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Measurements on the genotoxicity of nonthermal atmospheric plasma

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Abstract

This thesis describes research done to investigate the effect of plasma on the integrity of cellular DNA. Nonthermal plasma is a state of matter, which can be described as an ionized gas containing reactive particles such as ions, electrons and radicals. A low power (<200 mW) atmospheric pressure plasma has been used to treat cultures of 3T3 and HCT 116 cells. The plasma is sustained in helium and contains a small admixture of air (<1%). DNA damage has been assessed by analysis of the cell cycle and by looking at the repair mechanism of the cell.

The plasma is generated at the tip of a sharp needle. This needle is powered at a radiofrequency of 13.56 MHz. The tip of the needle is located at the end of a Teflon tube through which helium flows at a rate of typically 2 l/min. Temperature measurements show that the plasma is nonthermal and does therefore not thermally damage the cells.

Flow cytometry was used to analyze the cell cycle. Arrest in the G2/M phase of the cell cycle was observed, which shows that the plasma is able to induce DNA damage. Furthermore immunofluorescence was utilized to detect Double Strand Breaks (DSBs) in DNA. When DSBs are formed, histone proteins H2AX surrounding the site of the fracture are phosphorylated to γH2AX. Recently an antibody for γH2AX has been produced. This allows detection of DSBs within minutes after formation.

DNA damage induced by the plasma is comparable to damage induced by UV irradiation with an irradiance on the order of 10 mJ/cm². The amount of damage is linearly dependent on plasma power and increases with duration of treatment. Furthermore the amount of damage increases when the needle is brought close to the sample (<3mm). Measurements on H₂O₂ concentrations show that the plasma increases these concentrations to physiological damaging levels (0.1 mM). It is hypothesized that the formation of H₂O₂ works as a sensitizing agent that acts in conjunction with UV to damage the DNA.
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Chapter 1 Introduction

This thesis describes research done to investigate the effect of plasma on the integrity of cellular DNA. In this introduction some background information will be provided, which aims to clarify what plasma is and how this is created. Plasma holds the promise of becoming a revolutionary tool in medicine. The introduction will explain the possible role of plasma in medicine and some problems for medical applications of plasma, leading to the research questions of this work.
1.1 Plasma: the fourth state of matter

Plasma is often referred to as the fourth state of matter. The other states of matter are solid, liquid and gas. The fourth state, plasma, is an ionized gas. Plasma consists of freely moving electrons, ions, radicals and of course gas molecules. Plasma is defined as a state of matter with enough free charged particles for its dynamics to be dominated by electromagnetic forces. [1] This state of matter was first identified by Sir William Crookes in 1879 and was named plasma by Dr. Irving Langmuir in 1923. Most of the visible matter in the universe is plasma. For example huge objects like stars and nebulae are plasmas. Plasmas also exist on earth for example in the form of lightning and ‘Northern Lights’. These are natural phenomena, but there are also man-made plasmas, daily used in industry. In industry plasmas are used in numerous technologies such as lighting systems, displays and deposition systems.

A wide variety of plasmas exists and they can therefore not easily be characterized. This explains why plasmas are used in a wide variety of application fields. Plasmas possess some unique properties which are exploited in many applications. A few of these unique properties are the ability to radiate, electrical conductivity and chemical reactivity.

Because of differences in appearance, the particles in a plasma usually have different temperatures. The temperature of electrons is usually above 10,000 K. The temperature of neutral particles and ions is strongly dependent on the type of plasma and can vary from room temperature to 10^7 K. On the basis of temperature, one normally categorizes plasma as thermal or nonthermal. When full ionization of the neutral particles takes place, the ions reach a very high temperature approximately equal to the electron temperature. However when these particles are a minority, heating of neutral molecules will be limited. If this situation occurs the plasma is termed non equilibrium. Thus (diffuse) plasmas where the fraction of ionized species is below 0.1 % are usually nonthermal. This situation is readily achieved under reduced pressures, in the range of 10-1000 Pa. The effect of low pressure is twofold: in a rarefied gas ionization events are scarce, which keeps the charge density low. Moreover, the frequency of elastic collisions between electrons and molecules is low, so electrons do not have much chance to convey their energy to the gas. As a consequence many plasmas on earth are created inside a vacuum vessel at low pressure.

In the past two decades nonthermal atmospheric pressure plasmas have made a revolutionary appearance in solid state processing technology. They allow processing of heat sensitive materials and reduction of operating costs because of the absence complicated vacuum reactors.

1.2 Nonthermal atmospheric Plasmas

Energy is needed to sustain a plasma, to provide a continuous presence of sufficient charged particles. This energy can come from a number of different sources like electric fields, electromagnetic waves and gravity. The most common source of energy is the electric field. Charged species gain energy from the field and sustain the plasma by conserving the balance of charged particles. In atmospheric plasmas gas heating is a problem because the gas density is high enough to allow efficient gas heating by electrons.
To reduce gas heating a few tricks can be employed, which can be divided into two distinct approaches (after [2]): 1) reducing the input power and 2) enhancing the heat losses. To reduce the effective power, the following principles can be employed:

- **Reduction of the operating voltage by optimizing the gas composition**
  The well-known Paschen breakdown curves predict the lowest breakdown voltage for noble gases: helium, neon and argon. Many nonthermal atmospheric sources are operated in helium. Sources fed with argon tend to be hotter and more prone to arcing. In contrast to noble gases, plasmas operated in air require high breakdown voltages and are usually filamentary and unstable.

- **Reduction of the discharge current**
  This is the principle employed in dielectric barrier discharges (DBDs). The dielectric layer covering the electrode acquires charge upon contact with plasma. This charge reduces influx of charged plasma species and thus limits the plasma current.

- **Reduction of the duty cycle**
  Plasma ignition requires only a certain field strength, but the electric signal need not be applied continuously. An effective method to reduce the power is to apply short (microsecond) pulses. The major advantage of this approach is that the actual voltages can be quite high (1-10 kV), which helps to sustain high ionization rates and a high plasma activity.

To enhance heat losses from the source one can consider the following construction features:

- **Increase of plasma surface**
  Microplasmas (plasmas with dimensions smaller than a mm) have a large surface to volume ratio and this serves to increase heat losses by thermal conduction.

- **Introduction of a gas flow**
  In a microplasma it becomes possible to make the residence or dwell time $\tau$ of source gas very short, say one ms to a $\mu$s, in a small size and at a moderate gas flow rate. This is a very effective way to reduce the temperature of the plasma glow. Many designs employ large forced flows of several l/min.

- **Introduction of volatile matter**
  Plasma can be operated in the vicinity of water or other liquids. For example electric breakdown can be created under water, in a bubble of evaporated liquid. Alternatively liquid can be introduced into the plasma jet, or the plasma can be sustained in a chamber with a suspension of liquid droplets. The heat required for evaporation of the liquid forms a substantial heat sink.

For medical applications a low pressure plasma is impractical, even harmful to the patient. One of the recent trends focuses on developing new plasma sources, which operate at atmospheric pressure, but retain properties of low-pressure media such as their chemical reactivity and low temperature. Diffuse plasmas tend to be unstable at high pressures due to their susceptibility to filamentation and the transition to an arc, which limits their practical utility. At atmospheric pressure and electrode separation of centimeters breakdown voltages lie typically in the range of several kV. The high breakdown voltage leads to a high current density after the discharge is ignited, particularly in the cathode fall of the discharge. The high current density is the source of discharge instabilities in the cathode fall region, which quickly leads to the formation of an
undesirable arc. Since arcing leads to an uncontrollable behavior of the discharge, a more controlled operation mode is desired.

One approach to generate and maintain stable high pressure plasmas is based on the recognition that microplasmas display a remarkable stability towards arcing. There are several factors that contribute to the stabilization of microplasmas, not all of them fully understood at this point in time. One stabilizing factor can be explained in terms of pd scaling as discussed above. The voltage required to ignite a discharge, the so-called breakdown voltage, depends on the product of pressure and electrode separation. If one increases the pressure for a fixed value of d the required breakdown voltage increases. This relation is described by a Paschen curve. The typical operating parameters of microplasmas correspond to pd values of between 1 and 10 Torr cm. These pd values are similar to those for large volume, low pressure diffuse plasmas. Another factor that at least in part contributes to the stability of high pressure microplasmas are high losses of charge carriers to the surrounding walls. Therefore we can conclude that microplasmas are these weakly-ionized discharges that represent several scientific issues, such as the potential breakdown of pd scaling and the role of boundary based phenomena.

To prevent arcing several schemes have been devised such as the use of pointed electrodes in corona discharges and an insulating insert in the dielectric barrier discharges. The use of pointed electrodes confines the discharge to a small area surrounding the electrode tip and so keeps the ‘pd’ value small. The insulating insert in the dielectric barrier discharge plays an important role in suppressing the current: the layer is charged by incoming electrons which reduces the electric field and hinders charge transport towards the electrode. Paschen curves show that breakdown is easiest obtained in noble gases such as neon, argon and helium. Therefore optimization of the gas composition should help decreasing breakdown voltage and thus prevent arcing.

1.3 The plasma needle: an atmospheric pressure RF discharge

At the Tu/e the plasma needle project has been started in 2001 to study biomedical applications of plasma. The plasma needle is a device intended for such applications, developed by Stoffels et al. [4] It generates a small atmospheric plasma at the tip of a sharp needle. A picture of the needle tip and a schematic of the plasma needle setup are shown in Figure 1. The needle is powered at a radiofrequency of 13.56 MHz. Around the needle is a tube which guides helium past the needle tip into the surrounding environment. Using helium as a carrier gas has several advantages: the plasma is easiest to ignite, helium is not toxic and the thermal conductivity is very high. Gas flows are typically 1-2 l/min. A matching network ensures maximum power input into the needle.
1.4 Cells and tissues

The ultimate goal of research into medical plasmas is the development of a method of treatment which allows disinfection of tissues, suppression of inflammation and possibly stimulation of wound healing i.e. to develop a surgical tool. Key to this enterprise is a fundamental understanding of the effects of plasma on the building blocks of human tissue: cells.

A cell is a complex structure of organelles in cytoplasm surrounded by a membrane that shields its contents from the outside environment. Organelles are specialized sub units, which perform special functions within the cell. These organelles are generally protected by their own membrane. The membrane is a continuous sheet of phospholipid molecules in which various proteins are embedded. Cytoplasm is a gel-like fluid in which the organelles are embedded. One organelle that deserves some special attention is the nucleus. The nucleus holds the cell’s DNA.

Cells are the basis of each organism, whether it is a unicellular microorganism like a bacteria or a multicellular organism such as a human. There are however huge differences between the composition of bacteria and the human being. Cells in human beings are organized in tissues, whereas in bacteria by definition this organization does not exist.

Tissue is a complex material that usually consists of several cell types with connecting extracellular matrix (ECM). The ECM consists of several components. For example fibroblasts secrete various proteins and polysaccharide chains; these chains then become part of the ECM. The ECM has in total five components that make up a stable complex: collagens, basement membranes, elastic fibers, structural glycoproteins and proteoglycans. The exact composition of the extracellular matrix depends on the cell type. The direct application of plasma to tissues of any kind will lead to complicated interplay between plasma particles and the constituents of the tissue.

On top of differences on a macro scale there are also important differences on a micro scale between bacterial cells and animal cells. Bacteria are prokaryotes. Unlike cells of animals and other eukaryotes, bacterial cells do not tend to have membrane-bound organelles in their cytoplasm and contain few intracellular structures. They consequently lack a nucleus present in...
eukaryotic cells. Also a typical human cell is 10 to 20 μm in diameter and bacteria are about one tenth the size of eukaryotic cells: typically 0.5 to 5 micrometers in diameter.

1.5 Plasma sterilization

Plasma possesses several properties which are detrimental to bacteria. Therefore the development of nonthermal plasmas sparked the idea of using plasma for disinfection purposes. In the previous decade research was done to assess the disinfection power of these nonthermal plasmas. [5, 6] Nonthermal plasmas are very effective in killing bacteria. This makes these plasmas very useful for various biological and medical applications such as sterilization of medical instruments, decontamination in biological warfare and filtering of air or water.

Understanding of the working principles of plasma sterilization will give valuable insights in the effects of plasma on biological material. Research has shown that four inactivation factors are involved in the destruction of pathogens and chemical agents: heat, ultraviolet radiation, reactive neutral species and charged particles.

- **Heat**
  It has long been known that heat has detrimental effects on living cells. Heat leads to the ultimate death of all living forms by destroying the cellular metabolic system, which includes enzymatic components. In heat-based conventional sterilization methods both moist heat and dry heat are used. In the case of moist heat, such as in an autoclave, a typical temperature of 121°C at a pressure of 15 psi is used. Dry heat sterilization, such as in an oven, requires temperatures close to 170°C and treatment times of about 1 hour. Wet-heat systems require water content sufficient to produce 100% relative humidity at the sterilization temperature.

- **Ultraviolet radiation**
  Ultraviolet (UV) radiation is known to kill bacteria. Wavelengths in the 220-280 nm range and doses of several mJ/cm² are known to have to optimum effect. However UV radiation is said to play a significant role in low pressure sterilization, but it is of less importance in atmospheric plasmas. In particular VUV photons (<180 nm) are strongly absorbed by air at atmospheric pressure, which prevents them from reaching the sample.

- **Reactive neutral species**
  In high pressure non equilibrium plasma discharges, reactive species are generated through various collisional pathways, such as electron impact excitation and dissociation. Reactive species play an important role in all plasma-surface interactions. Many researchers concluded that reactive species play an important role in the sterilizing mechanism of plasma. [5, 6] Research has shown that discharges containing oxygen have a strong germicidal effect. This is due to the presence of reactive oxygen species (ROS).

- **Charged particles**
  Charging of the cell membrane is believed to have effect on cells. Mendis suggested that charged particles might play a significant role in the rupture of the outer membrane of bacterial cells, although this is disputed. [7] They showed that the electrostatic force caused by charge accumulation on the outer surface of
the membrane could overcome the tensile strength of the membrane and cause its rupture. Another explanation might be that electrons attach to the membrane structure and change the membrane via electron reduction. This increases permeability of the membrane and eventually leads to the destruction of the cell. Research is needed to validate these hypotheses.

Plasma sterilization research provides some insight in the mechanisms that play a role in the effect of plasma on cells. Although it is very important to realize that the aim of sterilization research is very different than that of medical research. Plasmas used in sterilization research are designed to maximize bacterial cell death and thus possesses lethal intensities. Furthermore sterilization can be done under low pressure conditions while treatment of tissue can only be done at atmospheric pressure.

One step beyond sterilization of non living material is the idea of disinfecting or treating living tissue. Plasmas used for these purposes are designed to keep cells alive. Effects induced by such plasmas are therefore termed non lethal effects or sub lethal effects. Intensities of these medical plasmas are much lower (~100 mW) than those used in sterilization research (~ 1 W). And because of the extreme non linearity of plasmas and the complex cellular machinery one cannot easily extrapolate effects from sterilization research. Furthermore bacterial and mammalian cells are completely different, which justifies this statement even more. It is therefore impossible to predict the effects of low intensity plasma on tissue.

A biocompatible plasma is defined here as a plasma that is able to induce non lethal effects on cells or tissue. In general one expects from medical treatment that damage to the living organism should be avoided or at least minimized and that the final outcome of the treatment is beneficial to the organism in general. A plasma that complies with these demands is difficult to obtain. It has to be 1) nonthermal with a temperature preferably around 37°C, 2) atmospheric, 3) nontoxic and 4) electric fields cannot be too high.

1.6 Scope of this thesis

In order to examine potential biomedical applicability, several studies have been done on cell cultures. [8] These studies have shown some insight into the possibilities of the plasma needle. Tentative applications of the device are disinfection of skin and mucous membranes and caries treatment as research pointed out that for certain intensities bacteria can be killed by plasma treatment, while cells appear unaffected. On the other hand a lot of the processes are poorly understood and it is unclear what plasma does to cells. One issue that has not been completely addressed before is the effect of plasma on DNA: the genotoxicity of plasma. A potential hazard for the biomedical application of plasmas is the fact that plasma treatment may induce DNA damage.

From previous research there are some indications that the plasma from the plasma needle alters DNA. Cell necrosis and apoptosis have been studied by staining cells and imaging them with confocal laser scanning microscopy (CLSM) and with a light microscope. [8] Stains that were used include propidium iodide (PI) and cell tracker green (CTG) in case of CLSM and trypan blue in case of the light microscope. Necrotic cells were reported to have preserved their shape and
internal structure. However they displayed an abnormal DNA distribution in their nucleus, which could indicate that the DNA has been damaged by exposure to the plasma needle.

In this thesis the effect of plasma on DNA will be investigated. The goal of this research is to determine if DNA damage occurs, if so under which conditions and what the final effects are for the cells. Chapter 2 provides some theoretical background to understand which mechanisms could play a role in the interaction between plasma and biological material. Chapter 3 will discuss the design of the experiment and chapter 4 will show an investigation into treatment conditions. In chapter 5 the effect of plasma on cells will be investigated and measurements on DNA damage will be presented. And in chapter 6 a conclusion will be drawn from these measurements.
Chapter 2 Theory

An important property of plasma is that it is chemically active. Inside the plasma reactive chemical species like radicals or excited molecules are generated, that can be used to process surfaces: for example to change wettability or adhesion properties of a surface or to deposit material. To human cells the property of chemical activity presents a risk: it means that plasma could potentially be toxic for cells.

Toxicity of the plasma consists of two components: electromagnetic radiation and reactive species which for a large part consist of radicals and charged particles. From sterilization research it is known that plasma-cell interactions takes place via heat, UV radiation, radicals and charged particles. Another mechanism which does not play a significant role in plasma sterilization and may do so in medical plasmas is the electric field. In this chapter each mechanism will be studied individually. The effects of each mechanism on cells will be discussed in section 2.1.

Furthermore the structure of DNA will be studied. DNA is a complex structure which is constantly perturbed. Consequently DNA repair is very important to maintain DNA integrity. The makeup of DNA and DNA repair will be discussed in section 2.2. What happens to the DNA when it is damaged will be examined. When DNA is damaged the cell will react to this damage and this response will be discussed in section 2.3.
2.1 Plasma-cell interaction

Five mechanisms through which plasma effects cells will be treated here and in particular their effect on DNA.

Heat

Nonthermal plasmas for sterilization purposes should have a gas temperature below say 100 °C to prevent destruction of the target material. For a biocompatible plasma this limit is even stricter. As a general rule gas temperature of the plasma should be around body temperature. For a good understanding of the thermal limits of the plasma one should look at the effects of heat on cells. The effects of heat can be divided in several regimes. A list of these regimes is given in Table 1.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Biological effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>Normal</td>
</tr>
<tr>
<td>45</td>
<td>Hyperthermia</td>
</tr>
<tr>
<td>50</td>
<td>Reduction in enzyme activity, cell immobility</td>
</tr>
<tr>
<td>60</td>
<td>Denaturation of proteins and collagen, coagulation</td>
</tr>
<tr>
<td>80</td>
<td>Permeabilization of membranes</td>
</tr>
<tr>
<td>100</td>
<td>Vaporization</td>
</tr>
</tbody>
</table>

Table 1: Several regimes for effects of heat on cells.

For temperatures until 41°C there is no effect on the cell. The first regime in which tissue is thermally affected can be attributed to conformational changes of molecules. The first effects show between 42 °C to 50°C and include changes in molecules, membranes and the destruction of chemical bonds. These effects are summarized in the term hyperthermia. A healthy cell will at first try to protect itself against overheating by producing heat shock proteins. This protein allows the cells temporarily to cope with the elevated temperatures until the temperature has returned to its original value. However, if the temperature increase persists for too long the cell cannot protect itself anymore and dies. In the second regime above 50°C enzyme activity will decrease dramatically, so that there is less energy transport in the cell. This prevents the cell from functioning normally. Also several repair mechanisms stop functioning. Over 60°C denaturation of proteins and collagen will occur, which leads to coagulation of tissue and cell necrosis. Above 80°C membranes will have higher permeability which causes chemical imbalance in the cell. At 100°C water molecules start to vaporize which ruptures cell membranes definitely destroying cells.

The state of hyperthermia is reversible and temperature of the cells should therefore not surpass 50 °C. Hyperthermia is however also not the ideal situation for cell treatment, since it is known to have adverse effects on the cellular integrity. Hyperthermia affects fluidity and stability of cellular membranes and impedes the function of transmembranal transport proteins and cell receptors in vitro. Furthermore hyperthermia has been demonstrated to induce various changes in cytoskeletal organization and cellular proteins, specifically nuclear proteins. [9] Most importantly, hyperthermia has been known to induce DNA damage. However this effect is only
observed for relatively long (~10 minutes) periods of hyperthermia and can thus not seriously contribute to DNA damage in plasma treated cells. [10]

Temperatures above 41 °C can already pose a threat to human tissue. For long exposure times there are hardly any noticeable effects below 41°C. Above 41°C cell death occurs. Part of the cell death between 41 and 49°C is caused by necrosis, another by apoptosis. [11] It appears that apoptosis occurs mainly in the region between 41 and 45°C with a maximum at 43°C. For temperatures above 45°C, cells die mainly because of necrosis. However susceptibility to apoptotic cell death induced by heat differs for some cell types. [12]

Not only temperature plays a role for the induction of damage, but also the duration of this temperature plays a significant role. [13] Figure 2 shows a curve, indicating which temperatures and exposure times lead to irreversible tissue damage. This curve can be used as a rough orientation, but one has to keep in mind that sensitivity to heat differs for various tissues.

![Figure 2: Curve indicating the critical temperature for occurrence of irreversible tissue damage.][13]

**UV radiation**

Plasma generally emits electromagnetic radiation in a broad spectrum. Depending on the frequency EM radiation is called ionizing because it can disrupt molecular or atomic structures. Ionization energies are typically in the order of 10 eV, corresponding to radiation wavelengths of 100 nm. Therefore radiation with frequencies higher than 300 GHz is generally considered as ionizing radiation. The short wavelength limit of the UV region is often taken as the boundary between the ionizing radiation spectrum (wavelengths < 100 nm) and the non ionizing radiation spectrum.

However most organic molecules exhibit efficient absorption of light in the UV range, since photons in this region possess sufficient energy to break selected chemical bonds in these molecules. This process is known as homolytic cleavage. UV radiation is therefore a well-known factor that induces cell injury. When organic molecules are irradiated, however, bonds are seldom broken at random. Instead, the excited molecules undergo fairly selective bond breaking rearrangements or bimolecular reactions. UV radiation with a wavelength between 200 to 300 nm has been known to cause damage to the DNA molecule. [13]
UV is usually split into three regimes:

- UV-A (315-380 nm) - the least harmful; causes tanning but may also accelerate skin aging.
- UV-B (280-315 nm) - absorbed by the DNA, causes DNA damage; carcinogenic.
- UV-C (100-280 nm) - more aggressive than UV-B, damages DNA, proteins and cellular lipids.

An action spectrum is a parameter that describes the relative effectiveness of energy at different wavelengths in producing a particular biological response. Biological response may refer to effects at a molecular level, such as DNA damage, or at a whole organism level, such as plant growth. An action spectrum is used as a weighting factor for the UV spectrum to find the actual biologically effective dose (BED) for a given effect. This relation is described mathematically by:

\[ \text{BED} = \int_{\lambda} \text{UV}(\lambda) A(\lambda) d\lambda, \]

where UV and A are the ultraviolet irradiance and action spectrum values at a given wavelength \( \lambda \), respectively. The total dose is then found by integrating over the entire wavelength range. The action spectrum of DNA damage has been calculated by Setlow [14] and is shown in Figure 3.

![Figure 3: DNA action spectrum.](image)

It is known that UV radiation in the B and C range can efficiently inactivate bacteria. [15] The lethal UV dose for E. Coli bacteria is 50 mW/cm\(^2\) for 222 nm and 150 mW/cm\(^2\). The lethal doses for fibroblasts were substantially higher: irradiation with 0.5 W/cm\(^2\) did not result in direct cell damage. Cell death occurred from 0.7 W/cm\(^2\) at 206 nm to 1 W/cm\(^2\) at 282nm. [16] These results show that living cells are much more resistant to UV than bacteria. Low power UV exposure can accelerate wound healing, probably due to disinfecting and thus reducing the inflammation. [17]
Reactive species

Atmospheric nonthermal plasmas are a rich source of chemically active species. Plasmas with an admixture of air are excellent sources of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Especially radicals are of concern because of their chemical toxicity. Radicals are molecules or atoms with an unpaired electron. The term ROS is a collective one that describes both oxygen radicals (O₂⁻; OH⁻) and free radicals (RO•) and non-radical derivatives (H₂O₂, HOCl, singlet O₂, ozone). They are generally very small molecules and are highly reactive due to the presence of unpaired electrons.

Free radicals can be formed via addition or release of an electron, or by homolytic cleavage of a covalent bond. There are various intra- and extracellular sources of oxygen radicals. The major intracellular source of oxygen radicals is probably leakage associated with the reduction of oxygen, superoxide radicals, hydrogen peroxide and hydroxyl radicals. Other intracellular processes resulting in the release of reactive oxygen include the peroxisomal metabolism, the enzymatic synthesis of nitric oxide and the metabolism of phagocytic leukocytes. Extracellular sources include radiation (especially ionizing radiation and near-UV light), heat, various drugs and redox cycling compounds. Table 2 provides an overview of various reactive forms of oxygen, the way in which they are formed, their oxidizing potential and lifetimes.

The half-life and diffusibility of the generated radicals are important parameters that can influence the potential for DNA damage. Diffusion range \( x \) of these species can be estimated by the following relation:

\[
\bar{x} = \sqrt{6Dt},
\]

where \( D \) is the diffusion constant, which is \( 2 \times 10^{-9} \) m\(^2\)/s for oxygen in water at 20°C, and \( t \) is the time.

<table>
<thead>
<tr>
<th>Reactive oxygen species</th>
<th>Common mode of formation in cells</th>
<th>Oxidizing potential (V)</th>
<th>Lifetime in water (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Singlet oxygen O(_2^*)</td>
<td>Excitation of O(_2)</td>
<td>1.78 from [18]</td>
<td>( 10^6 ) from [18]</td>
</tr>
<tr>
<td>Ozone O(_3)</td>
<td>From O(_2) through photolysis</td>
<td>2.07 from [18]</td>
<td>600 from [19]</td>
</tr>
<tr>
<td>Hydroxyl radical OH(^*)</td>
<td>From H(_2)O under the influence of ionizing radiation and from H(_2)O(_2) through metal-catalyzed disintegration</td>
<td>2.56 from [18]</td>
<td>( 10^9 ) in water [18] 1 in air [20]</td>
</tr>
<tr>
<td>Hydrogen peroxide H(_2)O(_2)</td>
<td>Dismutation of the superoxide anion radical</td>
<td>1.77 from [18]</td>
<td>30000</td>
</tr>
<tr>
<td>Superoxide anion radical O(_2^\cdot)</td>
<td>One-electron reduction of O(_2) and auto-oxidation of reduced heme proteins.</td>
<td>1.58 from [21]</td>
<td>( 10^6 ) from [22]</td>
</tr>
</tbody>
</table>

Table 2: Various forms of reactive oxygen species, their mode of formation as a part of the human metabolism, their oxidizing potential and their lifetime. Note that the lifetime of the species is dependent on other solutes and that these numbers represent merely orders of magnitude.

Reactive species have the capability of causing severe cell damage through chemical reactions with the cell constituents. Radicals can induce a wide range of effects: membrane damage, inactivation of enzymes, cell death and cancer. The major types of interaction of reactive species are covalent binding and oxidative interactions, which include oxidative stress and lipid peroxidation.
Reactive species may bind covalently to cellular molecules such as nucleic acids, proteins, cofactors, lipids and polysaccharides. The potential effects are manifold: decreased energy production, changes in membrane permeability, inhibition of the synthesis of macromolecules etc. At this moment in time little is known about the implications of covalent binding to cofactors, lipids and polysaccharides. Experiments of covalently bound xenobiotics to proteins have shown that this can lead to a disturbance in homeostasis which will eventually cause to cell death. [23]

Since the plasma causes an increase in oxidizing agents it is assumed that oxidative interactions will dominate over covalent binding effects. The human body has been adapted to eradicate small amounts of ROS, since ROS are a byproduct of the human metabolism. [24] However if concentrations of ROS in a cell become too high various types of damage occur, a situation known under the common name of oxidative stress. In this situation there is a continuous interaction with oxidizing agents inside the organism. In other words: in this state consistently more reducing equivalents are used than would be the case for normal functioning of the cell, hence the term stress. Oxidative stress is caused by an imbalance between the production of reactive oxygen species and the ability of a biological system to readily detoxify the reactive intermediates or easily repair the resulting damage.

Oxidative interaction with cellular components follows a chain of chemical reactions. Any oxidation chain reaction involves three steps: initiation, propagation and termination. In the initiation phase radicals are formed through for example homolysis of molecules containing weak bonds (C-C, O-O, C-N=N) or enzymic processes playing an important role in the production of radicals in vivo. Two common examples of radical initiation are:

\[
\begin{align*}
\text{H}_2\text{O}_2 + \text{hv} & \rightarrow 2 \text{OH}^* \\
\text{O}_3 + \text{OH} & \rightarrow \text{HO}_2^* + \text{O}_2^*
\end{align*}
\]

(3) (4)

In the propagation phase these radicals can abstract electrons from residues of organic molecules, indicated by the letter R, according to the following general reaction:

\[
\text{RH}_2 + \text{OH}^* \rightarrow \text{RH}^* + \text{H}_2\text{O}
\]

(5)

and can initiate chain reactions that result in damage at considerable distances (μm) from the initial chemical event. Termination is the process by which radicals are quenched which is described by the following general reactions:

\[
\begin{align*}
\text{S} + \text{OH}^* & \rightarrow \text{SOH} \\
\text{RH}^* + \text{OH}^* & \rightarrow \text{ROOH}
\end{align*}
\]

(6) (7)

where S denotes a radical scavenger such as vitamin C. Termination occurs when two radicals come into contact and couple together. These radical coupling or radical bond forming reactions are rather rare processes. The reason is that radicals are normally present at low concentrations in a reaction medium, and it is statistically more likely they will abstract a hydrogen atom, or undergo another type of a substitution process, rather than reacting with each other by coupling. And as radicals are uncharged, there is little long range Coulomb attraction between two radicals.
A notable example of a type of damage is peroxidation of unsaturated lipids initiated by reactive free radicals such as the hydroxyl radical. Peroxyl (ROO•) and alkoxyl (RO•) radicals are likely intermediates in many chain reactions, while peroxides (ROOH and H₂O₂) and hydroxylated (ROH) and carbonylated (HR=O) residues are likely end products. H₂O₂ is probably the most significant of these in terms of diffusion range. H₂O₂ by itself is relatively inert but can give rise to highly reactive OH• radicals in a process catalyzed by transition metal ions, typically Fe²+. This reaction is called the metal-catalyzed Haber-Weiss reaction or the Fenton reaction. Iron is essential for DNA synthesis and oxidative metabolism and is compartmentalized under normal conditions by the protein ferritin, which protects the cell by keeping iron in a non reactive crystallized core as ferric ion.

Superoxide radical and H₂O₂ are relatively non toxic but they are precursors of the most powerful oxidant occurring in biological systems: the hydroxyl radical. The hydroxyl radical is considered as one of the strongest oxidants, with an oxidative potential of 2.57 eV. The OH• radical is extremely reactive with a half-life in water of approximately 10⁻⁹ s and a radius of diffusion of 20 Å. [25] It reacts very quickly with almost all organic compounds through electrophilic reactions affecting the unsaturated bond of e.g. benzene ring. Unlike superoxide, which can be detoxified by superoxide dismutase, the hydroxyl radical cannot be eliminated by an enzymatic reaction, as this would require its diffusion to the enzyme's active site. As diffusion is slower than the half-life of the molecule, it will react with any oxidizable compound in its vicinity. It can damage virtually all types of macromolecules: carbohydrates, nucleic acids (mutations), lipids (lipid peroxidation) and amino acids. The only means to protect important cellular structures is the use of antioxidants such as glutathione and of effective repair systems.

On the cellular level, several effects leading to cell injury have been identified resulting from oxidative interactions:

- **Protein oxidation**
  Protein oxidation is generally not so harmful, because damaged proteins are efficiently replaced. A state of oxidative stress leads to a depletion of reducing equivalents. Reducing equivalents is a collective term for substances, which directly reduce or help reducing oxidants. Examples are: NADPH, glutathione, protein thiols and vitamins C and E. Depletion of reducing equivalents may result in increased Ca²⁺ concentrations, which will lead to changes in the composition and permeability of the cellular membranes. And these changes in the membranes will eventually cause cell death.

- **Lipid peroxidation**
  The reactive species mentioned above have direct impact on the cells, especially on their outermost membranes. Fatty acid chains in biological membranes are mostly unsaturated. They are therefore highly sensitive to oxidation by singlet oxygen or hydroxyl radicals. The oxidation consists of a chain of reactions which is known as lipid peroxidation. Lipid peroxidation causes serious membrane damage and may therefore lead to cell death. Membrane damage can compromise the function of the membrane lipids whose role is to act as a barrier against the transport of ions such as Ca²⁺ and Mg²⁺ and polar compounds in and out of the cells. Lipid peroxidation and cell death often go hand in hand and it is not always clear whether cell death results from lipid peroxidation or vice versa.
DNA damage

The number of different modifications resulting from ROS acting on DNA include both Single Strand Breaks (SSBs) and Double Strand Breaks (DSBs), DNA-protein cross-links, and a wide variety of base and sugar modifications. [26] Ames and Shigenaga have estimated that roughly $2 \times 10^4$ lesions occur per day per human genome because of oxidative damage to DNA. [27] Fortunately most of these are SSBs which can readily be repaired.

While possibly all these effects play a role in plasma tissue interaction, this thesis focuses on elucidating the effect of plasma on DNA. Therefore a more detailed description of DNA will be given in section 2.2.

The living cell disposes of a number of enzymatic and non enzymatic defense mechanisms against reactive oxygen species to counter the state of oxidative stress.

Enzymatic defense against ROS

There are three classes of enzymes known to provide protection against reactive oxygen species: the catalases and peroxidases that react specifically with hydrogen peroxide, and the superoxide dismutases. To detoxicate hydrogen peroxide the cell has two enzymes at its disposal: catalase and glutathione peroxidase. Catalase converts hydrogen peroxide to water and oxygen. In fact, catalase cooperates with superoxide dismutase in the removal of the hydrogen peroxide resulting from the dismutation of the superoxide anion radical. Catalase occurs in most tissues, encapsulated in subcellular organelles, the so-called peroxisomes. Maximum catalase activity requires very high concentrations of hydrogen peroxide. This distinguishes catalase from other peroxide-transforming enzymes. Catalase acts only on hydrogen peroxide and not on organic hydroperoxides.

Gluthation peroxidase catalyzes not only the conversion of hydrogen peroxide but also that of organic peroxides. Gluthathione peroxidase is found in most tissues, both in the cytosol and in the mytochondria. In the cytosol the enzyme is present in special vesicles.

The superoxide dismutases are metal-containing proteins that catalyze the conversion of the superoxide anion radical to molecular oxygen in the ground state and hydrogen peroxide. Superoxidase dismutase is almost exclusively found to be an intracellular enzyme. It can only be demonstrated in very small amounts in the plasma or the lymph. This is of great importance, since any metabolic disorder involving the release of the superoxide anion radical, or any increase in the concentration of the superoxide anion radical in an extracelluar fluid may be very harmful to the organism.

Non enzymatic defense against ROS

The antioxidant enzymes discussed above can be seen as a first-line defense. After all they prevent the conversion of the less reactive oxygen species, superoxide anion radical and hydrogen peroxide, to more reactive species such as the hydroxyl radical. The second line of defense largely consists of substances that eliminate radicals. The major radical scavengers of the cell are the vitamins E and C. Vitamin E is lipophilic and is incorporated in membranes. Vitamin C is water soluble and occurs in the cytosol. Vitamin C (ascorbic acid) reacts quite rapidly with
the superoxide anion radical and with hydrogen peroxide, but even faster with hydroxyl radicals. Furthermore it can eliminate singlet oxygen. This antioxidant exerts its protective effect for example in the lens of the eye, which does not contain any superoxide dismutases.

Electricity

Plasma and electricity are inevitably coupled. At atmospheric pressure breakdown voltages may be quite high: from several hundreds of Volts to even 10 kV, depending on the type of discharge, electrode gap and gas composition. Such high electric fields may interact with nervous system, disturb the heartbeat and cause damage to individual cells. Interactions of the electromagnetic field with biological tissue is a wide field of research. Since this thesis focuses on cellular effects a short summary of these effects will be given. For more information on the tissue level the reader is referred to [28].

One should differentiate between effects caused by electric currents and electromagnetic fields. Applying plasma to tissue presents a conducting path for an electric current. Effects of electric currents in human tissue have been thoroughly studied. Excessive electric power dissipation by electric currents causes cells to heat up and die eventually due to heating. This method is actually not unfamiliar in medicine. Electrosurgery uses the fact that electric power dissipation in living tissue results in coagulation and cutting of tissue. [29] One important discovery that made electrosurgery possible is the fact that muscles and nerves show a decreased sensitivity to high frequency currents. The threshold current that causes irritation is only 1 mA at 10 Hz, but increases dramatically with frequency. At 100 kHz it is as high as 0.1 A. Plasma dissipates almost all electrical power as resistance is much higher (~400 kΩ) than resistance of tissue (~50 kΩ). So heating of tissue due to electrical energy dissipation is negligible. Heating by conduction from the plasma poses a bigger problem.

Research into biological effects of electromagnetic fields is a relatively new and unexplored territory in science. Cellular effects have been studied although a univocal theory on the effects of EM radiation is presently non existent. It has become clear though that frequency of the radiation has a large effect on cellular effects. First of all there is the difference between ionizing and non ionizing radiation. Whereas ionizing radiation clearly does have an effect on biological materials, many other biological effects require photon energies well below the level of ionizing potentials, such as heating, dielectrophoresis, depolarization of cell membranes and piezoelectric transduction. Considered here are the effects of non ionizing radiation. It is found that threshold field strength for ‘any effect’ increases in general with frequency.

The basic effects of an electric field on a living cell can be described by considering the cell to be a conductive body (the cytoplasm) surrounded by a dielectric layer (the surface membrane). When an electric field is applied to this cell (by placing the cell in a conductive medium between two electrodes and applying a unipolar voltage pulse to the electrodes), the resulting current causes accumulation of electrical charges at the cell membrane and consequently a voltage across the membrane. If the membrane voltage exceeds a critical value, structural changes in the surface membrane occur that cause pore formation/increased permeability, a process known as electroporation. [30] If the membrane voltage is not excessive and the duration of the pulse is limited, the increased membrane permeability can be reversible and the cell survives, an effect that is used for electrochemotherapy and gene delivery into cells. [31]
Extremely high electric fields can cause cell membrane rupture. The rupture occurs when the potential difference across the cell membrane is 1 V. [32] Since the membrane has a thickness of several nm, external fields must be on the order of 10^6 V/m. In man-made plasmas electric fields are typically much lower: in extreme cases (plasma sheath at the powered electrode) they are about 10^5-10^6 V/m. During plasma treatment a sheath is formed around the exposed grounded object, but the electric field in this sheath is lower than 10^5 V/m. Thus plasma induced electric fields should not inflict much membrane damage.

A voltage difference is established across a cell membrane as a result of separating charge across the membrane. This is the only way a voltage difference is established. The only way the transmembrane voltage can be changed is to change the charge separated across the membrane. The membrane, because of its large lipid content, is relatively impermeable to ions. Phospholipids provide the capacitive property of the membrane (C_m), which allows charge, once separated, to remain separated across the membrane. A change in V_m with time dV_m/dt requires a change in charge separation across the membrane with time dq/dt. This change in charge with time is defined as current I.

\[
I = \frac{dq}{dt} = C_m \frac{dV_m}{dt} \tag{8}
\]

Therefore, an observed change in V_m indicates that there is a net transmembrane current flowing. The amount of current that typically flows across a membrane during bioelectric activity is small (~3000 Ca^{2+} ions), too small to significantly change either the intracellular or extracellular concentration of the ion moved.

The time required to charge the surface membrane is dependent upon the electrical parameters of both the cell and the medium in which it is suspended. For a spherical cell with a surface membrane that is an ideal dielectric layer (no leakage currents) and for low volume concentration of cells in suspension, the charging time constant is [33]:

\[
\tau_c = \left( \rho_c - \rho_a \right)/2 C_m D/2 \tag{9}
\]

where C_m is the capacitance of the surface membrane per unit area, D is the cell diameter, \( \rho_c \) is the resistivity of the cytoplasm, and \( \rho_a \) is the resistivity of the medium in which the cell is suspended. For a cell with a diameter of 10 μm, resistivities of cytoplasm and medium of 100 Ωcm, and a membrane capacitance of 1 mF/cm², t is 75 ns. The charging time constant is a measure of the time during which the cell interior is exposed to the applied pulsed electric field. This is equivalent to the statement that the outer membrane becomes increasingly transparent for oscillating electric fields when the angular frequency of the oscillation exceeds a value given by the inverse of the charging time. So for RF plasmas (f=13.65 Mhz, \( \tau_c=73 \) ns) the cell interior is not really shielded anymore from electrical fields. However field strengths for electroporation are in the order of 10^6 V/m [34] and as was pointed out earlier field strengths resulting from plasma treatment are typically not so high.

Experiments on low-level (<10^5 V/m) RF and microwave exposure of cell cultures are usually difficult to reproduce and do not show any effect in most cases. [28, 35] Some experiments suggest genotoxicity of low-level RF and microwave radiation but at long (at least several
minutes) exposure times. [36] It can therefore be concluded that any electromagnetic effects in cells as a result of plasma treatment are small in comparison to other effects (chemical and thermal).

**Charged particles**

A sheath is a layer of several Debye lengths thick formed when a plasma contacts a material surface. This layer is positively charged and balances an opposite negative charge in the surface of the material, with which it is contact. A Debye sheath arises in a plasma because the electrons are faster than the ions and therefore diffuse outward faster, charging the surface negative. As a result, positively charged ions are accelerated towards the surfaces.

Energetic particles originating from the plasma typically carry several eV of energy. These particles therefore are non-ionizing radiation as they do not possess enough energy to ionize particles in their target. The cell membrane will protect the DNA from attacks by energetic particles directly impinging on the cell and will absorb these particles before they reach the target of interest: DNA. The charged ions that impinge a water surface can ionize and excite the water molecules if they carry enough energy. Ionization of water occurs at 13 eV and excitation at 7.4 eV. Below those energy they will thermally equilibrate the water.

It is hypothesized by Stoffels et al. that charging of cells in a dry environment, directly exposed to the plasma leads to cell detachment. [37] It has been shown that the cell’s surface charge and cell attachment are coupled [38], however not much is known about this subject.

### 2.2 DNA, Damage and Repair

DNA is a long polymer of simple units called nucleotides, which are held together by a backbone made of sugars and phosphate groups. This backbone carries four types of molecules called bases and it is the sequence of these four bases that encodes information. A picture of the chemical structure of two paired DNA molecules is shown in Figure 4. In living organisms DNA does not usually exist as a single molecule, but instead as a tightly associated pair of molecules. These two long strands entwine like vines in the shape of a double helix. The nucleotide repeats contain both the backbone of the molecule, which holds the chain together, and a base, which interacts with the other DNA strand in the helix. In light of this thesis it is important to remember that DNA in human cells is located inside the nucleus. The nucleus has its own membranes surrounding it and therefore provides additional protection to the DNA molecules. On the other hand bacteria lack this protection and are therefore more vulnerable.
DNA damage due to environmental factors and normal metabolic processes inside the cell, occurs at a rate of 1,000 to 1,000,000 molecular lesions per cell per day. [39] While this constitutes only 0.000165% of the human genome's approximately 6 billion bases (3 billion base pairs), unrepaired lesions in critical genes (such as tumor suppressor genes) can impede a cell’s ability to carry out its function and appreciably increase the likelihood of tumor formation.

Damage to DNA consists of any change from the usual double-helical structure. We can divide such changes into two general classes: Single base changes and structural distortions.

Single base changes affect the sequence but not the overall structure of DNA. They do not affect replication, when the strands of the DNA duplex are separated. So these changes exert their damaging effects on future generations through the consequences of the change in DNA sequence. Such an effect is caused by the conversion of one base into another that is not properly paired with the partner base. The common feature is that the mismatch persists only until the next replication. Unfortunately mismatch repair can also lead to replacement of the old, correct nucleotide rather than the mutated one, resulting in a (point) mutation. The impact of a single base mutation can be highly variable. While most point mutations will not cause any harm to the functioning of the DNA, some point mutations lead to the loss of function of a vital gene, resulting in inherited diseases, cancer or cell death.

Structural Distortions may provide a physical impediment to replication or transcription. Introduction of covalent links between bases on one strand of DNA or between bases on opposite strands inhibits replication and transcription. A well studied example of structural distortion is the pyrimidine dimer. UV irradiation introduces covalent bonds between adjacent thymine bases, giving an intrastrand pyrimidine dimer. Similar consequences could result from addition of a bulky adduct to a base that distorts the structure of the double helix. A single-strand nick or removal of a base prevents a strand from serving as a proper template for synthesis of RNA or DNA. The common feature in all these changes is that the damaged adduct remains in the DNA, continuing to cause structural problems and/or induce mutations, until it is removed. The ease with which these distortions are repaired depends on the type of damage but in most cases repair is difficult and not without errors.

Another example of a structural distortion is double strand breaks. Double strand breaks (DSBs) can occur when DNA is distorted by bulky lesions caused by ionizing radiation or carcinogens.
that add large molecules onto bases. DSBs also arise endogenously during replication and as a result of the oxidative metabolism. [40] DSBs are difficult for the cell to repair accurately, but can be fixed by recombination with the second undamaged copy of the chromosome or by non homologous end joining of the broken strands, which causes deletions or insertions of bases surrounding the breaks. Homologous recombination (HR) is the most accurate mechanism for mending DSBs and relies on repeated sequences in the genome (e.g. on sister chromatids or homologous chromosomes). If these DSBs are not repaired efficiently they can lead to genomic rearrangements causing the activation of proto-oncogenes or inactivation of tumor suppressor genes and the start of cancer.

The eukaryotic nucleus is a complex organelle that contains not only the cell’s genetic information in the form of DNA, but also the molecular machinery for transcribing, replicating and repairing the genome. In order to fit the enormous DNA molecules in the cell nucleus, the DNA of all eukaryotes is compacted into chromatin, the primary unit of which is the nucleosome. [41] They are made up of DNA and four pairs of proteins called histones, and resemble ‘beads on a string of DNA’ when observed with an electron microscope. A picture of DNA wound around histones is show in Figure 5. Although the structure of the nucleosome core bound to DNA is known to atomic resolution, the higher order, compacted structure of chromatin is less clear. The compaction of DNA occurs at the level of the nucleosome and with non histone chromosomal proteins it is compacted further to varying degrees along its length in a given chromosome.

![Diagram of DNA and histones](image)

Figure 5: Organization of DNA. Picture taken from [42].

In addition to this extensive DNA compaction, each of the twenty-three pairs of chromosomes occupies a discrete territory within the nucleus. To efficiently repair DNA lesions within chromatin the cell has evolved a number of recently elucidated mechanisms for marking chromatin for nucleosome remodeling, often dependent on post-translational modifications of core histones, to allow access to DNA repair machinery.

In the mid to late 1990s several groups identified DNA damage-dependent focal accumulations in nuclei of both DNA repair factors and the phosphorylated form of the histone variant H2AX. [43] The term ‘repair foci’ has since been used to describe these protein accumulations. The histone variant histone H2AX comprises up to 25% of the H2A complement in mammalian cells. [44] H2AX is evenly distributed throughout the genome with approximately one to two H2AX molecules every ten nucleosomes. [45] It is rapidly phosphorylated following exposure of cells to
double-strand break (DSB) inducing agents such as ionising radiation. Within minutes of DSB generation, H2AX molecules are phosphorylated in large chromatin domains flanking DNA double-strand breaks (DSBs). [46] These domains can be observed by immunofluorescence microscopy and are termed H2AX foci. H2AX phosphorylation is believed to have a role mounting an efficient cellular response to DNA damage. The number of phosphorylated H2AX molecules increases linearly with the severity of the damage for small amounts of damage and saturates for high levels of damage. [44]

In a relaxed double-helical segment of DNA, the two strands twist around the helical axis once every 10.4 base pairs of sequence. To add or subtract twists, as some enzymes can do, is to impose a strain. If a DNA segment under twist strain were to be closed into a circle by joining its two ends and then it is allowed to move freely, the circular DNA would contort into new shape, such as a simple figure-eight. Such a contortion is a supercoil.

Although understanding of chromosome structure is incomplete, evidence suggests that chromosome abnormalities are a direct consequence and manifestation of damage at the DNA level. For example chromosome breaks may result from unrepaired double strand breaks in DNA and chromosome rearrangements may result from misrepair of strand breaks in DNA. An event which is linked to the formation of abnormal chromosomes is the formation of small nuclei separated from the central nucleus. These so called micronuclei (MNi) are expressed in dividing cells that either contain chromosome breaks lacking centromeres (acentric fragments) and/or whole chromosomes that are unable to travel to the spindle poles during mitosis. An example of a cell exhibiting micronuclei can be found in Figure 6. At telophase a nuclear envelope forms around the lagging chromosomes and fragments, which then uncoil and gradually assume the morphology of an interphase nucleus with the exception that they are smaller than the main nuclei in the cell, hence the term ‘micronucleus’.

![Figure 6: A mitotic cell showing two micronuclei. Picture taken from [47].](image)

2.3 Cell injury and the cell cycle

If the genetic and metabolic adaptive responses are inadequate for a given injury, the cell will die. Two types of cell death can be distinguished: necrosis and apoptosis. In the first cell death is manifested by cytoplasmic swelling, plasma membrane blebbing, dissolution of the chromatin and finally interruption of cell membrane integrity. Apoptosis is manifested by cytoplasmic...
shrinkage, larger plasma membrane buds and nuclear chromatin condensation. Figure 7 is a picture showing the morphological differences between the two types.

![Figure 7: Two distinctive types of cell death: apoptosis and necrosis.](image)

Apoptosis, or programmed cell death, is a normal component of the development and health of multicellular organisms. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells and inflammatory responses. Apoptosis, by contrast, is a process in which cells play an active role in their own death, which is why apoptosis is often referred to as cell suicide. The molecular mechanisms that regulate the cell cycle and apoptosis are inextricably linked. A schematic of the cell cycle is depicted in Figure 8.

![Figure 8: Schematic of the cell cycle. Two checkpoints are shown: the G1 and G2/M checkpoint.](image)

Normal healthy cells cycle through an ordered set of phases leading to cell growth and division into two daughter cells. The phases are G1, S, G2, and M. The G1 phase stands for ‘GAP 1’. The S phase stands for ‘Synthesis’. This is the phase when DNA replication occurs. The G2 phase stands...
for ‘GAP 2’. The M phase stands for ‘mitosis’, and is when nuclear (chromosomes separation) and cell membrane (cytoplasmic) division occur. The latter process is called cytokinesis. Mitosis is typically divided further into 4 phases (prophase, metaphase, anaphase, and telophase) or more. The transmission from one phase to another is under the control of a set of proteins able to delay or arrest the cell cycle progression at several points known as ‘checkpoints’.

Checkpoints (shown as R in Figure 8) are used by the cell to monitor and regulate the progress of the cell cycle. If a cell fails to meet the requirements of a phase it will not be allowed to proceed to the next phase until the requirements have been met. [48] Several checkpoints are designed to ensure that damaged or incomplete DNA is not passed on to daughter cells. Experiments have shown that cells in which DNA has been damaged are arrested in either the G1 or G2 stage. The delays that cells experience in G1, S, or G2 phases of the cell cycle after damage to DNA are collectively called DNA integrity checkpoints.

There are two manifestations of these delays. The first is the transient arrest seen at G1, S, or G2 (independent of the key tumor suppressor protein p53) that gives the DNA repair machinery time to shore up the damage before division continues. The second (dependent on p53) is apoptosis or prolonged, probably permanent, G1 delay that results in removal of damaged cells from the population. A failure to halt at these checkpoints leads to genomic instability and an increased likelihood that the cell will become cancerous.

There are a number of mechanisms through which apoptosis can be induced in cells. The sensitivity of cells to any of these stimuli can vary depending on a number of factors such as the expression of pro- and anti-apoptotic proteins, the severity of the stimulus and the stage of the cell cycle. In some cases the apoptotic stimuli comprise extrinsic signals such as the binding of death inducing ligands to cell surface receptors called death receptors. This occurs when T-cells recognise damaged or virus infected cells and initiate apoptosis in order to prevent damaged cells from becoming neoplastic (cancerous) or virus-infected cells from spreading the infection. In other cases apoptosis can be initiated following intrinsic signals that are produced following cellular stress. Cellular stress may occur from exposure to radiation or chemicals or to viral infection. It might also be a consequence of growth factor deprivation or oxidative stress caused by free radicals.

The p53 protein mediates cell cycle arrest in response to DNA damage, providing the cell with the opportunity to repair the damage before proceeding with DNA replication during cell division. Alternatively, p53 may mediate entry of the cell into apoptosis, thus ensuring that potentially tumorigenic mutations are not propagated from mother to daughter cells. The critical importance of this anti-tumour activity of p53 is highlighted by the number of cancers that exhibit mutation of this gene. [49]
Chapter 3 Experimental setup

In this chapter the experimental equipment, the model that was used to study the genotoxic effects of the plasma needle and measurement techniques are described. The overall schematic of the plasma needle setup can be found in figure 2. The individual parts will be described in this chapter. The existing plasma needle was modified for experiments in 96 well plates. In section 3.1 the design of the plasma needle used in this study will be discussed. To increase precision of treatment, the scanner was built. The scanner allows more precise positioning of the plasma needle and treatment of large surfaces. The design of the scanner will be discussed in section 3.2. The design of the matchbox which for a large part determines the performance of the plasma needle is described in section 3.3. During the experiments power was continuously measured. Equipment for these measurements is described in section 3.4. The model system which was used to experiment on is described in section 3.5. Measurement techniques for the analysis of the model system will be discussed in 3.6.
3.1 Plasma needle design

The plasma needle was rebuilt for treating cells in 96 well plates. The tip of the needle was changed so that it fits into the wells of 96 well plates. The head of the needle consists of three pieces: the housing, an insulator and a gas tube. The housing is made of steel and the other two pieces consist of Teflon. A schematic of these pieces is shown in Figure 9, Figure 10 and Figure 11. A picture of the needle is shown in Figure 12. The needle is made of tungsten and is put in a SMC-connector, which is fastened to the housing with a small screw. This version of the needle is sometimes referred to as the needle with the small tip.

Figure 9: Schematic of the needle housing.

Figure 10: Schematic of the insulator. The dashed lines represent small bore holes, which allow helium to pass through the insulator to the tube.
A second gas tube piece has been made to solve some problems encountered during plasma treatment in 96 wells plates. A schematic of this piece is shown in figure 13. This tube piece features a narrow tip and a broader base to enable a more stable position in the well. This version of the needle is sometimes referred to as the fixed tip.
The sharpness of the needle can be characterized by the angle $\theta$. Definition of the angle $\theta$ is depicted in Figure 14. The sharpness of the needle makes ignition of the discharge easier by increasing the field strength around the tip. The needle was initially designed with a sharpness angle $\theta$ of 60±10 degrees. This angle was decreased to 20±10 degrees. This roughly caused an increase of plasma density of a factor 1.5.

![Figure 14: Definition of the sharpness angle $\theta$.](image)

3.2 Scanner Design

The small size of the plasma generated by the plasma needle makes accurate positioning difficult. On top of that, the optimal treatment distance lies between 1 and 2 mm. This makes manually manipulating the plasma needle a difficult task. Manually scanning large areas is not possible without introducing large variations in gap size and discharge power. Furthermore it has been found very difficult to monitor all variables during experiments. An automatic scanner has been developed at the GTD to increase the position accuracy of the needle and to allow reproducible treatment of large areas.

A schematic drawing of the scanner is given in Figure 15. The scanner consists of an x-y platform mounted on a solid frame of granite with a positioning arm at one corner. The x-y platform consists of two Physik Instrumente M-403.2PD linear translation stages. Both stages have a travel range of 50 mm. Each of the stages is driven by a Physik Instrumente Mercury DC-servo/stepper motor controller. A round container for Petri dishes has been attached to the upper stage.

![Figure 15: Schematic of the scanner. Two translation stages are stacked to form an x-y platform.](image)
The positioning arm can be used to control the z position of the needle. The holder of the needle can roughly be positioned by the sliding mechanism on the back of the arm. Precise positioning of the needle is done with a construction of two leaf springs on top of the arm. One revolution of the screw equals 0.5 mm of z-displacement. The needle slides through an opening at the end of the arm and is fastened with a screw.

The controllers receive their instructions from a custom built Labview program. The program consists of three parts: manual control, scanning mode and a programmable mode. Manual control can be used to move the motors manually. Only one motor can be controlled at a time. The programmable mode consists of a table which contains positions and speeds that can sequentially be fed to the motors. However this part of the program contains bugs and is unreliable. Therefore it has been chosen to work with the scanning mode. In the scanning mode one can define the size of the scan area, the width between scan lines and the scan speed. Once activated the plasma needle follows a zigzag pattern with the predefined parameters.

3.3 Matchbox design

The needle is powered by a RF source designed and made by the General Technical Service (GTD). To assure optimal power dissipation in the plasma, the impedance of the load must be matched to the impedance of the source. This is achieved by inserting a tunable matching network between the source and the plasma needle. A schematic of the tunable matching network is given in Figure 16.

![Figure 16: Electronic schematic of the matchbox.](image)

3.4 Power measurements

The electrical energy that goes into the plasma can be measured with a bidirectional power meter. Discharge power measurements were done with a SWR power meter (Amplifier Research PM 2002) with a dual directional coupler. (Amplifier Research Power coupler DC3002 0.1 - 1000 MHz with Amplifier Research PH2002 power heads) The meter was inserted between the source and the matching network to monitor forwarded and reflected power. The nominal power (forwarded power minus reflected power) contains the power consumed by the plasma, but also RF radiation and losses due to ohmic heating in the matching network and cables. In microplasmas losses in the electronics are overwhelming compared to the power dissipated in the discharge. The plasma power can be evaluated using the subtraction method proposed by Horwitz. [50] The difference in the nominal power with and without plasma at the same electrode voltage is a good estimate for the dissipated power.
In mathematical form this is:

\[ P_{\text{plasma}} = \left( P_{\text{fwd}} - P_{\text{refl}} \right)_{\text{on}} - \left( P_{\text{fwd}} - P_{\text{refl}} \right)_{\text{off}} \]  

(10)

where \( P_{\text{plasma}} \) is the power dissipated in the plasma, \( P_{\text{fwd}} \) is the forwarded power, and \( P_{\text{refl}} \) is the reflected power. With this method, the discharge voltage must be measured using an attenuating voltage probe placed as close as possible to the powered electrode. The voltage power curves must be determined and used in the subtraction of the powers. However, for the micro-plasma circuit, the voltage probe is a substantial dissipative element and its usage is therefore not recommended. Fortunately, the needle voltage does not change much when the plasma is extinguished and the condition for using the subtraction method is fulfilled. This is because the discharge forms a relatively small load, which does not have a large influence on the circuit. Measurements of the voltage at the tip with and without plasma were reported in [51].

Accuracy of this method is dependent on the quality of the matching box. If the matching box is not stable enough, measurement of the electrical power is impossible. Matching ratio \( P_{\text{refl}}/P_{\text{fwd}} \) should be as low as possible to increase power to the needle. However, this contradicts with the tunability of the matching box and also the robustness of the matching box.

\[ 3.5 \text{ Cells} \]

Understanding of the effect of plasma on tissues begins with an understanding of the effect of plasma on individual cells. For research purposes, it is therefore logical to start using cultured cells as a model system. Due to the connection of cells with the ECM and group effects, it can be expected that the effect of plasma on tissues differs from that on cultured cells. However, for studying the genotoxicity of plasma cell cultures, they are the ideal model system, since it is easier to measure DNA damage in these cultures.

Cell biologists have developed ways of culturing cells in the laboratory. This technique allows culturing a single type of cell as a relatively homogenous population of cells in a medium. Most media used are derived from horse serum or fetal calf serum. The medium provides nutrients and growth factors to the cells, which are normally delivered to the cells by the blood.

Cell lines used in this work are 3T3 and HCT-116. The 3T3 line has been chosen for its ease in culturing and its high growth rate. The 3T3 cell line is an important fibroblast culture, widely utilized in laboratory research, which was established from disaggregated tissue of an albino Swiss mouse embryo. Medium for the 3T3 cell line consists of Dulbecco’s Modified Eagle’s medium, 2 mM L-Glutamine and 10% Fetal Bovine Serum (FBS). The HCT-116 is a cell line taken from a colorectal carcinoma, with characteristics of epithelial cells, such as the expression of cytokeratins. During apoptosis, vital intracellular proteins are cleaved. HCT-116 cells contain cytokeratins, which are cleaved during apoptosis. These cleavage sites are recognized by the M30 antibody. The M30 assay consequently is an easy and reliable determination of early apoptotic events in single cells. The HCT 116 cell line has been chosen to allow simple detection of apoptosis with the M30 antibody. Medium for the HCT-116 cell line consists of McCoy’s 5A medium and 10% Fetal Bovine Serum (FBS).
Cells are treated in the plates or Petri dishes in which they are cultured. Culture plates that have been used are Corning Costar 12, 24 and 96 well plates. (Cat. no. 0400-3513, 0400-3526 and 0400-3596 respectively) These plates contain wells with a growth area of 3.8, 1.9 and 0.32 cm² respectively. The plates are made of polystyrene, a non conducting polymer. The Petri dishes that have been used are 5 cm Greiner Bio-one Cellstar tissue culture dishes. (Cat. No. 628160)

3.6 Techniques for measuring DNA damage

There are several techniques to measure the amount of damage DNA has sustained. The techniques are explained here and in section 3.7 it will be explained why flow cytometry and immunofluorescence have been chosen.

The comet assay

The single cell gel electrophoresis assay, also called comet assay, is described by Östling and Johanson [52] and has been used in many applications to evaluate irradiation or compound induced genotoxicity. The comet assay is an easy protocol that can be used to detect double stranded DNA breaks in single cells. Cells are lysed with non ionic detergent and high-molarity sodium chloride. This treatment removes membranes, cytoplasm and nucleoplasm, and disrupts nucleosomes, almost all histones being solubilized by the high salt. What is left is the nucleoid, consisting of a nuclear matrix of scaffold composed of ribonucleic acid (RNA) and proteins, together with the DNA, which is negatively supercoiled as a consequence of the turns made by the double helix around the histones of the nucleosome. When the negative supercoiling is unwound by for example ionizing radiation the loops extend out from the nucleoid core to form a halo - one single strand break being sufficient to relax the supercoiling in that loop. For SCGE the cells are lysed in agarose, so that the DNA is immobilized for subsequent electrophoresis. When subjected to an electric field the relaxed loops are pulled to one side and form a tail. The length of this tail is a measure of the extent of DNA damage. The tail needs to be visualized by a fluorescent marker such as ethidiumbromide, DAPI or PI. Sensitivity of the comet assay can be high. Singh et al. demonstrated that detection of DNA damage following irradiation of 0.25 Gray is possible. [53]

Micronucleus assay

In the classical cytogenetic techniques, chromosomes are studied directly by observing and counting aberrations in metaphases. This approach provides the most detailed analysis, but the complexity and laboriousness of enumerating aberrations in metaphase and the effect of artefactual loss of chromosomes from metaphase preparations has stimulated the development of a simpler system of measuring chromosome damage. It was proposed independently by Schmid [54] and Heddle [55], that a simple approach to assess chromosome damage in vivo was to measure micronuclei (MNi) in dividing cell populations. MNi provide a convenient and reliable index of both chromosome breakage and chromosome loss. Because MNi are expressed in cells that have completed nuclear division, they are ideally scored in the binucleated stage of the cell cycle [56]. It is evident from the above that MNi can only be expressed in dividing eukaryotic cells. In other words, the assay cannot be used efficiently or quantitatively in non dividing cell populations or in dividing cell populations in which the kinetics of cell division is not well understood or controlled.
In the CBMN assay cells that have completed one nuclear division are blocked from performing cytokinesis using cytochalasin-B (Cyt-B) and are consequently readily identified by their binucleated appearance. Cyt-B is an inhibitor of actin polymerisation required for the formation of the microfilament ring that constricts the cytoplasm between the daughter nuclei during cytokinesis. [57] The use of Cyt-B enables the accumulation of virtually all dividing cells at the binucleate stage in dividing cell populations regardless of their degree of synchrony and the proportion of dividing cells. MNI are then scored in binucleated cells only, which enables reliable comparisons of chromosome damage between cell populations that may differ in their cell division kinetics.

**Cell cycle analysis by flow cytometry**

Flow cytometry is a technique for counting, examining and sorting of cells suspended in a stream of fluid. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of single cells. The most important feature of flow cytometric analysis is that large numbers, for example 10000 particles or more are analyzed one after the other typically in about 1 minute.

Figure 17: A schematic drawing of a flow cytometer. The cells in the reservoir flow one by one through the small tube. The laser then excites the fluorescent markers with which the cells have been stained. Detectors pick up this fluorescence signal and this data is then processed by a computer.

A small suspension of cells is mixed with a larger amount of cell-free buffer (called ‘sheath fluid’). The fluids come together under laminar flow conditions so that they flow together evenly without mixing. The ‘sheath’ fluid surrounds a thin core thread of sample. A beam of light produced by a laser of a single wavelength is then directed onto this hydro-dynamically focused stream of fluid containing the cells. The configuration of the stream spaces the cells out so that ideally only one passes the laser beam at a time. Also it keeps the cells centered in the flowing stream so that they pass the laser beam optimally centered. A number of detectors are aimed at the point where the stream passes through the light beam; one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter or SSC and one or more
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fluorescent detectors). Each suspended particle passing through the beam scatters the light in some way and fluorescent chemicals found in the particle or attached to the particle is excited into emitting light. This combination of scattered and fluorescent light is picked up by the detectors and by analyzing fluctuations in brightness at each detector (one for each fluorescent emission peak), it is then possible to extrapolate various types of information about the physical and chemical structure of each individual particle. FSC correlates with the cell volume and SSC depends on the inner complexity of the particle (i.e. shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness).

Usually data only from single, viable cells is desired. Typically one wishes to eliminate data from cell debris (particles smaller than cells), dead cells and clumps of 2 or more cells. Subcellular debris and clumps can be distinguished from single cells by size (estimated by the intensity of low angle forward scatter). Dead cells have lower forward-scatter and higher side-scatter than living cells. These differences are accurately preserved following fixation, despite the fact that after fixation, all the cells are dead.

The computer can be configured to display the fluorescence signals only from those particles with a specified set of scatter properties. This is called a scatter-gated fluorescence analysis. In some cases, it may be desirable to gate on a combination of fluorescence and scatter values.

Propidium Iodide (PI) is a fluorescent chemical that binds with DNA. PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye molecule per 4-5 base pairs of DNA. PI is membrane impermeant and generally excluded from viable cells. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20- to 30-fold, the fluorescence excitation maximum is shifted to ~30-40 nm to the red and the fluorescence emission maximum is shifted ~15 nm to the blue. The absorption and emission profiles of PI bound to dsDNA are shown in Figure 18.

![Figure 18: Absorption and emission profiles of the fluorescent dye propidium iodide bound to double stranded DNA.](image)

While flow cytometry cannot detect DNA damage itself, this method is capable of detecting alterations in the distribution of cells within the cell cycle, which is indicative for the response of cells to DNA damage. When cells are stained with PI, the DNA content of a single cell can be
measured. Cells in the G2 stage contain a double amount of DNA and therefore fluoresce twice as intense. The largest part of the cells in a sample is generally in the G1 phase, while only a fraction has doubled its DNA and is thus in the G2 phase. Another fraction is busy replicating its DNA, which makes up the S stage. Figure 19 shows an image of the general shape of a DNA profile of a normal proliferating population of cells. The relative distribution of cells in the various stages is cell type specific. For instance some cell lines have a longer G1 phase, resulting in an increased number of G1 phase cells in the DNA profile. When cell cycle arrest due to DNA damage occurs, the DNA profile will change shape and the ratios of the areas below the curves belonging to each phase change.

![Figure 19: Typical example of a DNA profile. The data is fitted with four distributions corresponding to four different cell populations. From left to right: the apoptic population or SubG1 phase, the G1 phase, the S phase and the G2/M phase.](image)

**Immunofluorescence**

Immunofluorescence is a technique, which labels or stains specific molecules with fluorescent dyes. This technique is used to visualize the subcellular distribution of the biomolecules of interest. Immunofluorescent labeled tissue sections or cultured cells are studied using a fluorescence microscope or by confocal microscopy. Labeling is most commonly done in two steps. First a primary antibody is bound to the molecule of interest (antigen) and then a secondary dye coupled antibody is attached to the primary antibody. Antibodies are an important tool for demonstrating both the presence and the subcellular localization of an antigen. Cell staining is a very versatile technique and, if the antigen is highly localized, can detect as few as a thousand antigen molecules in a cell. Cell staining can be divided into three steps: fixation, application of antibody and evaluation.

The first step is to fix and permeabilize the cells to ensure free access of the antibody to its antigen. Perfect fixation would immobilize the antigens, while retaining authentic cellular and
subcellular architecture and permitting unhindered access of antibodies to all cells and subcellular compartments. A wide range of fixatives is commonly used and the correct choice of method will depend on the nature of the antigen being examined and on the properties of the antibody used. Fixation methods fall generally into two classes: methods using organic solvents and methods using cross-linking reagents. Organic solvents such as alcohols and acetone remove lipids and dehydrate the cells, while precipitating the proteins on the cellular architecture. Cross-linking reagents (such as paraformaldehyde) form intermolecular bridges, normally through free amino groups, thus creating a network of linked antigens. Cross-linkers preserve cell structure better than organic solvents, but may reduce the antigenicity of some cell components and require the addition of a permeabilization step to allow access of the antibody to the specimen. Fixation with both methods may denature protein antigens and for this reason antibodies prepared against denatured proteins may be more useful for cell staining. Four different fixation methods are described. The appropriate fixation method should be chosen according to the relevant application. The second step of cell staining involves incubation of cell preparations with the primary and subsequently the secondary antibody. Unbound antibody is removed by washing. In the third step, the staining is evaluated using fluorescence microscopy.

An antibody specific to the phosphorylated form of H2AX was produced recently and used to show that γH2AX colocalizes with sites of double stranded DNA damage. [58] This antibody has been used to visualize DSBs. The specific antibody that has been used is anti-phospho-histone H2AX (Clone JBW301 Millipore Cat. No. 05-636).

3.7 Choice of experimental setup

Choice of cell culture conditions

In the past experiments were done in 96 well plates. [8] The first series of experiments on DNA integrity presented in this thesis (section 5.4) have also been done in 96 well plates. The reason for treating cells in 96 well plates is, that it was assumed easy to obtain a homogeneous and thus reproducible treatment in the entire well. Furthermore 96 well plates are widely used in cell research. However it was found that reproducibility of these experiments is very low for a number of reasons:

- Fluid is absorbed due to capillary forces between the needle and the wall of the wells. A new gas tube piece was made to counter this problem, but other problems still remained.
- The fluid on top of the cells does not have a flat surface due to formation of a meniscus. The meniscus makes the water surface in the well highly irregular, almost parabolic in shape. This means that more liquid is needed to prevent dehydration, because a large part of the liquid is at the sides.
- Cells dry up in the middle of the well because adhesion forces pull small remaining amounts of fluid to the sides of the well.
- The size of the discharge is smaller than the well. Therefore fluid evaporation caused by the plasma is local.
- The discharge does not always ignite due to turbulent flow of helium.
- It is difficult to see if the discharge is on, because the wells are spaced very close to another.
• Plasma power fluctuates when the discharge is ignited in the well. This is probably caused by the fluids moving in the well and by the manual positioning of the needle.

It can be concluded from the first series of experiments that treatment of cells in 96 well plates is not as conveniently simple as it at first seems. Therefore characterization of the plasma needle has been performed in chapter 4 to gain more understanding into the processes that influence plasma needle operation. To counter the problem of inaccurate positioning and liquid crawling up between the gas tube piece and the wall of the well in 96 well plates, a second gas tube piece was made. However problems still remained and therefore the scanner was built. With the scanner it is possible to treat large areas. Being able to treat larger samples means that all problems mentioned above are solved. The experiments reproduce better.

The program that has been used is a program written for x-y scanner that was used in a microscope. Some features in the program are therefore not really useful or do not work properly. Unfortunately there was no time to adapt the program to our needs.

The method of measuring power dissipation with an SWR power meter was chosen because equipment was easily available. This method has been used in the past. [59] It was noticed that warming up of the electronics influences the matching ratio. Ideally the plasma needle should be allowed equilibrate for at least 30 min before usage.

**Choice of method of treatment**

On the matter of the cell treatment procedure: the best choice for liquid to keep cells wet is medium. It was found that the plasma needle is able to denature the proteins in the medium. However if plasma power is below 200 mW temperature this effect does not appear. In the past PBS was sometimes used. PBS is isotonic and does not form clots when subjected to the plasma. A disadvantage of PBS is the fact that PBS does not contain any compounds that uphold the cells’ ability to attach such as Ca\(^{2+}\) ions. For attachment experiments it is therefore advisable to use medium when treating cells. It has been noticed, that cells round up after as soon as 10 minutes after treatment.

**Choice of DNA damage measurement technique**

One of the simplest methods to indirectly detect DNA damage is to measure the response of cells to DNA damage on the cell cycle. Analysis by flow cytometry is one of the most powerful techniques in terms of the number of cells that can be analyzed and the volume of data generated. Flow cytometry offers very good statistics and that is the reason it was used in this thesis as analytical technique. Disadvantage of the method is that any spatial information in the sample is lost, since trypsinisation removes all cells in the wells.

The reasons for the use of immunofluorescence are plentiful. Immunofluorescence is a more direct DNA damage measurement. Immunofluorescence provides more information than flow cytometry, as it specifically detects one type of DNA damage namely DSBs. Furthermore it is very sensitive. [60] It has been reported that each \(\gamma\)H2AX focus corresponds to a single DSB. [61] It is a simple and very fast method to detect DNA damage. Another advantage of this method is
that spatial information in the sample is retained, so response of individual cells within treated areas can be visualized.
4 Characterization of the plasma needle

Doing measurements on plasma treated cells is not easy. It is important, that cell treatment is reproducible and thus results in good measurements. There are many parameters which influence the quality of the measurements. These parameters can be divided in mechanical and biological parameters. Table 3 gives a list of both mechanical and biological parameters.

<table>
<thead>
<tr>
<th>Mechanical parameters</th>
<th>Biological Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas temperature</td>
<td>Cell viability</td>
</tr>
<tr>
<td>Flow speed</td>
<td>Cell density</td>
</tr>
<tr>
<td>Needle tip sharpness</td>
<td>Medium Poisoning</td>
</tr>
<tr>
<td>Materials/surroundings</td>
<td>Fluid levels</td>
</tr>
<tr>
<td>Plasma power</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: A list of parameters that influence experiment quality.

Chapter three presents an investigation into the mechanical parameters. Insight in these parameters will help improving the experimental setup. Also the influence of biological parameters will be discussed.

One other issue that will be addressed in this chapter is the issue of standardization. It is difficult to compare this experiment to previous experiments, because the electric setup is constantly changing. Furthermore discharge behavior is depending on experimental conditions. Therefore a good description of the conditions in the experiment is necessary to be able to compare results.
4.1 Treatment modes

There are two modes of plasma: unipolar and bipolar. In the unipolar mode the plasma surrounds the tip of the needle and is small. In close vicinity of a grounded object the plasma spreads over its surface and increases in brightness. This is called the bipolar mode. Figure 20 shows a graph of the gap size as function of the forwarded power.

![Figure 20](image)

Figure 20: Distance at which transition of unipolar to bipolar mode occurs for different powers. A PVC substrate was used.

4.2 Plasma power measurements

It was observed that power dissipation of the plasma fluctuates and that plasma power in different experiments can not be compared. To validate the need for measurement during plasma treatment plasma power was measured under various conditions. The intensity of the plasma is dependent on the surroundings. The surroundings determine the strength of the electric field at the tip of the needle and thus the size of the ionization zone(s). This then determines the size of the entire plasma, which consists of an ionization zone and a recombination zone.

To investigate the influence of substrate material plasma power was measured as described in section 3.4 for different substrates. Results are given in Figure 21.
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The influence of the presence of cells in a well was investigated more in depth. The difference of the presence of cells for various powers is graphed in Figure 22.

In order to estimate the fluence, the energy deposited by the plasma per area, the size of the plasma spot on the substrate was measured. The results of these measurements are shown in Figure 23. The width of the plasma bundle was measured with a marking gauge by aligning the gauge with the sides of the plasma outside the discharge zone, so that the gauge did not influence the discharge.
Calorimetry was employed to measure the energy that is transferred to the sample. A small Eppendorf tube was cut in half and the lower part was put into polystyrene foam for thermal isolation. The tube was filled with 0.25 ml water and the thermocouple was inserted. The needle was positioned at 1 mm from the water surface. Helium flow rate was 2 l/min. From the observed temperature rise after 30 seconds, the power needed for this change in water temperature was calculated. Thermal losses were low and therefore neglected. The ratio of this power and the plasma power (measured as indicated in 3.4) can then be calculated. A graph showing this ratio as a function of needle-to-surface distance for different input powers is shown in Figure 24.

Figure 23: Width of the plasma at the bottom, where it touches the surface.

Figure 24: Part of the energy generated by the plasma that is absorbed by the water.
4.3 Temperature measurements

To avoid thermal effects gas temperature has been measured. A type K (chromel-alumel) thermocouple has been used to measure the gas temperature of the plasma. The thermocouple was connected to a Fluke 87 multimeter with a Fluke 80TK Thermocouple module. Gas temperature of the plasma was measured at various needle to surface distances. These measurements are shown in figure 21. It can be seen that all measurements lineup on a single line. The gas temperature was found to scale linearly with power dissipated in the plasma. A graph of the measurement of the gas temperature at various helium flow rates is shown in Figure 26.

Figure 25: Gas temperature as a function of power dissipated in the plasma and distance to the substrate. It can be seen that all points line up on a straight line on the left side. It can thus be concluded that temperature is only dependent on power dissipated in the discharge. Flow speed was 1.5 l/min. The substrate was a polystyrene Petri dish.
The same setup has been used to measure the temperature of the liquid layer on top of the cells. Procedures for these measurements are somewhat different. The conduction current interferes with the measurement. Temperature can therefore only be measured after the discharge has been switched off. Results for a phosphate buffered saline (PBS) layer of 2 mm are shown in Figure 27. Unfortunately this is only possible for liquid layers bigger than 1.5 mm. Smaller layers cannot be created because the water will not form a layer but a drop instead. Furthermore the tip of the thermocouple is about 1.5 mm thick and will stick out of the liquid for smaller layers.
In vitro the situation is different. The cells on the floor of the well constitute an irregular surface and thus surface tension of the liquid is broken and liquid can still crawl in between cells. As a result a small (<1mm) layer of liquid is formed. Measurement of temperatures in this small layer is very difficult.

A method for measuring temperatures in these small layers of liquid is to solidify the water by adding a small amount of agarose. Pieces of 1x1x0.8 cm 1%wt agarose in water were cut. The temperature sensor was inserted into the block at 0.25 and 0.5 cm below the surface. Results of these measurements are shown in Figure 28. It was found that for low plasma powers the jet of helium cools the block.

![Figure 28: Water temperature as a function of power dissipated in the plasma. The plasma was ignited above a 1x1x0.8cm block of agarose. Ambient temperature of the agarose was 21.4±0.3 °C.](image)

**4.4 Fluid levels**

Evaporation of liquid by the plasma needle was studied by weighing a treated Petri dish with a Sartorius BP 110 S scale. The evaporation rate in open air was measured at 1.9 μl/min. Figure 29 shows the evaporation rate of the plasma needle for different needle to surface distances.
4.5 Effects on medium pH and H2O2 concentrations

The pH of several fluids after plasma treatment has been measured with pH test strips (Indigo Instruments 33812-5070 for mildly acetic fluids and Indigo Instruments 33812-7010 for basic fluids). 50 µl of fluid was pipetted into a well of a 96 well plate. Fluids were then treated by plasma. Power dissipated in the plasma was 50±30 mW and the helium flow was 2 l/m. The distance between the discharge and the fluid level in the center of the well was 3 mm. Measurements for this distance showed no change in pH of all tested media within 4 minutes. DMEM (Dulbecco’s modified Eagle’s medium, 10% FBS and 0.1% streptomycine) measured pH 8.5±0.3, PBS measured 7.5±0.3 and demineralized water measured 5.8±0.2. Measurements for a distance of 1 mm are shown in Figure 30. pH for demi water changed slightly from 5.8 to 4.8 in 4 minutes while the pH for PBS and DMEM did not change.
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4.8 5.0 5.2 5.4 5.6 5.8 6.0 6.2 6.4 6.6 6.8 7.0 7.2 7.4 7.6 7.8 8.0 8.2 8.4 8.6

Figure 30: The effect of plasma treatment on the pH value of the medium. Measurements were done with 50 μl of fluid.

Measurements on the effects of plasma treatment on H₂O₂ concentration are shown in Figure 31. The H₂O₂ concentration increases from 0 to 200 ppm in minutes for small amounts of liquid. The plasma used in this experiment is similar to the one in the pH experiment. The concentration graphs for DMEM and PBS showed similar profiles as the one shown for demi water.

Figure 31: The effect of plasma treatment on the concentration of H₂O₂.
4.6 Discussion

It was noted that the discharge sometimes intensifies when the tip of the needle is inserted in the well of 96 well plate. The walls of the well contain the helium and probably increase the helium purity which makes igniting the plasma easier.

Experiments show that both the plasma and the helium flow contribute to the evaporation of fluid. The plasma increases the evaporation rate with a factor 2. The evaporation rate does not change much when the plasma is moved farther away from the liquid. This suggest that the jet of heated helium stretches quite far outward, possibly more than 1 cm. If one takes a 5 mm plasma bundle this means that the plasma will be able to evaporate a hole in 1 mm of fluid in 30 seconds.

The fact that the evaporation rate lowers when the discharge is moved away from the substrate, can be understood by looking at figure 24. Less energy is transferred to the liquid at larger distances, thereby lowering the evaporation rate.

Temperature of the cells should preferably not rise above 41 °C, to minimize thermal effects of the plasma to the cells. Temperature measurements show that heat does not play a significant role in the experiments. When a flow rate of 2 l/min is used, the gas temperature does not increase above 50 °C for plasma powers up to 150 mW. This is in case of direct treatment: the plasma physically touches the cells. In previous experiments treatment times of roughly 60 seconds were found to be effective. [8] From Figure 2 it can then be concluded that damage is reversible if the gas temperature is kept below 50°C. Techniques to lower the temperature of the plasma have already been discussed in section 1.2.

When cells are treated indirectly with a layer of fluid of at least 2 mm temperature does not increase significantly. In fact when a low power plasma (<100 mW) is used the helium flow cools the fluid. Even for small layers of fluid temperatures are still very modest and do not rise above body temperature (37°C).

One can debate whether the thermocouple accurately measures the gas temperature of the plasma. The wires of the thermocouple conduct heat away from the measuring point. This means that the measured temperature will be lower than the actual gas temperature. However since temperatures are so low this heat leak is assumed to be very small. Furthermore it could be argued that temperatures are underestimated due to ion bombardment. However ion energies are so low (~5 eV), that contribution of the ion bombardment can be neglected.

Evidently there is no risk of poisoning the medium or PBS by plasma treatment at a distance of 3mm with a 40 mW plasma. At a distance of 1mm the plasma does induce changes in the medium. The plasma has then also shifted from the unipolar mode to a bipolar mode and touches the surface of the medium. From power measurements it is known that plasma power in this mode increases roughly by a factor 1.5. Whether the inability of the plasma to increase the pH was due to the distance or just low power could not be investigated at that time. The configuration at that time had a maximum of 40 mW. It can be understood that pH values for PBS and DMEM hardly change since these media are buffered. Usually a CO₂-bicarbonate buffer or an
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organic buffer such as HEPES is used to help keep the medium pH in a range from 7.0 to 7.4 depending on the type of cell being cultured.

Based on results shown in Figure 21 it can be calculated that the amount of H₂O₂ particles that are created or introduced to the medium per second is $10^{15}$ or roughly 2 nMol. This experiment was done with a very weak plasma (50 mW). Unfortunately it was not possible increase the plasma power. But it can be expected that the amount of H₂O₂ that is introduced to the medium increases as the power of the discharge increases.

It was hoped that all cells in the well would be treated homogenously. For 96 well plates, treated wells under the microscope show a nice circular area of detached cells, that has roughly the same size as the borders of the plasma needle tip. This means that the size of the plasma inside the wells is too small to guarantee homogenous treatment of all cells. Measurements of the size of the discharge confirm that it is too small to treat the entire well at once.

The first hurdle to overcome towards a good dose response measurement is that of finding a measure for the intensity of treatment. The choice of a sensible measure of intensity depends on what the active ingredients of the plasma are. At this moment it is not clear what each mechanism (UV, radicals, charged particles) contributes to the effects demonstrated by the plasma needle. Ideally one would use radical concentrations and absorbed doses of UV radiation to as a measure of ability to induce DNA damage. But insight in these mechanisms is required for a good choice. Lack of data on this subject means that a simpler and more generalized measure has been chosen for the intensity of treatment: plasma power. Plasma power seems like a logical choice for describing the intensity of the treatment. Kieft [8] showed that radical concentrations and UV intensities scale linearly with power.

Matchbox design strongly influences the discharge intensity. Since a number of different plasma needle and matchbox designs have been used in the past, we should use parameters to describe experiments that are not influenced by the design i.e. plasma power.

With regard to biological parameters: cell density should not exceed 500,000 cells/cm² as the cells will then very easily detach from the substrate in the form of large sheaths. Furthermore the amount of necrotic and apoptotic cells will increase when cell density is too high. Typically the fraction of necrotic and apoptotic cells in the entire population lies somewhere between 1 to 3 %, but can rise to somewhere between 5 and 10%.

Main objective of the characterization of the plasma needle was to find the best experimental conditions for in vitro plasma treatment. From the data presented in this chapter the following can be concluded:

- It is advisable to use a flow of more than 1.5 l/min. Preferably 2 l/min. These high flows are necessary to minimize thermal effects of plasma treatment.
- The amount of liquid that is needed to prevent dehydration can be estimated using the measured evaporation rate.
- Plasma power is an simple measure for the intensity of treatment and should be measured simultaneously to have accurate values. Determination of plasma power afterwards is difficult.
• With regard to the issue of the best measure for treatment intensity the following can be concluded. Since the effect of the plasma treatment is global (as shown in section 5.6), the best measure for treatment intensity would be either ROS concentrations or UV intensity. It does not seem logical to use the fluence, which can be determined by dividing the plasma power by the treatment area of the plasma.
Chapter 5 Measurements on DNA damage: Results

Section 5.1 will describe what was observed during manual treatment of cultured cells. This section consists of two parts: visual observations and viability assay. The first part will show what was observed during treatment of the cells. Viability of the cells was assayed with trypan blue, a stain that colors dead cells blue. Results of this assay are shown in the second part. Section 5.2 will elaborate on what was observed during treatment with the scanner. Section 5.3 will present measurements on cell counting. Dose response measurements with flow cytometry will be presented in section 5.4. Optimization and calibration of the immunofluorescence method will be discussed in section 5.5. The first part of section 5.6 will show pictures of plasma treated samples, which have been stained with anti-γH2AX. The second part will show the results of the dose-response measurements. In section 5.7 observations of measurements on samples, which were cultured for days after treatment. These results will then be discussed in section 5.8.
5.1 Manual cell treatment

Visual observations

3T3 wild type cells have been grown in 24 well plates for two days. (starting concentration 5000 cells/well) Flow rate was 2 l/min and discharge power was 150±50 mW. Helium purity was 99.996% (helium 4.6). Cells were treated for 10 seconds. Photos were taken with a Canon Powershot A720 IS with an Axiocam zoom lens locked in a Zeiss Axiovert microscope.

A typical picture of a treated well a few minutes after treatment is shown in Figure 32. 100 μl of PBS was added prior to treatment. A large void can be seen with necrotic cells in the middle. The large group in the middle is still fixed to the bottom of the well. The cells in the empty area are floating. Visually most of them appear dead however some living cells can also be identified. A close-up of these cells is shown in Figure 32.

![Figure 32: Left: Cellular void after treatment. The large group of cells in the middle is necrotic and fixed to the bottom of the well. The rest of the cells in the void are floating. Right: Close-up of detached cells after 150±50 mW plasma treatment. On the upper half of the picture cells are mostly necrotic and on the bottom half cells are still viable.](image)

It was noticed that cells only detach when temporarily a small void in the liquid layer on top of the cells is created. Usually this void is quickly filled up with liquid again. A photo of such a void in the liquid can be seen in Figure 33. The photo was taken seconds after treatment. The void had already filled again for a large part. Cells around the void were detached from the bottom of the plate but still fixed, because the void kept them in place. Once the void was filled cells started floating around and breaking up. Size and shape of the liquid void match the cellular void seen under the microscope. Therefore the area where the void in the cells appears, is also sometimes called the ‘dry zone’. The area where the liquid remained during treatment is referred to as the ‘wet zone’.
Figure 33: Left: Liquid void after treatment. Right: Close-up of the dry area immediately after treatment. Cells lie dried up without any substantial liquid. On the right side several cells can be found which are completely dry.

Treatment with the plasma touching the surface will evaporate the remaining liquid very fast, leaving the cells dry. A picture of cells just after treatment is shown in Figure 33. Control samples were treated with the helium flow. A picture of such a sample can be seen in Figure 34. The pattern appears almost similar to the pattern in the plasma treated samples. A void in the liquid can be seen and at the edge detached cells float in the PBS. The necrotic cells in the centre of the void are still attached to the bottom of the plate and do not show much instigation for detachment.

Figure 34: Flow treated sample. The cells in the gap between the normal attached cells and the dry area are floating.

Viability assay

HCT-116 wild type cells have been grown for two days in 96 well plates. (starting concentration 5000 cells/well) Flow rate was 1 l/min and discharge power was 50±30 mW. Helium purity was 99.996% (helium 4.6). 20 μl of PBS were added prior to treatment. Cells were treated for 10 seconds. After treatment 20 μl trypan blue has been added to assess the viability of the cells. Photos were taken through a Leica DM Irbe microscope (Objective lens Zeiss 10x) with a Hamamatsu C4742-95 digital camera. Wells were treated in 4 different ways: 1) with helium,
2) with the fixed tip, 3) with the small tip fixed in the middle and 4) with the small tip making a circle to increase the treated area.

All treated wells showed a more or less circular void in the middle where the plasma had been. There was no obvious correlation between the geometry of the affected area and the type of needle. In the voids both necrotic and living cells could be found. A picture of the edge of such a void is shown in Figure 35. Strange patterns are sometimes visible, but no relation could be found between method of treatment and these patterns. For example a large group of round floating cells could sometimes be found. A picture of such a group is shown in Figure 35.

![Figure 35: Left: Edge of a void induced by plasma treatment, with seemingly unaffected cells at the right. Cells have been stained by trypan blue. Right: A large group of detached cells above the void.](image)

**5.2 Treatment of cultured cells with the scanner**

3T3 wild type cells have been grown in 5 cm diameter Petri dishes. (starting concentration 200,000 cells/dish) Flow rate was 2 l/min and discharge power was 50±10 mW. Helium purity was 99.996% (helium 4.6). The Petri dishes were then scanned using the scanner. Scanning was done with the x-y subprogram. Parameters for this program are given in table 4. 0.2 ml of medium was added prior to treatment. Needle-to-surface distance was 1 mm. Photos were taken through a Leica DM Irbe microscope with a Hamamatsu C4742-95 digital camera unless stated otherwise.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>x start</td>
<td>15mm</td>
</tr>
<tr>
<td>x stop</td>
<td>15 mm</td>
</tr>
<tr>
<td>y start</td>
<td>-15 mm</td>
</tr>
<tr>
<td>y stop</td>
<td>-15 mm</td>
</tr>
<tr>
<td>Step size</td>
<td>4 mm</td>
</tr>
<tr>
<td>Velocity</td>
<td>5 mm/s</td>
</tr>
</tbody>
</table>

Table 4: List of scanner parameters.

Direct treatment of the samples is difficult. During treatment some areas dry up. Especially areas where the needle makes a turn in the scanning procedure are inclined to dry up. When these direct treated samples were examined under the microscope it was found that the cells in the
center of the dried areas were necrotic. Cells on the edges of these areas had detached and were floating in the medium. Figure 36 shows an image of such an edge. Treating the samples with only a helium flow also results in these transition areas. Figure 36 shows an image of an edge created by flow treatment. However some significant differences can be observed. The gap sizes in case of the plasma treated cells are bigger and most of the time completely devoid of cells, whereas the flow treated cells show very narrow gaps with some cells in the gap or no gaps at all.

Moreover morphologically the cells in the plasma treated area differ from the cells in the flow treated area. A close-up picture of both plasma and flow treated cells is depicted in Figure 37. Plasma treated cells appear intact and the nucleus and their nucleoli are clearly visible, while the flow treated cells have been rumpled up and are not showing their organelles very clearly. Both appear flattened.

The cells that are floating in the medium after treatment are a mix of living and necrotic cells. A close-up picture of these cells can be found in Figure 38. The necrotic cells do not appear to be intact anymore. These cells fall apart in a matter of minutes. An enormous amount of debris can be found in samples with necrotic cells. Also on some microscope photos debris can clearly be
seen around necrotic cells whereas around living cells this debris appears absent. Such a photo is shown in Figure 39.

Figure 38: Left: Floating necrotic cells. Right: Floating living cells. Some cells show a peculiar bumpy cell membrane. Also a large amount of debris can be seen.

Figure 39: Left: The edge of a treated spot. Left are living cells at the edge of the area where the plasma has been. On the right are necrotic cells with a visible debris field in the middle. (photo taken with the Canon Powershot A720 IS)

When samples were nearly confluent, clumps of cells could be found as depicted in figure 31. These clumps contained both living and necrotic cells. In this condition edges also contained dangling clumps as shown in Figure 40.
5.3 Cell counting

Three cell counting experiments have been done. 1) The number of detached cells has been determined, 2) the mixture of detached cells has been studied and 3) cell growth studies have been performed.

Cell detachment

3T3 wild type cells have been grown in 5 cm diameter Petri dishes. (starting concentration 200,000 cells/dish) Flow rate was 2 l/min and needle-to-substrate distance was 1 mm. Helium purity was 99.996% (helium 4.6). Scanning was done with the x-y subprogram. Parameters for this program are given in Table 4. 0.2 ml of medium was added prior to treatment. Cell counting was done with a Coulter counter (Coulter Counter ZM). Protocols and settings for counting are described in Appendix A.4.

![Figure 41: Amount of attached and detached cells after plasma treatment. The amount of attached cells should be multiplied by 10. The experiment has been repeated three times.](image-url)
Detached cell mixture

HCT-116 wild type cells have been grown for 2 days (starting concentration 2000 cells/100μl in one well) and manually treated with the plasma needle in 96 well plates. Flow rate was 1 l/min and discharge power was 50±30 mW. Treatment reproducibility was low as described in paragraph 2.2. Helium purity was 99.996% (helium 4.6). 30 μl of PBS were added prior to treatment. 100 μl of medium (McCoys 5A, 10% FBS, 0.1 % gentamicin) was put onto the PBS and cells were put back in the incubator (5% CO₂, 37°C) for at least 15 minutes in case of the 0 hour batch. After incubation cells were trypsinized and harvested. Two wells were pooled to increase the amount of harvested cells.

5.4 Dose response measurements with flow cytometry

HCT-116 wild type cells have been grown for 2 days (starting concentration 2000 cells/100μl in one well) and manually treated with the plasma needle in 96 well plates. Flow rate was 1 l/min and discharge power was 50±30 mW. Treatment reproducibility was low as described in section 3.7. Helium purity was 99.996% (helium 4.6). 30 μl of PBS were added prior to treatment. 100 μl of medium (McCoys 5A, 10% FBS, 0.1 % gentamicin) was put onto the PBS and cells were put back in the incubator (5% CO₂, 37°C) for at least 15 minutes in case of the 0 hour batch. After incubation cells were trypsinized and harvested. Two wells were pooled to increase the amount of harvested cells. The protocols for seeding, treatment and harvesting are given in appendix A.2 and A.3. After harvesting samples were fixed in methanol and stored at -20°C. For analysis cells were taken up in PBS with BSA. The cells were stained with PI 5 μl (1mg/ml) and analyzed with a BD Facsort flow cytometer. Protocols for these procedures are described in appendix A.4.

To study both long term and short term effects two different series were prepared. One contained samples that were treated for 0, 5, 10, 20 and 30 seconds. For each treatment time 5 samples were incubated for 0, 2, 4, 6 and 8 hours. For series no. 2 samples were incubated for 0, 24, 48 and 72 hours.
Measurements on the genotoxicity of nonthermal atmospheric plasma

Figure 43: Histograms of plasma treated HCT cells. Samples have been incubated for 0, 2, 4, 6 and 8 hours. The red lines correspond to the control sample. Yellow to 5 seconds of treatment, Green to 10 seconds, Light blue to 20 seconds and dark blue to 30 seconds.
From the histograms in figure 43 the amount of cells in the G1 and G2 phase were determined by fitting in modfit, a program for cell cycle analysis. The ratio of the fraction of the cells in G2 and the fraction of cells in G1 was from these numbers and is plotted as function of time in Figure 44. It can be seen that no significant deviations occur.

![Figure 44: Ratio of cells in the G2 phase and cells in the G1 phase after treatment.](image)

This experiment was repeated with a higher power. For this experiment 3T3 wild type cells have been grown in three 5 cm Petri dishes. Helium flow rate was 2 l/min and discharge power was 150±10 mW. The samples were scanned with the parameters found in table 4. Helium purity was 99.996% (helium 4.6). 1 ml of medium was added prior to treatment. After treatment cells were put back in the incubator (5% CO$_2$, 37°C) for at least 15 minutes in case of the control sample. The other two samples were incubated for 8 and 24 hours after treatment. After incubation cells were trypsinized and harvested. The protocols for seeding, treatment and harvesting are given in appendix A.2 and A.3. After harvesting samples were fixed in methanol and stored at -20°C. For analysis cells were taken up in PBS with BSA. The cells were stained with PI 5 μl (1mg/ml) and analyzed with a BD Facsort flow cytometer. Protocols for these procedures are described in appendix A.4.

The results for this experiment are shown in figure 38. It can be seen that the amount of cells in the G1 phase decreases dramatically while the amount of cells in the G2 phase increases. Also the amount of cells in the S phase seems to diminish.
Measurements on the genotoxicity of nonthermal atmospheric plasma

Figure 45: Histogram of plasma treated 3T3 cells. Cells were scanned with a 150 mW plasma. Scanner parameters can be found in table 4. The blue histogram represents the control sample. The green histogram corresponds to the 8H sample and the pink histogram to the 24H sample. It can be seen that the amount of cells in the G1 phase decreases dramatically while the amount of cells in the G2 phase increases. Also the amount of cells in the S phase seems to diminish. This is indicative of DNA damage.

5.5 Optimization and calibration of the immunofluorescence method

Optimization of the immunofluorescence protocol

3T3 wild type cells were grown in 12 well plates. (starting concentration 50,000 cells/well) An 18 mm cover glass was sterilized and put on the bottom of the wells prior to cell seeding. For optimization of the protocol eight different samples were prepared. A list of these samples can be found in Table 5. After treatment cells were placed in the incubator for one hour to allow them to recover and form γH2AX. Wells were then fixed and stained with anti-γH2AX. The secondary antibody was RAMFITC (Dako F0261) diluted 1:100 in PBS/BSA. Each cover glass was mounted on a microscope glass using mounting fluid containing PI, used as a counterstain. Protocols for these procedures can be found in appendix A.4. The samples were analyzed with a Zeiss Axiophot microscope equipped with a Biorad MRC 600 imaging system. This system is equipped with an Argon/Krypton laser, which excites the PI at 568 nm and FITC at 488 nm.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Description/Treatment</th>
<th>Fixative</th>
<th>Dilution antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control sample/Untreated</td>
<td>ethanol/acetic acid 95%/5%</td>
<td>1:500</td>
</tr>
<tr>
<td>2</td>
<td>Control sample/Untreated</td>
<td>Formaldehyde 3.7%</td>
<td>1:500</td>
</tr>
<tr>
<td>3</td>
<td>Staurosporine 0.5 μM, 6 hours</td>
<td>ethanol/acetic acid 95%/5%</td>
<td>1:1000</td>
</tr>
<tr>
<td>4</td>
<td>Staurosporine 0.5 μM, 6 hours</td>
<td>ethanol/acetic acid 95%/5%</td>
<td>1:500</td>
</tr>
<tr>
<td>5</td>
<td>Staurosporine 0.5 μM, 6 hours</td>
<td>ethanol/acetic acid 95%/5%</td>
<td>1:100</td>
</tr>
<tr>
<td>6</td>
<td>Staurosporine 0.5 μM, 6 hours</td>
<td>Formaldehyde 3.7%</td>
<td>1:1000</td>
</tr>
<tr>
<td>7</td>
<td>Staurosporine 0.5 μM, 6 hours</td>
<td>Formaldehyde 3.7%</td>
<td>1:500</td>
</tr>
<tr>
<td>8</td>
<td>Staurosporine 0.5 μM, 6 hours</td>
<td>Formaldehyde 3.7%</td>
<td>1:100</td>
</tr>
</tbody>
</table>

Table 5: A list of samples used for optimization of the immunofluorescence protocol.
Photos of sample no. 1, 2, 4 and 7 samples are shown in Figure 46. Samples no. 3 and 6 were similar to sample no. 4. Samples no. 6 and 8 were similar to sample no. 7.

If one compares the control samples, it can be seen that ethanol/acetic acid fixed samples show less detail and green signal. Both control samples (no.1 and 2) show one cell with DNA damage. In case of the ethanol/acetic acid fixation cells shows are very diffuse signal. The formaldehyde fixed cells show small spots and damaged cells show more and more intense spots. In case of the treated ethanol/acetic acid fixed sample (no. 4) no γH2AX positive cells could be found. The staurosporine treated sample shows unambiguously distinct spots. However the signal on staurosporine treated samples is still not so bright, when compared to plasma treated samples in section 5.6.

**Calibration**

3T3 wild type cells were grown in 12 well plates. (starting concentration 50,000 cells/well) An 18 mm cover glass was sterilized and put on the bottom of the wells prior to cell seeding. Two series were prepared: a UV series and H₂O₂ series. After 2 days of incubation the UV series were exposed to 254 nm UV radiation of 0, 1, 10 and 100 mJ/cm² using a Stratagene UV stratalinker 1800. The medium of the H₂O₂ series was replaced with medium containing 0; 0,1; 0,5; and 1 mM H₂O₂.

After UV treatment cells were placed in the incubator for one hour to allow them to recover and form γH2AX. The H₂O₂ treated cells were incubated for 2 hours. Wells were then fixed with formaldehyde (3.7%) and stained with anti-H2AX. Protocols for these procedures can be found in appendix A.4.

Of each sample 5 confocal images (containing both the green and red emission separately) were taken under identical lighting conditions. These images were analyzed with ImageJ. Only colored parts of the images were selected by using a threshold. The integrated value (or integrated density) from the green channel and the area from the red the channel were then measured. Furthermore the amount of cells in each image was counted. The integrated density per area and the integrated density per cell were then determined and averaged over the 5 photos. On average these photos contained 250 cells. These values were then normalized with respect to the control sample.

Results for the UV series are shown in Figure 47 and results for the H₂O₂ series are shown in Figure 48. The amount of γH2AX increases very rapidly for low fluences and saturates around 20 mJ/cm². The relative intensity per cell has been fitted with an exponential decay function. Saturation constant for this function is 7±3 mJ/cm². For the hydrogen peroxide treatment a similar trend is visible. Extrapolation of this signal gives a saturation value of 0.5±0.3 mM.

UV irradiated cells clearly showed increasing amounts of spots for subsequent higher fluences. For 10 and 100 mJ/cm² the samples showed cells, where no longer distinct spots were visible. Instead parts or sometimes entire cells were fluorescing very intensely. But in the 1 mJ/cm² sample spots were clearly visible. H₂O₂ treated cells also showed individual spots for low concentrations and very intense fluorescent nuclei for high concentrations.
Figure 46: PI (red) and γH2AX (green) stained cells. Upper left: Untreated sample no.1, ethanol/acetic acid 95%/5% fixed. Upper right: Untreated sample no.2, formaldehyde fixed. Lower left: Staurosporine treated sample no.4, ethanol/acetic acid 95%/5% fixed. Lower right Staurosporine treated sample no.7, formaldehyde fixed.
Figure 47: Amount of phosphorylated H2AX as a function of the fluence. Cells were treated with 254 nm UV radiation. Cells at 100 mJ/cm² were retracted and the coverage area was therefore significantly lower. The relative intensity per cell has been fitted with an exponential decay function. Decay constant for this function is 7 mJ/cm².

Figure 48: Amount of phosphorylated H2AX as a function of concentration of hydrogen peroxide. Cells were treated for two hours with hydrogen peroxide.
5.6 Dose response measurements with immunofluorescence

3T3 wild type cells have been grown in 12 well plates. (starting concentration 50,000 cells/well) An 18 mm cover glass was sterilized and put on the bottom of the wells prior to cell seeding. Four series of experiments were performed. Each series investigated one of the four parameters considered important: time, needle-to-liquid distance, plasma power and liquid depth. Treatment conditions of each series can be found in Table 6.

<table>
<thead>
<tr>
<th>Series 1: Time series</th>
<th>Flow rate</th>
<th>Treatment time</th>
<th>Power</th>
<th>Distance</th>
<th>Liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 l/min</td>
<td>Variable</td>
<td>100 mW</td>
<td>1 mm</td>
<td>2.5 mm</td>
</tr>
<tr>
<td>Series 2: Distance series</td>
<td>2 l/min</td>
<td>20 s</td>
<td>Varied from 160 to 100 mW</td>
<td>Variable</td>
<td>2.5 mm</td>
</tr>
<tr>
<td>Series 3: Power series</td>
<td>2 l/min</td>
<td>20 s</td>
<td>Variable</td>
<td>1 mm</td>
<td>2.5 mm</td>
</tr>
<tr>
<td>Series 4: Liquid series</td>
<td>2 l/min</td>
<td>10 s</td>
<td>120 mW</td>
<td>1 mm</td>
<td>Variable*</td>
</tr>
</tbody>
</table>

Table 6: List of experiment series and experimental conditions. *1 ml of medium was pipetted onto the samples with 0.4 ml medium and less directly after treatment.

After treatment cells were placed in the incubator for one hour to allow them to recover and form γH2AX. Wells were then fixed with formaldehyde (3,7%) and stained with anti-H2AX.

Visual observations on plasma treated samples

Photos of one sample from series 4 are shown in figure 44. These are photos of the sample which was treated with 0.4 ml (1 mm) liquid. Samples with less than 0.5 ml developed a dry spot in the middle of the sample. Photos are shown of cells in the dry zone and of cells in the wet zone.

Furthermore 3T3 cells in 6 Petri dishes were scanned with 100 mW using settings given in Table 4. Prior to treatment medium was removed, samples were washed with PBS and 0.2 ml medium was added. Treatment distance was 1mm and flow rate 2 l/m. Detached cells of these 6 Petri dishes were collected and spun onto microscope slides with a Shandon cytospin 4. Control samples were trypsinized and also spun onto microscope slides. These slides were then fixed with formaldehyde (3.7%) and stained with anti-H2AX.
Cells in the dry zone show some diffuse $\gamma$H2AX spots. The PI is mostly concentrated in the nucleus of the cells. Cells in the wet zone show numerous bright and diffuse spots and PI is evenly distributed over the entire cell. The detached cells control sample shows mainly cells, which do not have $\gamma$H2AX spots. And the cells which stain green show only diffuse spots. Cell
blebbing is a result of the trypsinization. The detached cells which came from the plasma treated samples show bright γH2AX spots.

**Dose response measurements**

Methods for analysis of the 4 series are described in section 5.5. Results of the series are shown in Figure 50 to Figure 53.

![Figure 50](image1.png)

**Figure 50:** Amount of phosphorylated H2AX as a function of treatment time. The uncertainty bars display the standard deviation of the 5 images per measurement.

![Figure 51](image2.png)

**Figure 51:** Amount of phosphorylated H2AX as a function of plasma power. The uncertainty bars display the standard deviation of the 5 images per measurement.
5.7 DNA repair and end points

3T3 wild type cells have been grown in 12 well plates. (starting concentration 50,000 cells/well) An 18 mm cover glass was sterilized and put on the bottom of the wells prior to cell seeding. Four plates were prepared and cells were plasma treated on the second day of incubation. Plasma power was 150 mW and the helium flow rate was 2 l/min. Treatment time was 20 seconds and treatment distance was 1 mm. Plates were fixed at 1, 33, 55 hours after treatment. Wells were then
fixed with formaldehyde (3.7%) and stained with anti-phospho-histone H2AX and anti-tubuline. Protocols are identical to the protocols used in section 5.6.

Cells at 55 hours appeared to exhibit more cells in the G2/M phase. Also some cells with abnormal DNA distributions were visible. An example of a cell which appears different can be found in Figure 54. The elongated cell in the middle is mitotic and both pair of chromosomes can be seen on either side of the cell. The cell does not show any sign of cytokinesis. Some cells displayed a malformed spindle. Figure 55 shows the spindle of a normal cell and a cell with a damaged spindle. No abnormal amounts of apoptotic cells were found.

Figure 54: Two mitotic cells with both pair of chromosomes at either opposite sides or the same sides of the cell. However both cells shows no signs of cytokinesis.

Figure 55: Left: A normal cell in the metaphase. Cells are stained with PI and tubuline, showing in red the chromosomes and in green the microtubules that make up the mitotic spindle. Right: A cell 51 hours after treatment. The condensed chromosomes are scattered over a large volume and the microtubules are in the middle and do not form a proper spindle.
The amount of γH2AX in the samples was determined as described in 5.6. The results are displayed in Figure 56. The values were normalized on the values of the 1 hour sample. The amount of γH2AX decreases to 27% after 55 hours.

![Figure 56: The amount of phosphorylated H2AX after plasma treatment.](image)

### 5.8 Discussion

Observation of the fact that the size of the voids in layer of cells induced by plasma treatment are of the same order as the dry area in the liquid covering the cells was already reported in [8]. Also there it was reported that if the liquid layer was too thin a small necrotic zone was visible in the middle. These cells had not detached. These observations correspond with observations reported in this thesis. However cell detachment was also observed when HCT cells were treated with a helium flow in 96 well plates and 24 well plates without plasma. This effect has not been observed in 5 cm Petri dishes. Directly after treatment an accumulation of cells can be seen at the edge of the void. (see Figure 32) This leads to the conclusion that cells at the edge of the void are blown outward. But it cannot be concluded that the helium flow is solely responsible for the voids in he layer of cells. When plasma treated and flow treated Petri dishes are compared, plasma treated Petri dishes contain roughly twice as much detached cells than the flow treated and control samples. Thus ionization of the gas enhances the detachment of cells at the edges of voids in the liquid layer.

It is speculated that plasma charges small pockets of liquid containing cells, which lowers the strength of attachment. The helium flow then blows these pockets with loosely attached cells to the sides. The surface tension of the water could also help in this detachment process by exerting a force on the cells. When the liquid edges move due to the helium flow cells could be torn loose from their substrate. If the cells then reach a liquid environment fast enough the will not become necrotic. Some other cells are less fortunate and liquid surrounding them dries up before they reach the edge of the void. These are the necrotic floating cells seen after treatment. The cells in the middle of the treatment area are almost sure to dry out as evaporation of liquid is most
efficient in the middle. These cells are essentially fixed as all liquid is evaporated. There is evidence that the cytoplasm is evaporated. When examining the dehydrated spots, only the nucleus of the cells in the dry area stain red. Apparently the cytoplasm which does stain in cells that are not necrotic has disappeared. This effect can clearly be seen in the upper two pictures of Figure 49.

Increasing treatment time and power (see [8]) results in an increase in the amount of necrotic cells. It seems unlikely that this necrosis is caused by the same mechanism that induces DNA damage. The timescale of cell injury through that mechanism DNA damage induction is much longer than

The fact that the first series of experiments with flow cytometry on cells in 96 well did not clearly show cell cycle arrest, while the second set of experiment on 5 cm Petri dishes did show these effects, can be explained by that the fact that treatment conditions in the first experiment were not as severe as in the second experiment. Although the same treatment conditions (30 s, 50 mW) that were used during the first experiment showed clear signs of DNA damage when examined by immunofluorescence. But it could be that only a small fraction of the cells is damaged in such a way that it will arrest. This fraction might be too small to influence proliferation rate of the total cell population.

The reason that was chosen for two measures to describe the amount of phosphorylated H2AX was the fact that each measure has its problems and that both complement each other. The drawback of the integrated density per area is that determination of the size of the red area is dependent on threshold settings. For some samples the red fluorescence signal was weak making correct determination of the area difficult. The drawback of the integrated density per cell is the fact that cells size influences the integrated density per cell and that sizes of cells vary per sample.

To be able to compare the different series it was chosen to normalize the measurements on the untreated control sample. The control sample always shows some degree of fluorescence. This background level of γH2AX can be attributed to DSBs occurring naturally as a result of oxidative metabolism. The intensity of the control sample is for a large degree dependent on the method of fixation. It was found that ethanol/acetic acid 95%/5% and methanol give a very weak fluorescence signal. Therefore it was chosen to use formaldehyde as a fixative, which gives a better signal. This unfortunately also means that interpretation of the measurements is somewhat more difficult. With ethanol/acetic acid fixation a clear distinction could be made between samples without damage and with damage. For formaldehyde quantification of the intensity of the signal is necessary.

The amount of phosphorylated H2AX shows a saturation characteristic. This saturation can also be seen in the UV calibration curve. This means that the saturation is not caused by the characteristics of the plasma needle but by a process inherent to the measuring technique. This is consistent with what is reported in [44]. Rogakou et al. also found a saturation behavior for the conversion of H2AX to γH2AX as a result of irradiation. A graph of their results is shown in Figure 57. From the description of their methods the fluence can be estimated. 100 Grays corresponds to 2.1 mJ/cm². This is roughly the average saturation constant for the exponential saturation. This corresponds to the saturation constant derived from the UV curve in Figure 47. Especially if one considers the fact a large part of the UV energy is not deposited in the cells...
because the cells do not cover the entire well. On average the coverage in the UV experiment was 35%.

![Graph showing percentage of phosphorylated H2AX as a function of dose ionizing radiation.](image)

**Figure 57:** Percentage of phosphorylated H2AX as a function of dose ionizing radiation. Picture taken from [44]. The closed and open dots represent two different experiments.

The saturation constant for the plasma treatment (figure 44) is 34±5 s. Consequently, in those 34 seconds 7 mJ/cm$^2$ of UV radiation was deposited on the samples by the plasma. This amounts to 0.2 mW/cm$^2$. This means that 0.2 % of the energy from the plasma went to chemical changes of the DNA.

The fact that the amount of γH2AX increases linearly with plasma power is understandable. Kieft has already reported that radiation increases more or less linear with power and also electron densities increase more or less linear with power. [8] Although it is a priori not to be expected that radical densities increase linearly with electron densities, it stands to reason that this might be the case.

The amount of γH2AX is higher when the plasma needle is within 3 mm of the liquid layer. A power fit of the data shows that the intensity decreases as $1.2\pm0.2$ power of $1/d$, where $d$ is the distance. This behavior is understandable as the plasma increases in intensity in close proximity of the medium. It was found that plasma power varied from 160 mW at short distances to 100 mW at longer distances. This means that fluorescence intensities should be 1.6 times brighter for short distance than for long distances. The intensity of the fluorescence signal decreases very slowly for distance above 3 mm. However the intensity of the signal is still larger than the intensity of the control sample. This means that the working range of the active ingredients of the plasma is several mm in air.

The amount of γH2Ax is independent of the thickness of the liquid layer on top of the cells. This means that the active ingredients of the plasma have a penetration depth of at least several mm. Results of this measurement show bigger uncertainty bars. This is probably due to the fact that the treatment time for this experiment was 10 seconds. This means that the signal to noise ratio is higher than for the other experiments. But to be able to incorporate samples with very small liquid layers it was necessary to shorten treatment time. If treatment is too long for these samples, they dry out and do not yield any results. Samples with a liquid layer of 1 mm and less showed a dry spot in the middle of the well. During analysis it was made sure that only images of the wet zones were analyzed.
The amount of phosphorylated H2AX decreases linearly after plasma treatment. Unfortunately no control sample was available, but it seems fair to assume that after 72 hours the signal will have decreased back to the background level. First thought that comes to mind is that the repair of the damage takes 72 hours. However there is some debate as to whether the phosphorylated H2AX is removed upon repair of the nick. Fillingham et al. report that removal of γH2AX is not a sign that repair of the DSBs has been completed. [62] They state that loss of γH2AX is due to completion of a step, that normally leads to repair.

All in all, loss of γH2AX in 72 hours is far longer than the 2 to 24 hours that is reported in literature following UV irradiation. [62, 63] This might indicate that damage is severe. Reports in literature show that loss of γH2AX takes longer if cells are irradiated with higher doses of radiation. [64]
Chapter 6 General discussion and conclusion

The last chapter will round off by answering the three research questions posed in the introduction of this thesis. After that recommendations for improvements in the setup and ideas for future research will be given.
6.1 Is plasma needle treatment able to induce DNA damage?

The answer to this question is without a doubt positive. It has been shown both by looking at the cell cycle with flow cytometry and by looking at the formation of repair foci by immunochemistry, that cells exhibit DNA damage following plasma treatment. With flow cytometry it has been shown that cells display G2 arrest and possibly also S arrest at 8 hours after treatment. This indicates that damage is severe enough to force cells to immediately repair the damage. And the fact that the amount of bright γH2AX foci increases after plasma treatment proves that plasma treatment induces DSBs. DSBs is the most severe form of DNA damage and is not easily repaired.

It has been shown that the extent of DNA damage is dependent on distance, plasma power and treatment time. The amount of liquid put on the cells does not seem to influence the amount of DNA damage much. Apparently the active ingredients of the plasma are able to traverse the liquid without any significant absorption.

There are two distinct modes of treatment: direct and indirect. Direct treatment means that hardly any medium or other fluids are covering the cells. The cells lie on the bottom of the well in an almost dry environment. A very small amount of liquid remains always remains and cannot be removed. Furthermore the plasma is in direct contact with the cells. Cells are thus directly bombarded by particles present in the sheath that has formed above them. Indirect treatment means that the cells are covered with a small layer of liquid.

It has been shown that direct treatment causes a spot consisting of a necrotic zone in the middle, a ring shaped void around this zone and living cells on the outside of the plasma affected area. Necrotic cells do not have the time to respond to any obtained DNA damage and therefore do not show any repair foci. Cells on the outside of the treatment zone do demonstrate signs of DNA damage and it can therefore be concluded that the treatment is not localized to the discharge area. Cells that have detached and have been very close the discharge, possibly in direct contact with the discharge, do not demonstrate more DNA damage than the cells that were outside the treatment zone. Indirect treatment causes DNA damage in all cells in the samples.

6.2 What causes DNA damage?

It is well known that plasma can increase the acidity of the treated liquid. [65, 66] And change in the pH of the growth media has been known to disrupt cell processes. Both Sun et al. [65] and Fridman et al. [66] reported a significant drop in pH by plasma treating fluids. Sun et al. contribute their decrease to generation of hydrogen peroxide. Measurements have shown that the plasma needle is not able to change to pH of large amounts (ml) of fluid. This rules out the fact that plasma poisons the medium of the cells by changing the acidity.

From Figure 48 we can conclude that 0.1 mM of H₂O₂ is enough to induce DNA damage. And from the data in figure 24 one can calculate that the amount of H₂O₂ molecules introduced to the medium by the plasma is capable of raising the concentration of H₂O₂ to hazardous levels. For 1ml of fluid and treatment of 60 seconds the plasma needle would be able to raise the H₂O₂.
concentration to 0.1 mM. This is for a plasma of 40 mW. For higher powers it is to be expected that \( \text{H}_2\text{O}_2 \) levels increase.

Kieft [8] has measured radical concentrations and reported that the number of radicals reaching the liquid was \( 2.6 \times 10^{12} \text{ s}^{-1} \). The radical density increased linearly with time and increased by a factor 8 for needle-to-surface distance below 3 mm. There is a striking resemblance between the graph showing those measurements and Figure 52. But this it is too simple to conclude that the damage is primarily caused by the radicals. The fact that radical densities increase at short distances is more likely to be caused by a higher radical concentration in the plasma, which is a result of more power dissipation in the plasma. On the basis of free radical lifetimes it is not possible to conclude that free radicals are the main suspects for DNA damage. Liquid levels had no clear influence on the induction of DNA damage. This is contrary to what one would expect. It is to be expected that the liquid will act as a sort of filter and that the amount DNA damage will decrease with more liquid on top of the cells. But in light of the hypothesis above it is concluded that the influence of radicals can still not be eliminated as cause of the DNA damage.

If one looks at the absorption spectrum of water in figure 58 it is obvious that only a minority of the UV photons are absorbed in the water. With an absorption coefficient of \( 10^{-2} \) to \( 10^{-3} \text{ cm}^{-1} \) and a layer depth of 0.25 cm, the intensity of the UV diminishes by less than 1%. This is true for UV above 200 nm. Possibly the plasma emits radiation below 200 nm due hydrogen or helium lines in that region. However these wavelengths are efficiently absorbed by both water and air and thus cannot reach the cells. Radiation of the plasma needle is therefore non-ionizing.

![Absorption spectrum of water.](image)

There are no data on the intensity of the UV radiation, generated by the plasma needle. An estimate of the intensity of the UV radiation can be made by examining the emission spectrum of the plasma needle shown in Figure 59. Nonthermal plasmas under atmospheric pressures are generally poor sources of near UV [67]: the irradiated power in the 200-300 nm region is of the order of 1 mW/cm\(^2\). It has been determined by Sladek that only 10% of the radiation is transmitted through the liquid and that 30% is in the form of radiation (visible and near UV/IR). [59] This means that 3% of the input power is absorbed by the cells. This is, under the assumption that most radiation is emitted downward, equivalent to 1 mW/cm\(^2\) at input powers of 100-150 mW. 10% of the spectrum is in the UV-C region (100-280 nm), leading to doses of several mJ/cm\(^2\).
for treatment times of 10 seconds and more. UV-C radiation is very aggressive in terms of its ability to damage DNA as shown in figure 4. Hanasoge and Ljungman [64] report that treatment with UV-C at doses as low as 0.5 mJ/cm² shows damage to the DNA. Conclusion is that these doses are biologically significant, but still rather low.

Figure 59: Emission spectrum for the plasma needle. Emission lines were ascribed to He, N₂, N²⁺, OH⁻ and O⁻. Note that sensitivity drops notably for wavelengths greater than 750nm. From [8].

The lifetime of radicals is not long enough to diffuse through the liquid into the cell. Radicals will have to be formed in situ by other processes. It is hypothesized here that radicals that enter the liquid are converted to species with a longer lifetime, such as ozone and H₂O₂. These species then enter the cell and damage biomolecules in the cell by forming radicals through processes which have been well documented in literature. The efficiency of radical generation in the cells due to H₂O₂ is greatly enhanced by UV radiation, which promotes hydroxyl radical formation through the following reaction:

\[
\text{H}_2\text{O}_2 + \text{hv} \rightarrow 2 \text{OH}^-.
\]  

(11)

This process is known is photosensitization. Reports on UV irradiation of H₂O₂ photosensitized bacteria show that inactivation is enhanced sometimes by a factor 10. [68]

There have been reports on UV (254 nm) induced DNA damage, that does not give the typical spots, when stained with for γH2AX. [69] Instead these cells show a diffuse pan nuclear signal. Because of the absence of distinct foci, it is concluded that UV induced phosphorylation of H2AX cannot be attributed to the formation of DSBs. However this is disputed [60] and precise relation between phosphorylation of H2AX and DNA damage remains unclear. The cells in this work show distinct foci after treatment also as a result of UV irradiation. The intense nuclear fluorescence that encompasses the entire cell or large portions of it when the cells are subjected to intense UV irradiation or high concentrations of hydrogen peroxide, is believed to originate from multiple foci overlapping to form bright large domains. Therefore no conclusion about the origin of the DNA damage can be drawn by the appearance of γH2AX.

Both UV irradiation and elevated concentrations of long living ROS cause a global effect. It can therefore not be decided on that basis which mechanism is the predominant factor for DNA
damage induction. Particle radiation on the other hand would cause a local effect, so it concluded that charged particles are not the main cause of the observed DNA damage. Particle radiation can be divided in two categories: electrons and heavy particles or ions. The electron temperature is about 2-3 eV. [8] The energies of the ions has been calculated by Sakiyama and Graves, who have developed several models of the plasma needle. [37] They report ion energies of 5 eV. So one can tentatively conclude that electrons and ions do not possess enough energy to play an important role in damaging DNA.

This confirms the conclusion that for direct treatment necrotic cells, that appear in the middle of a treated spot, do not die because of extensive DNA damage. This conclusion was already drawn from the fact that cell death from DNA damage occurs on larger time scales (hours) than the observed cell death, which takes place within a second after treatment. It is still unclear however, whether these cells die because of particle bombardment or dehydration. Particle bombardment could cause membrane permeabilization or rupture. It seems unlikely that cells die within one second because of particle bombardment. Therefore it is more plausible that cells have died of dehydration, as they are particularly sensitive to dehydration. [70]

### 6.3 What are the final effects of plasma treatment?

The importance of DNA repair in maintaining genomic integrity and protecting against development of cancers has been shown in studies involving cancer patients and cancer-prone individuals as well as in studies involving genetically altered mice that exhibit deficiencies in DNA repair. The connection between DNA repair defects and human cancer predisposition was first recognized by Cleaver. [71]

Recently a genotoxicity study of the plasma needle has been done on human skin. [72] Explants of human skin were treated with the plasma needle and analyzed by gel electrophoresis and fluorescent imaging. It was concluded from the analysis of the gel electrophoresis experiments that no DNA damage was present in the cells as a result of plasma treatment. However the methods used are less sensitive than the γH2AX method, since this method allows detection of single DSBs. With fluorescent imaging some micronuclei were found in samples treated with a high intensity plasma. Unfortunately no data are presented on the intensity of treatment. Formation of these micronuclei indicates that the plasma has severely damaged DNA during mitosis. The amount of micronucleated cells is proportional to the extent of chromosome damage. [73]

The fact that some cells show abnormal nuclear events such as micronuclei and abnormal mitotic spindles, leading to mitotic catastrophies is a strong indication that plasma causes severe DNA damage in some cells. [74] Unfortunately it is not yet possible to draw any decisive conclusions on the end points of these cells, since the follow-up of these samples was not long enough to observe whether these cells are either removed from the population by apoptosis or if the damage is eventually repaired.
6.4 Recommendations

On the matter of practicality the following recommendations can be made:

- The labview program can be updated so that it is more practical for experimenting. The code has been copied from a program controlling a microscope stage and therefore lacks practicality. Also the programmable mode does not work. Ideally the program contains a mode for scanning (round) Petri dishes, another mode for automatically positioning above culture plates and a mode for manual control.

- Ideally the power that the plasma consumes and the time it is burning is automatically recorded. This takes makes the treatment procedure easier and eventually more reproducible. One can also improve the scanner setup and incorporate an automatic control that regulates the amount of energy that the plasma burns up.

- As this was a first attempt at measuring DNA damage by immunofluorescence it was chosen to analyze the cells with a fluorescence microscopy rather than by flow cytometry. Although flow cytometry offers detailed information regarding the total level of cellular H2AX phosphorylation, it does not reveal information regarding the distribution or appearance of H2AX within the nucleus. Thus immunofluorescence was very useful to analyze different treatment areas within each sample. However having this information, it is recommended that in the future mainly flow cytometry is used for making dose-effect curves, since it offers better statistics than the analysis of a few fluorescence microscopy images. Also it is a less laborious task this way.

On the scientific side several ideas for future research can be formulated:

- The endpoints of the damaged cells are still an open issue. Cells are arrested in the G2/M phase, but it is not clear whether they are able to repair the damage or eventually die through apoptosis. And if the cells continue following the cell cycle, is this damage correctly repaired?

- Radical density measurements should be performed in vitro after treatment to see if the plasma needle is able to induce a state of oxidative stress. Electron spin resonance or fluorescent probes could be used to measure radical densities. A nice overview of radical detection techniques can be found in [75]. Furthermore cells could be treated with a small piece of CaF2 in front of the plasma to allow only the UV radiation to reach the cells in order to discriminate between UV and radical induced effects.

- Removing the flow of the plasma needle will solve the problem of dehydration. This will also shed light on the issue whether plasma or the helium flow causes the necrotic spots.
Appendix A

A.1 Protocols for media

*Making medium for HCT cell line*

Contents (250 ml):  
- McCoy’s 5a (90vol%)
- BCS (Serum) (10vol%)
- Gentamicin (0.1vol%) final concentration 50μg/l

1. Thaw serum (put in warm water).
2. Measure 225 ml McCoy’s 5a in cylinder.
3. Sterilize membrane of gentamicin (antibiotics) with alcohol.
5. Pipet 25 ml of Serum in cylinder.
6. Pour into bottle.

*Making medium for 3T3 cell line*

Contents (250 ml):  
- DMEM (90vol%)
- BCS (Serum) (10vol%)
- Gentamicin (0.1vol%), final concentration 50μg/l
- L-glutamine (1vol%)

1. Thaw serum and L-glutamine (put in warm water).
2. Measure 225 ml DMEM in cylinder.
5. Pipet 25 ml of Serum in cylinder.
6. Pipet 2.5 L-glutamine in cylinder.
7. Pour into bottle.
**Making trypsine**

Contents (50 ml):

- 2.5 ml trypsine, final concentration 0.125 gr/100ml → dilute 20x
- 0.5 ml glucose, final concentration 0.02 gr/100ml → dilute 100x
- 0.5 ml EDTA, final concentration 0.02 gr/100ml → dilute 100x
- 46.5 ml PBS

1. Thaw trypsine.
2. Pipet 2.5 ml trypsine in measuring cylinder.
3. Add 0.5 ml glucose.
4. Add 0.5 ml EDTA.
5. Fill up with PBS to 50 ml (46.5 ml PBS).
6. Pour into bottle.

**Making PBS/BSA**

1. Take BSA powder from fridge.
2. Weigh powder (1mg/ml).
4. Add BSA powder to cylinder.
5. Cover with tape and mix.
A.2 Cell culture protocols

Transfer Cells (in 5ml flask)

1. Remove medium.
2. Add 1 ml trypsin.
3. Incubate for 1 minute.
4. Add 3.5 ml of medium.
5. Resuspend.
6. Leave ca. 1/5 in flask. (ratio depends on cell type HCT: 1/4 3T3: 1/5)
7. Fill flask with 5 ml of medium.

Seed cells (with or without microscope cover glass)

Steps 2 and 3 are only necessary when preparing samples for analysis under the microscope.

1. Prepare an empty tube.
2. Put cover glasses in empty container of a culture plate. (keep sterile)
3. Sterilize glasses one by one in flame with tweezers.
4. Let glass cool down and drop in well.
5. Remove medium of culture flask and put in tube
6. Trypsinize for 1 minute (1 ml trypsin for small 25cm² flask)
7. Add medium from the tube. Mix.
8. Leave ca. 1/5 in flask. The rest can be put into the tube for seeding.
9. Count cells.
10. Dilute tube with medium such that the concentration is 50,000 cells/ml.
11. Pipet cell suspension from the tube in the wells.
12. Incubate for 48 hours.
A.3 Protocols for cell experiments with the plasma needle

*Manually Treat cells (in 96 well plates)*

1. Adjust flow rate and power settings.
2. Remove medium.
3. Wash with PBS.
4. Remove PBS and small amount of PBS to prevent drying out during treatment.
5. Turn on plasma and treat with plasma.
6. Remove PBS (only controls).
7. Add medium.
8. Put plate back into incubator.

*Treating cells with the scanner (in 5 cm Petri dish)*

1. Clean Petri dish container and needle with alcohol.
2. Adjust flow rate and close helium bottle.
3. Adjust power settings.
4. Adjust scanner (define zero’s and adjust scanning parameters).
5. Remove medium from sample and add a known amount of medium.
7. Lower arm and position needle to 1 mm above fluid level.
8. Turn on generator.
9. Run scan.
10. Open helium bottle as soon as needle is at the right position. (gently)
11. Take note of the forwarded power and the reflected power.
12. Close helium bottle at the end of the scanning cycle.
13. Take not of the forwarded power and the reflected power.
14. Move arm up and close Petri dish.
15. Turn off generator.
16. Add 3 ml medium.

*Harvest and fix cells for flow cytometry*

1. Prepare tube(s).
2. Remove medium and put in tube.
3. Wash with 200 μl PBS and put in tube.
4. Add 50 μl trypsin and incubate 1 or 2 minutes.
5. Remove trypsin and put cells in tube.
6. Wash with 200 μl PBS.
7. Centrifuge (1200 rpm 5 min (400g)).
8. Remove supernatant and vortex cell pellet in the tube slightly.
9. Add a few drops of methanol (-20 °C) while stirring the tube.
10. Add additional 3 ml of methanol and put in freezer.
Harvest and fix cells for immunofluorescence

1. Remove cells from incubator 1 hour after treatment.
2. Wash with PBS.
3. Fix (methanol -20 °C 5 min, Formaldehyde 15 min or 95% ethanol 5% acetic acid 5 min at room temperature).
4. Wash with PBS.
5. Add PBS and store in Fridge.
A.4 Protocols for Analysis

Counting cells

Settings of coulter counter:
- Current: 999
- Full Scale: 1
- Polarity: Auto
- Low Threshold: 16.1
- Upper Threshold: 99.9
- Attenuation: 16
- Matching: 10
- Alarm: off

1. Fill jar with 10 ml isotonic solution (isoton).
2. Shake tube with cell suspension lightly and pipet 50 μl in isoton.
3. Turn on coulter counter (2x).
4. Flush with isoton.
5. Count cells.
6. Insert jar with cells and count.
7. Repeat a few times (3x).
8. Flush with isoton.

Stain cells with PI for flow cytometric analysis

1. Centrifuge at 400 g 5 min.
2. Remove the supernatant.
3. Loosen the pellet and add 0.5 ml PBS/BSA.
4. Centrifuge at 400g 5 min.
5. Remove supernatant.
6. Add 300 μl PBS/BSA.
7. Add 5 μl PI containing RNAse.

Flow cytometric analysis (BD Facsort)

1. Empty waste container and fill sheath fluid container.
2. Turn facsmachine on and then computer.
3. Put pressure on system (handle between waste and sheath reservoirs).
4. Put pressure difference at low.
5. Start Cellquest (CQ).
6. CQ: Acquisition → connect to cytometer.
7. CQ: Acquire → open settings.
8. Set Acquisition and Storage (amount of cells to count: ca. 10,000).
9. Set button to run and click acquire (for test tick setup).
10. Set gate (format histogram in plots) Make sure all events are saved. (acquisition and storage)
**Staining cells with γH2AX antibody**

1. In case of formaldehyde fixation wash with Triton 0.1% and then wash 3 times with PBS to remove the Triton
2. Prepare primary antibody dilution 1:500 in PBS/BSA 3%. (50 μl per cover glass)
3. Pipet a drop of primary antibody dilution of 50 μl on each cover glass and incubate for 1 hour
4. Wash 3 times with PBS
5. Prepare secondary antibody dilution 1:100 in PBS/BSA 3%.
6. Pipet a drop of secondary antibody dilution of 50 μl on each cover glass and incubate for 1 hour
7. Wash 3 times with PBS
8. Take cover glass out of the plate and gently dry with the sides of a tissue
9. Add a drop of glycerol/PI solution on a microscope glass and lower the cover glass slowly on the drop.
10. Fix cover glass with nail polish
References

Measurements on the genotoxicity of nonthermal atmospheric plasma


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