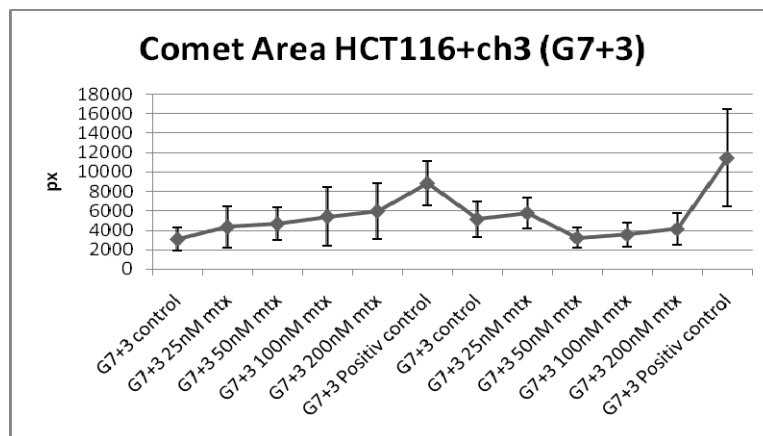
**Figure 4 and 5.** These two images show the obtained comets from the HCT116+chr3 cell line. To the left is a comet from the positiv control test and to the right is a comet from the cells treated with 200nM methotrexate.

### 4.2.1 HCT116 + chr3

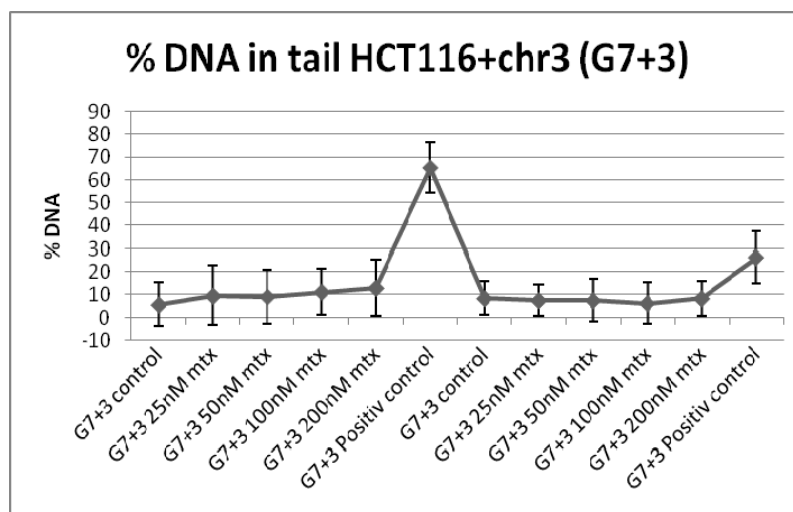
By looking at the figures that follows a modest increase in comet length, comet area, % DNA in tail and tail length can be seen for HCT116 in the first experiment; however the differences are not significant. In the second experiment no increase was seen.



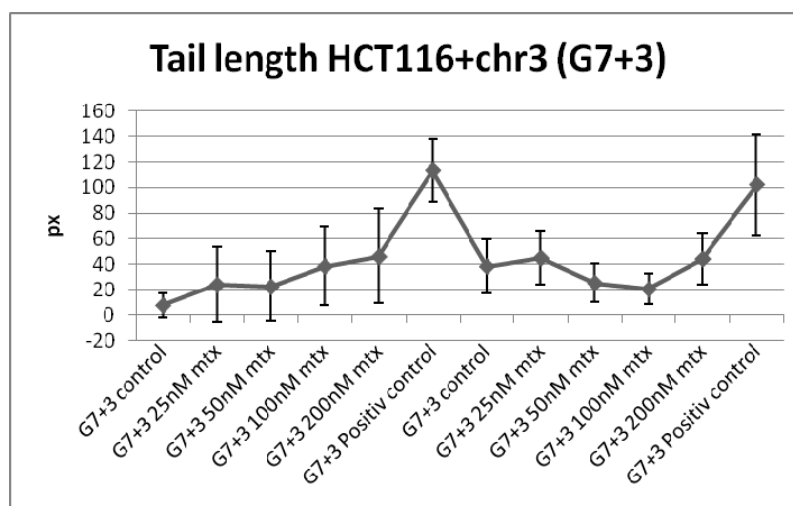
**Figure 6.** Cometlengths (px) for the cell line HCT116 + chr3. A small but not significant increase can be seen in the first experiment, although not in the second experiment.



**Figure 7.** Comet areas (px) for the HCT116 + chr3 cells. The comet area increases for the first experiment but not for the second.



**Figure 8.** This figure represents the measured % DNA in the tail of the HCT116 + chr3 cells. The % DNA remains quite similar for the different methotrexate concentrations and no significant increase can be distinguished.



**Figure 9.** The tail lengths (px) for the cell line HCT116 + chr3. The tail length varies and no increase can be seen.

#### 4.2.2 KU86

In the first experiment a very small increase in comet length was seen and in % DNA in tail, although in the other parameters no change in data was distinguished.

In the second experiment no increase for any of the parameters was seen. See appendix 3 and 4.

#### 4.2.3 BLM +/-

Looking at the data from the first and second experiments no change in the measured parameters could be distinguished. See appendix 5 and 6.

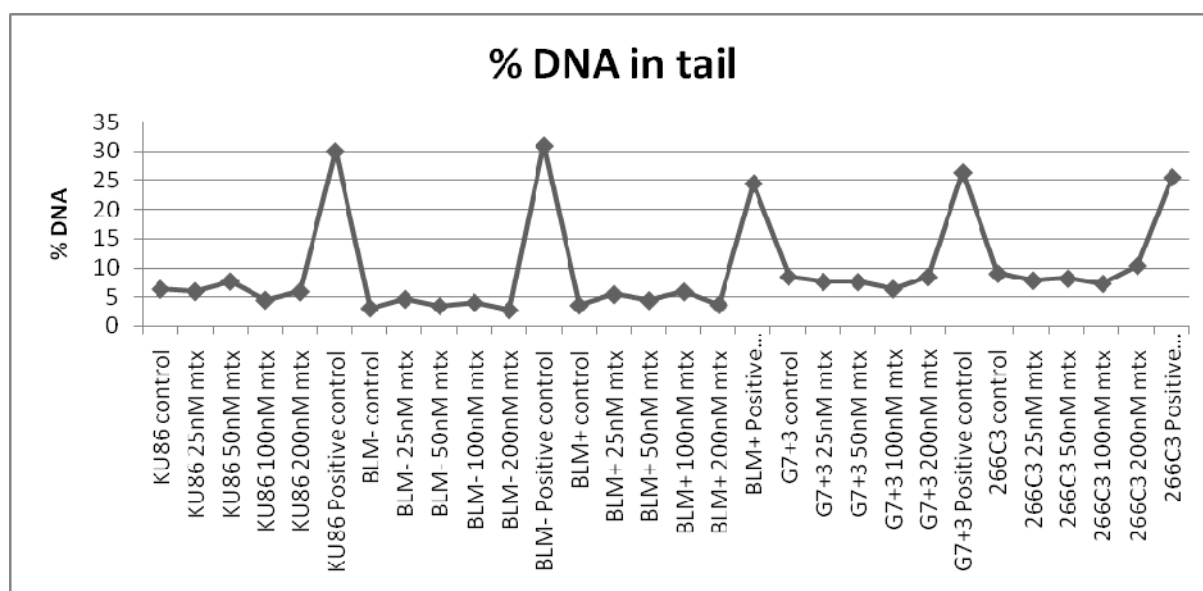
#### 4.2.4 BLM-/-

In both experiments no increase in any measurements could be detected. See appendix 7 and 8.

#### 4.2.5 266C3

The first experiment has unusually low values for % DNA in tail and tail length. It seems like the electrophoresis did not work as it should have. However, the second experiment appeared to have worked and in this experiment no increase in parameters could be demonstrated. See appendix 9 & 10.

#### 4.2.6 % DNA in tail



**Figure 5.** A schematic overlook of the average % DNA in tail parameter for all cell lines; KU86, BLM-/-, BLM+/-, G7+3(HCT116+ chr3) and 266C3. From left to right is the value for the control sample (no mtx), 25nM mtx, 50nM mtx, 100nM mtx, 200nM mtx and the positive control sample. The values are taken from the second experiment. No increase in parameter value for the methotrexate treated cell lines. Although the positive control shows that the comet assay does work.

The parameter % DNA in tail makes it possible to evaluate the degree of DNA damage. By looking at figure 5 it is clear that the amount of DNA that has migrated out of the cell is very little. Clearly is also that the positive control with hydrogen peroxide caused a lot of DNA damage.

This figure is also representative for the other parameters, no increase in parameter value but a positive control that does show that the comet assay works.

## 5. Discussion

In this study, potential DNA damage caused by methotrexate was examined in the colon cancer cell lines HCT116 +chr3, KU86, BLM +/-, BLM-/- and 266C3 by performing the cytokinesis-block micronucleus assay and comet assay.

For the comet assay 6 samples were tested for each cell line; one sample that had not been exposed to methotrexate, 4 samples that had been treated with 25nM, 50nM, 100nM and 200nM methotrexate respectively and one positive control sample which was treated with hydrogen peroxide to obtain comets.

Looking at the possible outcomes for the cytokinesis-block micronucleus assay I would assume that I could have seen micronucleus and maybe some nucleoplasmic bridges but it is impossible to say to what extent. As I earlier mentioned I did see binucleated cells with micronucleus. I did not see any nuclear bridges or nuclear buds though. Calculating the micronucleus index would have given an indicator of genotoxicity caused by methotrexate. Looking at the results from my Comet assay I would suspect that the index should have been low also.

By looking at the results from the Comet assay I can only draw the conclusion that methotrexate does not give the genome as much damage as we thought before the experiments were done.

The parameter “% DNA in tail” is the one I find most important since it clearly shows how much DNA that has migrated out of the cell. Looking at the tables in appendix 1 and 2 and comparing the data for % DNA in tail I find that methotrexate does not seem to cause any damage to the DNA.

The positive control with hydrogen peroxide does show that the Comet assay is working. Despite that, no detectable effect can be distinguished for methotrexate. Possible reasons for that could be that the methotrexate exposure time was too short, that the methotrexate concentration was too low to cause any damage or that methotrexate just does not have a damaging effect on these carcinoma cell lines.

## 6. Acknowledgements

I would like to thank my supervisor Donna Albertson for giving me the opportunity to do my degree project in her lab. I would also want to thank Bing Huey for taking the time to help me and answer all my questions. Many thanks to everyone in the Albertson lab for making me enjoy my stay at the Cancer Center. Finally I would like to thank my scientific reviewer Anders Isaksson at Uppsala University for helping me with the writing of this report.

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### Websites

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## Appendix 1

First experiment	Cometlength (px)	Comet Area (px)	%DNA in Tail	Tail Length (px)
KU86 control	61,16 ± 11,64	2256,16 ± 771,45	7,72 ± 15,75	6,31 ± 9,42
KU86 25nM mtz	61,72 ± 11,96	2271,09 ± 736,65	8,19 ± 17,26	6,03 ± 9,8
KU86 50nM mtz	63,72 ± 11,55	2673,84 ± 926,83	9,01 ± 19,16	5,74 ± 10,66
KU86 100nM mtz	64,3 ± 11,99	2566,95 ± 887,2	9,8 ± 21,68	7,1 ± 13,67
KU86 200nM mtz	65,53 ± 12,07	2552,41 ± 803,21	11,28 ± 23,01	7,83 ± 12,83
BLM- control	55,34 ± 10,28	2242,95 ± 792,32	4,39 ± 13,02	1,92 ± 5,84
BLM- 25nM mtz	74,33 ± 17,06	3409,74 ± 1214,95	7,01 ± 18,96	5,53 ± 13,66
BLM- 50nM mtz	52,65 ± 8,97	1971,48 ± 685,36	3,73 ± 13,17	1,65 ± 5,71
BLM- 100nM mtz	83,83 ± 17,12	3715,87 ± 1419,33	10,16 ± 18,85	9,85 ± 13,6
BLM- 200nM mtz	65,66 ± 16,54	2766,69 ± 1240,39	9,13 ± 17,75	6,31 ± 10,34
BLM+ control	71,09 ± 15,76	3106,99 ± 1090,1	9,27 ± 21,25	6,38 ± 12,76
BLM+ 25nM mtz	69,43 ± 13,79	2898,79 ± 1076,8	6,31 ± 13,97	5,29 ± 8,87
BLM+ 50nM mtz	67,96 ± 11,84	2649,1 ± 840,49	7,74 ± 17,45	6,56 ± 11,54
BLM+ 100nM mtz	88,35 ± 22,84	3606,48 ± 1469,44	11,74 ± 14,88	18,97 ± 19,41
BLM+ 200nM mtz	76,19 ± 17,93	3025,04 ± 976,89	10,72 ± 22,6	10,74 ± 16,21
G7+3 control	69,78 ± 13,22	3072,46 ± 1206,57	5,7 ± 9,6	7,68 ± 9,45
G7+3 25nM mtz	94,77 ± 30,4	4338,53 ± 2118,12	9,6 ± 13,01	24,09 ± 29,46
G7+3 50nM mtz	99,25 ± 31,99	4664,53 ± 1688,96	9,22 ± 11,82	22,54 ± 17,6
G7+3 100nM mtz	114,67 ± 37,19	5409,93 ± 2985,35	11,31 ± 9,91	38,62 ± 31,17
G7+3 200nM mtz	126,38 ± 43,89	5966,41 ± 2906,3	13,07 ± 12,23	46,24 ± 36,91
G7+3 Positive control	169,91 ± 22,96	8835,89 ± 2271	65,51 ± 11,05	113,19 ± 24,35
266C3 control	63,96 ± 15,69	3120,63 ± 1621,92	1,9 ± 2,57	0,44 ± 0,9
266C3 25nM mtz	105,6 ± 27,22	6952,38 ± 2689,19	1,64 ± 3,12	0,46 ± 1,84
266C3 50nM mtz	99,76 ± 22,48	6010,93 ± 1789,64	1,98 ± 3,71	0,3 ± 1,08
266C3 100nM mtz	94,56 ± 31,19	5667,4 ± 2655,67	1,86 ± 4,03	0,45 ± 1,71
266C3 200nM mtz	72,78 ± 33,73	3493,84 ± 3114,65	1,24 ± 3,66	0,13 ± 0,49
266C3 Positive control	149,64 ± 45,62	10711,54 ± 5989,58	0,28 ± 1,47	0,27 ± 0,49

**Table 1.** The obtained average- and standard deviation values for the different cell lines and comet score parameters for the first comet assay experiment.

## Appendix 2

Second experiment	Cometlength (px)	Comet Area (px)	%DNA in Tail	Tail Length (px)
KU86 control	73,73 ± 14,49	3395,26 ± 939,87	6,33 ± 7,31	27,02 ± 13,79
KU86 25nM mtx	73,62 ± 15,41	3565,66 ± 1020,91	5,94 ± 8,5	25,62 ± 14,46
KU86 50nM mtx	90,24 ± 19,34	5195,38 ± 1809,3	7,67 ± 8,58	33,71 ± 16,39
KU86 100nM mtx	69,44 ± 23,16	3797,49 ± 2521,56	4,45 ± 6,53	2,03 ± 4,03
KU86 200nM mtx	80,96 ± 20,11	4293,21 ± 1597,08	5,88 ± 6,86	29,01 ± 17,17
KU86 Positive control	154,35 ± 52,5	8624,51 ± 3896,02	30,07 ± 12,6	101,18 ± 45,26
BLM- control	83,96 ± 16,43	5064,96 ± 1653,33	3,05 ± 5,12	24,84 ± 12,45
BLM- 25nM mtx	91,88 ± 14,98	6241,52 ± 2018,58	4,6 ± 6,77	25,45 ± 8,21
BLM- 50nM mtx	94,91 ± 17,67	5669,61 ± 1910,5	3,44 ± 4,27	36,58 ± 11,73
BLM- 100nM mtx	107,9 ± 19,52	8536,4 ± 2932,13	4 ± 5,06	30,51 ± 12,89
BLM- 200nM mtx	89,35 ± 14,76	4925,94 ± 1593,15	2,76 ± 3,36	35,41 ± 9,26
BLM- Positive control	176,1 ± 17,38	13616,56 ± 2841,92	31,05 ± 8,1	99,38 ± 14,01
BLM+ control	75,34 ± 11,87	3568,12 ± 969,62	3,55 ± 4,71	28,63 ± 9,26
BLM+ 25nM mtx	99,76 ± 15,4	7465,89 ± 2094,21	5,42 ± 4,96	26,15 ± 10,13
BLM+ 50nM mtx	80,65 ± 13,33	4243,15 ± 1206,59	4,37 ± 5,02	28,73 ± 10,86
BLM+ 100nM mtx	72,19 ± 12,29	3718,32 ± 1169,71	5,89 ± 5,62	21,65 ± 8,22
BLM+ 200nM mtx	90,1 ± 15,25	4992,76 ± 1258,74	3,66 ± 5,02	35,21 ± 13,27
BLM+ Positive Control	175,11 ± 58,1	12893,72 ± 5989,76	24,53 ± 11,68	105,21 ± 47,7
G7+3 control	92,92 ± 24,38	5138,02 ± 1827,74	8,48 ± 7,46	38,57 ± 21,79
G7+3 25nM mtx	101,6 ± 20,87	5773,99 ± 1548,94	7,59 ± 6,72	45,14 ± 20,71
G7+3 50nM mtx	70,45 ± 16,75	3206,3 ± 1049,09	7,56 ± 9,16	25,53 ± 15,07
G7+3 100nM mtx	70,1 ± 14,99	3541,25 ± 1266,48	6,36 ± 9,06	20,6 ± 12,32
G7+3 200nM mtx	89,45 ± 23,59	4122,89 ± 1654,56	8,43 ± 7,73	44,43 ± 20,17
G7+3 Positive control	167,85 ± 47,15	11448,35 ± 5036,06	26,42 ± 11,28	102,2 ± 39,53
266C3 control	95,4 ± 20,51	5008,44 ± 1455,14	9 ± 9,73	43,5 ± 18,87
266C3 25nM mtx	104,22 ± 29,75	6043,14 ± 2307,41	7,81 ± 8,1	47,13 ± 27,22
266C3 50nM mtx	102,36 ± 24,78	5800,89 ± 1853,28	8,15 ± 7,7	46,3 ± 23,58
266C3 100nM mtx	110,63 ± 46,68	6606,52 ± 3851,83	7,27 ± 6,96	52,93 ± 42,46
266C3 200nM mtx	115,42 ± 37,31	6886,77 ± 2931,42	10,37 ± 7,77	57,01 ± 34,06
266C3 Positive control	161,78 ± 47,34	10154,44 ± 4095,88	25,58 ± 11,88	101,16 ± 41,11

Table 2. Average- and standard deviation values for the second comet assay experiment.

### Appendix 3

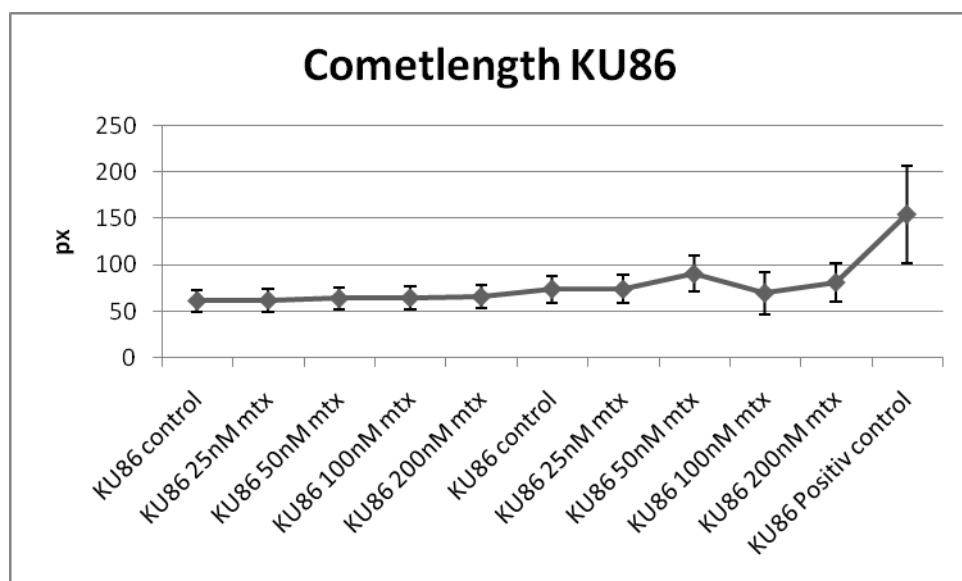


Figure A. Cometlengths (px) for the KU86 cells. A very small increase in comet length can be seen in the first experiment but not in the second experiment..

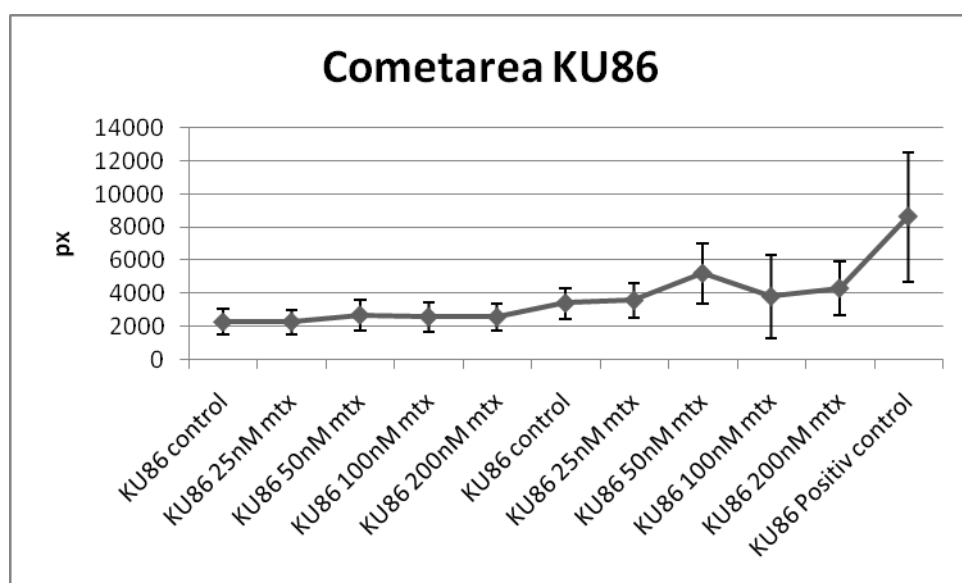


Figure B. Cometareas (px) for the cell line KU86. No increase can be distinguished for the cometarea in KU86 cells.

## Appendix 4

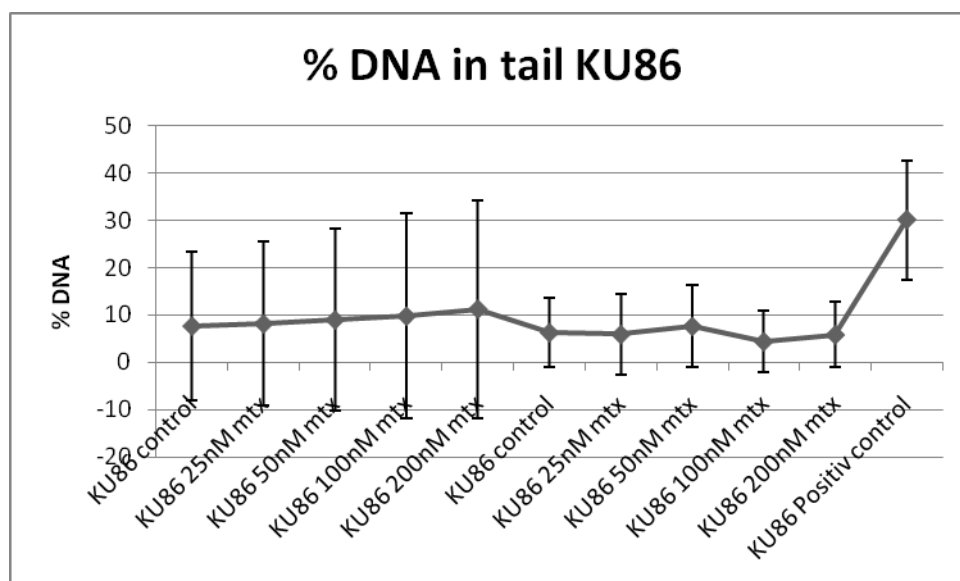


Figure C. An overview of the % DNA in the tails of the KU86 cells. A slight increase in the first experiment, with high standard deviations. No increase in the second experiment.

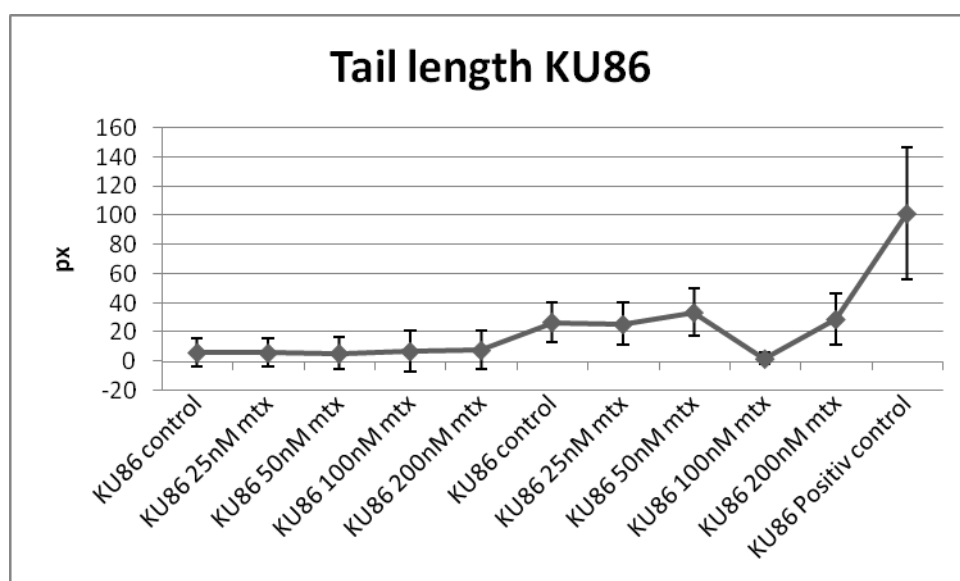


Figure D. The tail lengths (px) in the KU86 cells. The tail length does not increase in either of the experiments for KU86.

## Appendix 5

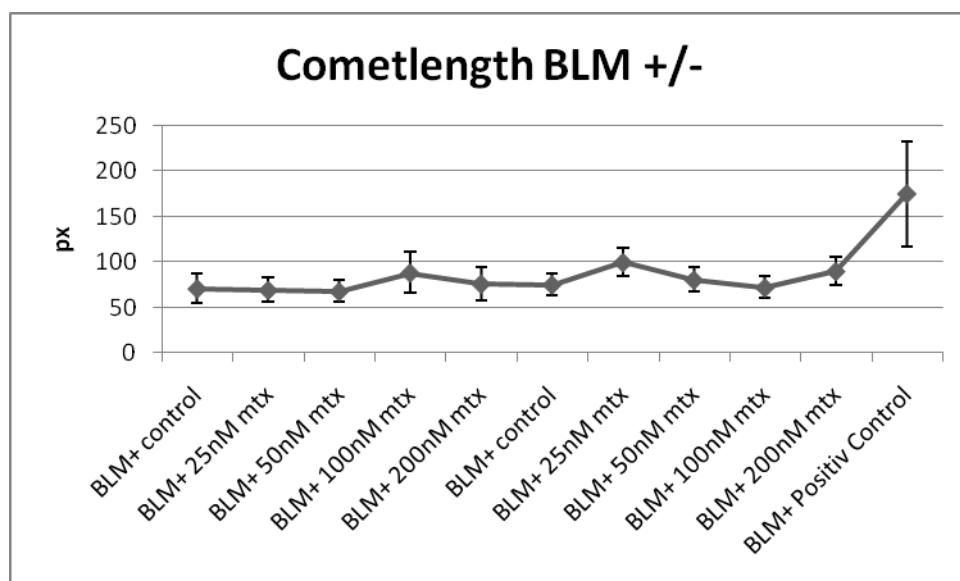


Figure E. Cometlengths (px) for the BLM +/- cells. The cometlength does not increase in either of the experiments.

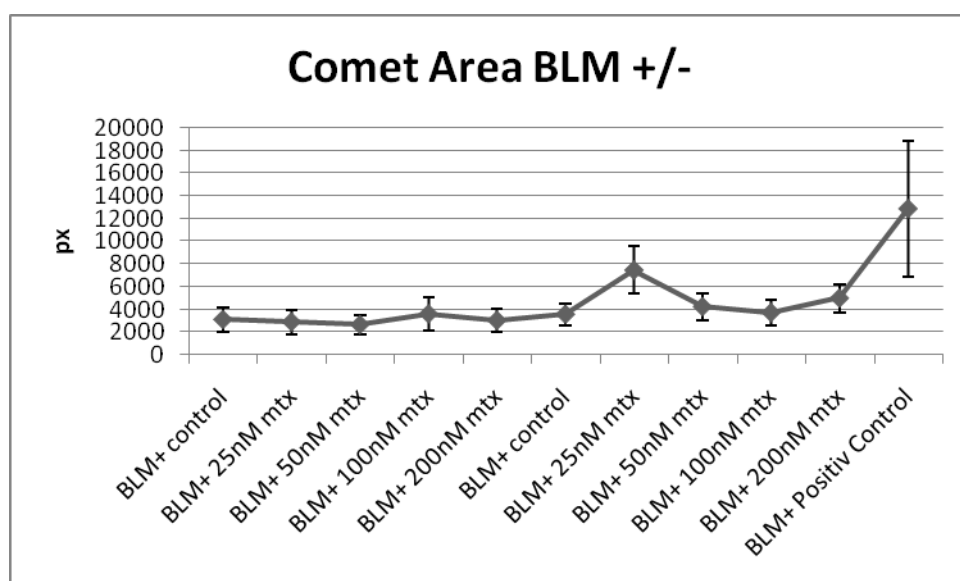


Figure F. An overview of the obtained cometareas (px) for the cell line BLM +/- . No increase in comet area for either of the experiments.

## Appendix 6

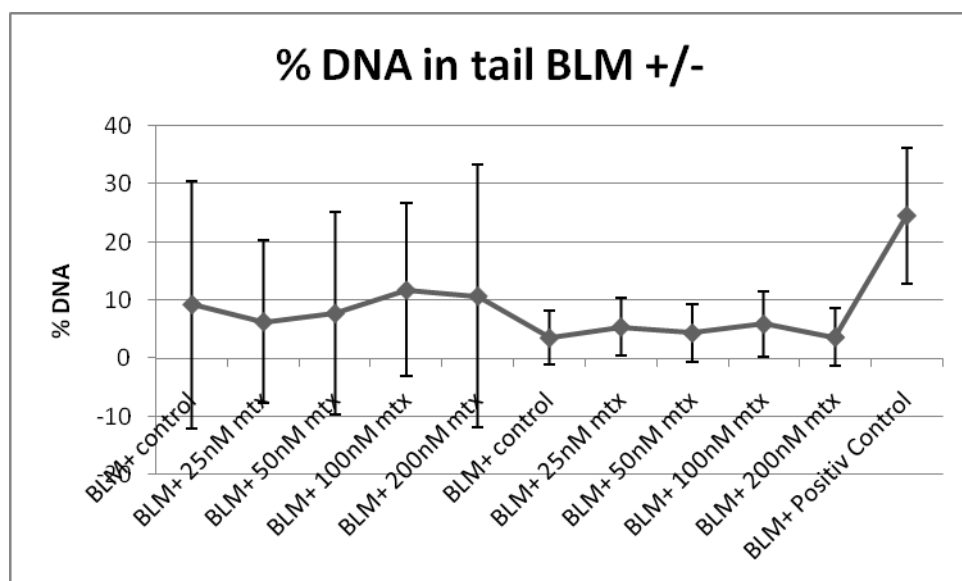


Figure G. A figure over the % DNA in the tails of the BLM +/- cells. High standard deviations for the first experiment but no increase in %DNA in the tail for the both experiments.

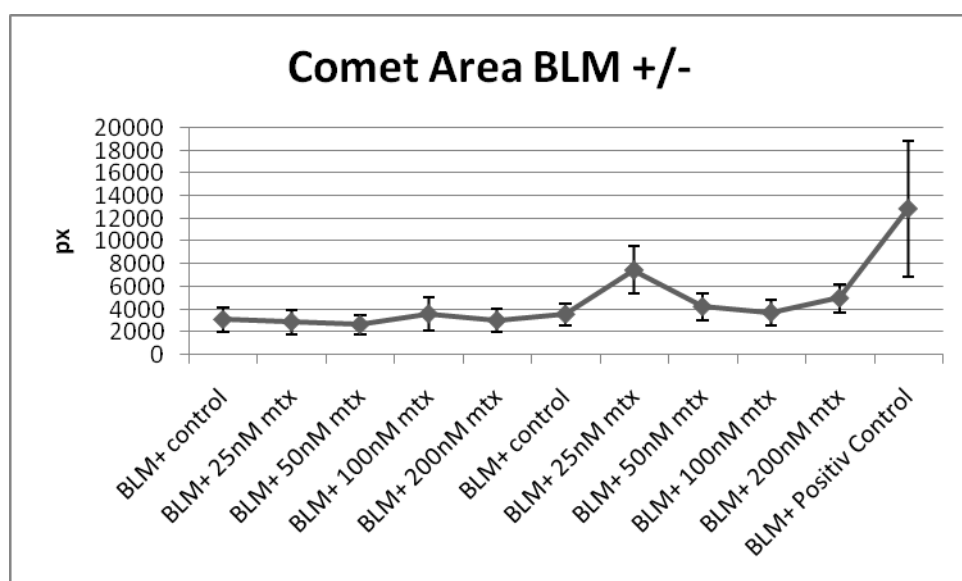


Figure H. The obtained comet areas (px) for the BLM +/- cells. The Comet area for the BLM +/- cells does not increase in either of the experiments.

## Appendix 7

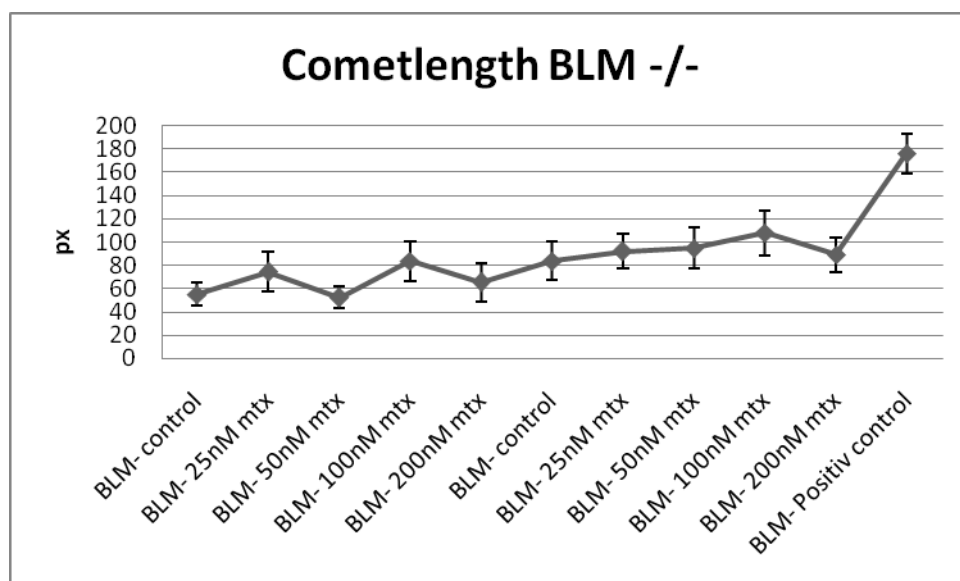


Figure I. Here is a figure over the cometlengths obtained for the cell line BLM -/-. The cometlength does not increase for the different methotrexate concentrations in either experiment.

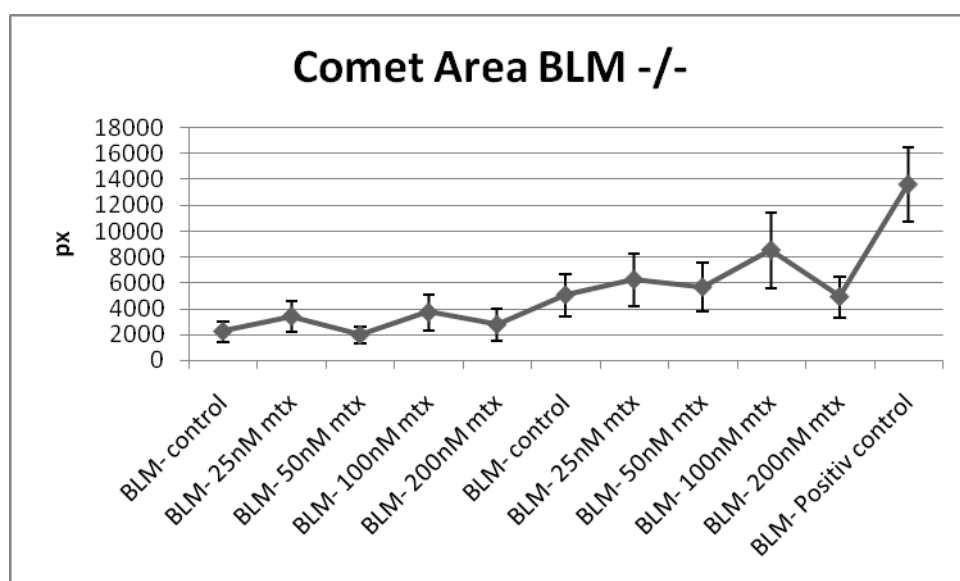


Figure J. The resulting comet areas (px) for the cell line BLM -/-. Neither of the experiments show an increase in comet area.

## Appendix 8

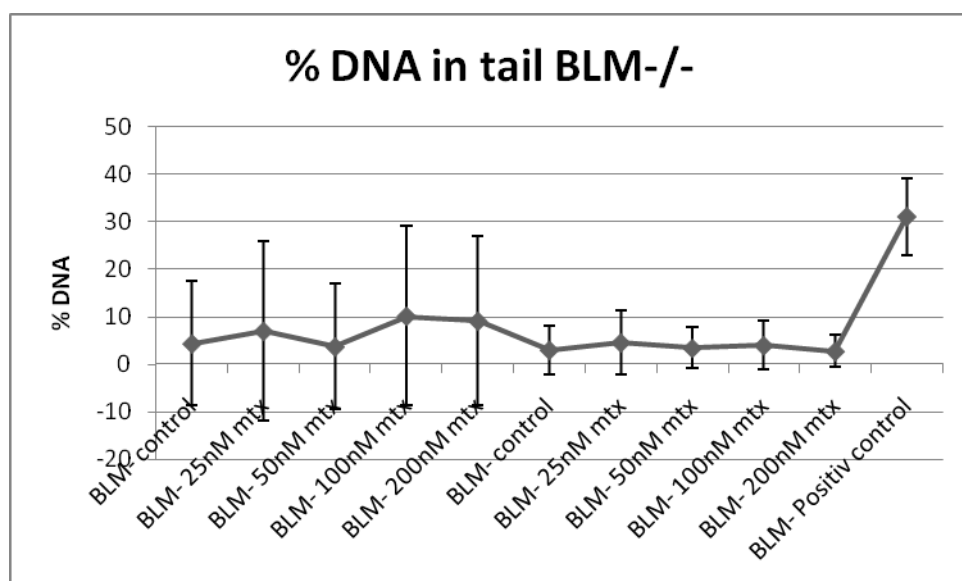


Figure K. This figures shows the obtained % DNA in tails of the BLM  $-/-$  cells. The % DNA in tail does not increase for the different methotrexate concentrations in either experiment

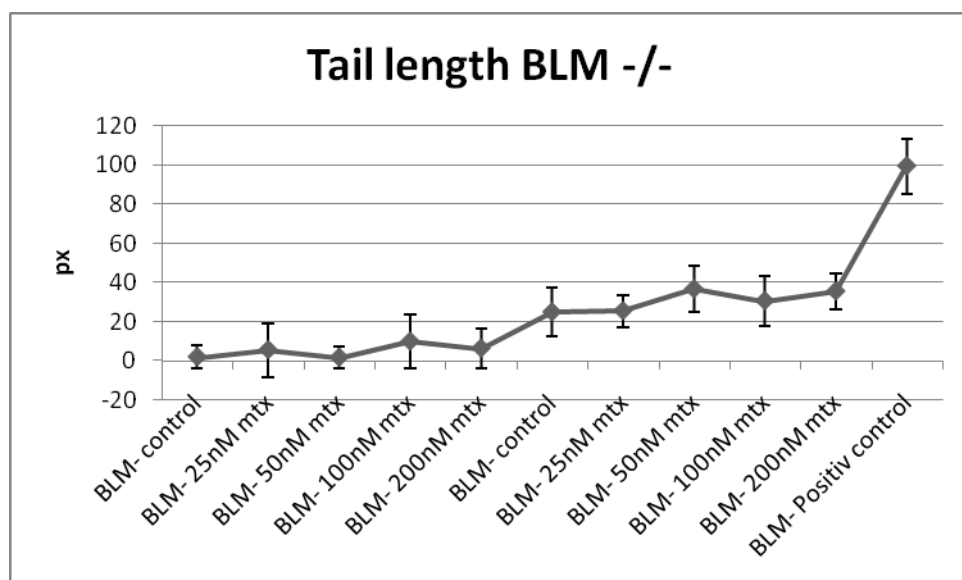


Figure L. The tail lengths (px) for the BLM  $-/-$  cells. The tail length for the BLM  $-/-$  cells does not increase in either of the experiments.

## Appendix 9

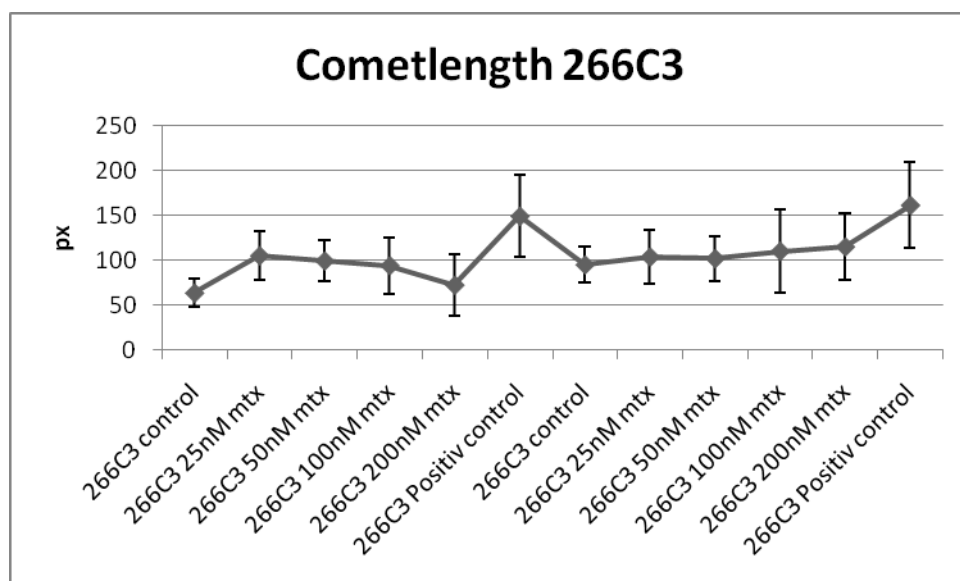


Figure M. Cometlengths (px) for the cell line 266C3. Neither of the experiments show an increase in comet length.

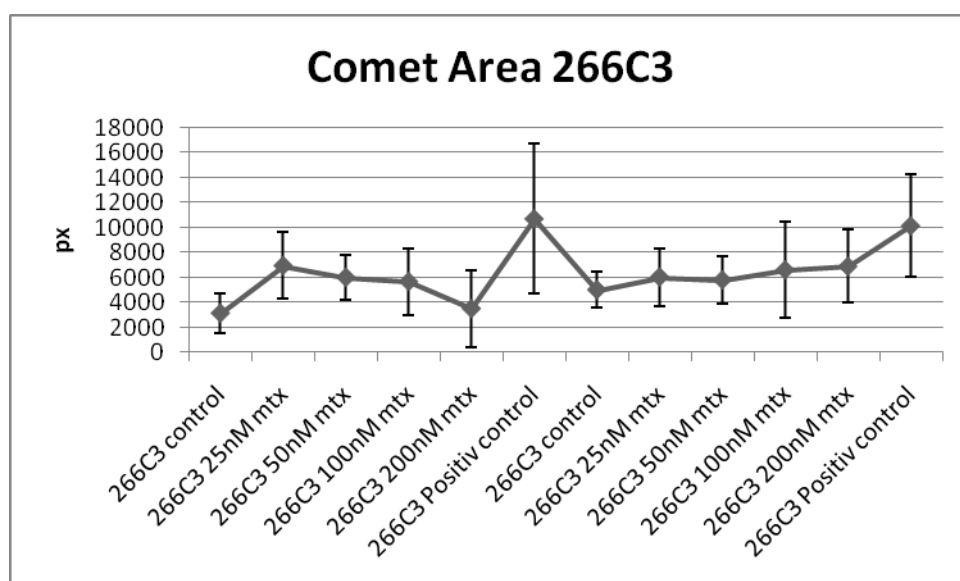


Figure N. The obtained comet areas for the 266C3 cells. The cometarea for the 266C3 cells does not increase in either of the experiments.

## Appendix 10

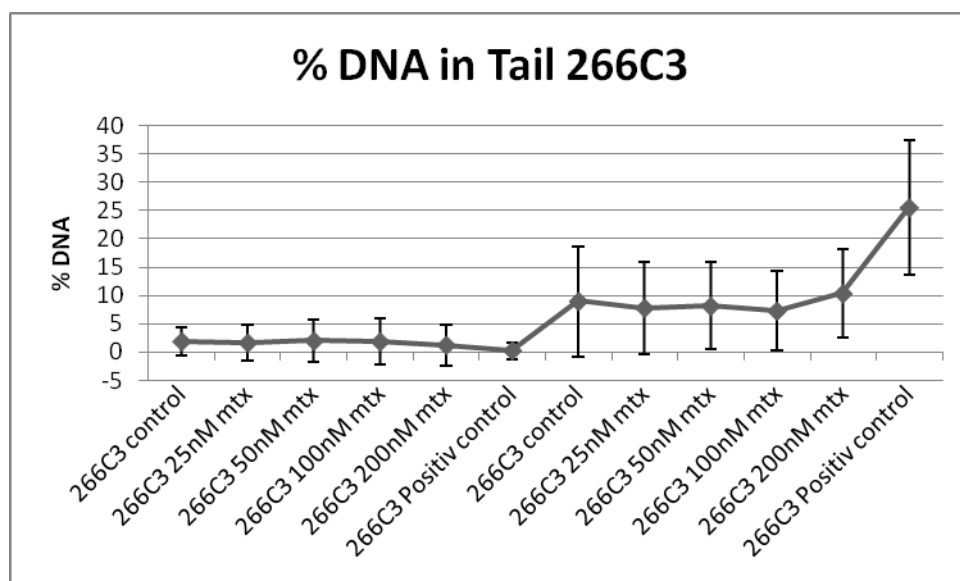


Figure O. This figure shows the % DNA in the tails of the 266C3 cells. Unusually low values for the first experiment. No increase in either of the experiments.

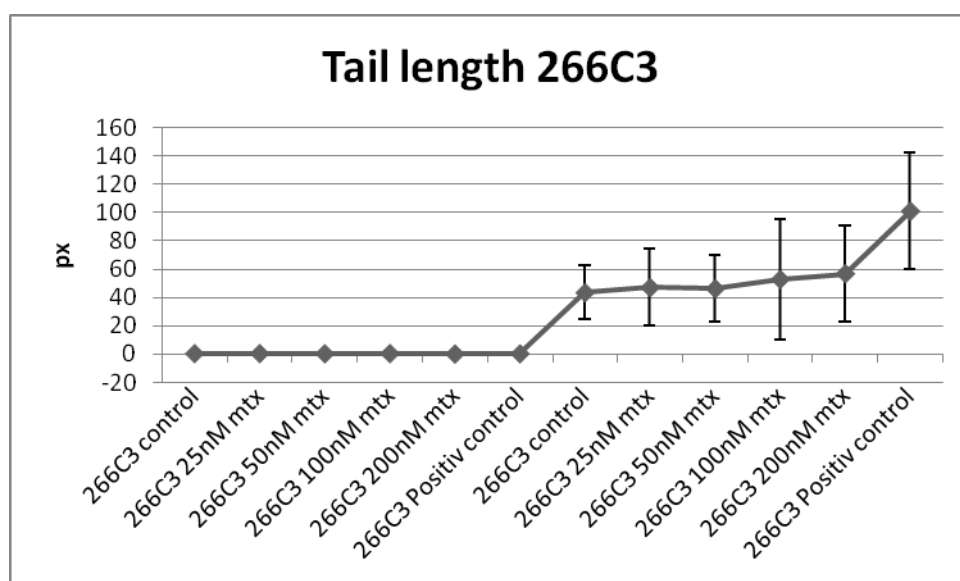


Figure P. Tail lengths (px) for the 266C3 cells. Unusually low values for the first experiment. No increase in tail length can be distinguished for the experiments.