Comet assay on the anhydrobiotic *Richtersius coronifer*

Environmental Biology, RUC, spring 2009.
Supervisor - Hans Ramløv
Abstract

The tardigrade, *Richtersius coronifer* has an ability to withstand desiccation by going into anhydrobiosis. During anhydrobiosis, metabolism is arrested, which leads to an accumulation of DNA damage, since repair systems are inactive. The understanding of desiccation tolerance in tardigrades is still superficial, and it is rather unclear if desiccation tolerance in tardigrades is due to an effective repair system or if it is connected to an efficient protection of DNA and other cell components. The repair system of *R. coronifer* upon rehydration is investigated by using comet assay to detect DNA damages in animals at 0 and 24 hours after rehydration.

There was found no evidence for an efficient repair system in *R. coronifer*. Still, it seems likely that *R. coronifer* possesses a complex combination of adaptations, including a considerable repair system, to exhibit successful anhydrobiosis and revival. Inevitably, more investigations must be done to demonstrate this claim.
# Abstract

# Introduction

# Materials and methods

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tardigrade preparation</td>
<td>4</td>
</tr>
<tr>
<td>Comet assay</td>
<td>5</td>
</tr>
<tr>
<td>Positive controls</td>
<td>6</td>
</tr>
<tr>
<td>Scoring of comets</td>
<td>6</td>
</tr>
<tr>
<td>Statistical treatment of data</td>
<td>6</td>
</tr>
</tbody>
</table>

# Results

# Discussion

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Results and method</td>
<td>9</td>
</tr>
<tr>
<td>Repair or protection</td>
<td>10</td>
</tr>
</tbody>
</table>

# Acknowledgements

# References

# Appendix 1 - detailed comet assay procedure

Page: 2
Introduction

Tardigrades are known to exhibit remarkable tolerance against physical extremes through their ability to go into a state of reversible metabolic halt, known as cryobiosis (Ramlof & Westh 2001; Hengherr et al. 2008). Cryobiosis can be induced by several environmental stresses - when induced by desiccation it is termed anhydrobiosis (Neumann et al. 2009). In this state, the organism can be referred to as a biological crystal, that according to the usual definition of life, where metabolism has to take place, cannot be considered as being alive (Ramlof & Westh 2001). In cryobiosis, an animal can cope with environmental conditions that would otherwise kill it in its active state (Wright et al. 1992). Thus, cryptobionts are able to occupy harsh and variable environments and live in a diversity of niches in marine, freshwater and terrestrial environments (Hengherr et al. 2008; Nelson 2002). *Richtersius coronifer* is a terrestrial anhydrobiotic tardigrade found on lichens and mosses, but should rather be called semi-terrestrial, since it needs its habitat to be covered by a water film to stay in an active state (Ramlof & Westh 1992).

During anhydrobiosis, serious damages to DNA are often induced (Jönsson 2007), since metabolism is arrested, and energy consuming repair systems are not working (Neumann et al. 2009). Hence, DNA damage will accumulate in the anhydrobiotic organism (Neumann et al. 2009). The kind of damage that is likely to occur in the genome are single and double strand breaks (SSB and DSB) and chemical modification of bases and sugars (Maynard et al. 2009). The cause of these damages to the DNA, as well as proteins and lipids, can be reactive oxygen species (ROS), UV light, ionizing radiation, high temperatures and chemicals (Maynard et al. 2009). To deal with the damage, organisms have evolved several repair systems. Base excision repair (BER) locates and repairs base modifications and SSB which are primarily caused by ROS attacks (Maynard et al 2009). The nucleotide excision repair (NER) removes bulky DNA adducts caused by UV light, chemicals and ROS. In cases where ionizing radiation, ROS and chemicals cause DSB, these are repaired by the recombination repair system (Maynard et al. 2009).

As in all other anhydrobiotic organisms, it is unclear if the tolerance of desiccation in tardigrades is connected to an efficient DNA repair system, or to a sophisticated system for protecting their DNA during their ametabolic state (Jönsson 2007; Neumann et al. 2009). It has been suggested that the accumulation of the disaccharide trehalose, plays an important role in the anhydrobiotic state (Westh & Ramlof 1991). Trehalose is known to stabilize membranes, proteins and other biological structures, protecting them against damage caused by exogenous conditions (Elbein et al. 2003). Westh & Ramlof (1991) have demonstrated a 20-fold increase in the trehalose level upon induction of anhydrobiosis in *R. coronifer*, which indicates the protecting role of trehalose. Besides trehalose, stress proteins are believed to play important roles in protecting organisms in anhydrobiosis from damage (Goyal et al. 2005). In this context, an increased activity of chaperone proteins of the hsp70 class has been identified in the tardigrade *Milnesium tardigradum* during the transition to anhydrobiosis and the following rehydration (Schill et al. 2004). Additionally, an unknown protein with a molecular weight of approximately 71KDa has been identified by proteome analysis of *R. coronifer*. It is likely that this protein belongs to the hsp70 family (Ramlof & Westh 2001). At the same time, studies show that there is a synergetic relationship between trehalose and a small heat shock protein, p26 (Collin & Clegg 2004; Ma et al. 2005; Viner & Clegg 2001).
The elevated presence of stress proteins and polar disaccharides during cryptobiosis, suggests that these animals have evolved a sophisticated system for protection of cellular components during their ametabolic state. But in the case that the damage arising during anhydrobiosis, cannot be completely prevented, biochemical systems for repairing DNA damages must be present in order for the organism to revive successfully (Jönsson 2007). Thus, biochemical systems for repair of damage, including DNA damage, may likely be present in anhydrobiotic organisms for them to remain viable after rehydration (Jönsson 2007; Neumann et al. 2009). The role of DNA repair in tardigrades is still unknown, and though not verified, processes of DNA repair may play a role in the tolerance to desiccation (Jönsson 2007).

The upper limit for survival of tardigrades in anhydrobiosis is not known. However, Guidetti & Jönsson (2002) have reported that the upper limit under atmospheric oxygen conditions is unlikely to exceed ten years. In a study of Hengherr et al. (2008) on M. tardigradum it is indicated that the time spent in anhydrobiosis is ignored by the internal clock. Thus, they suggest that it seems likely that desiccation produces a time shift in the age of tardigrades.

On the basis of the above-mentioned, the working hypothesis for this study has been that when R. coronifer exits anhydrobiosis, DNA damage will be excessive. Within a reasonable time frame, much of the DNA damage will be corrected by repair pathways, which require an active metabolism to function.

In this study, the ability of R. coronifer to repair its DNA upon rehydration is investigated by comparing DNA damage in storage cells collected from tardigrades rehydrated for 0 hours and 24 hours by using comet assay. Comet assay is a very sensitive method for determining gaps on the DNA sugar backbone due to the loss of a nucleotide base, as well as for detecting single and double stranded DNA breaks (Palmqvist et al. 2003).

This study should be considered as a pilot study for future experiments concerning comet assay with R. coronifer. For this reason the discussion will be rather extensive, including potential improvements in method.

Materials and methods

Tardigrade preparation

Dry mosses containing anhydrobiotic R. coronifer were collected at Öland, Sweden, in August 2008 and stored dry until use in April 2009. Tap water and a sieve column with nets of declining mesh sizes were used for extraction of tardigrades from the moss. The mosses were crushed over the sieve column and the filtrate from the 125 µm fraction of the sieve column was transferred to petri dishes containing tap water. Following a rehydration period of approx 45 min, the tardigrades, which were moving, were collected using an Irwin sling.

Two groups of tardigrades, apart from the positive controls, were used – a 24 hours group and a 0 hours group. The 24 hours animals were transferred to new petri dishes containing tap water at room temperature, where they were stored for 24 hours until use, whereas 0 hours
animals were used directly following the rehydration period. After the first 24 hours the survival of the animals was close to 100%.

To isolate storage cells for comet assay, the rehydrated animals were transferred individually to approx 30 µl PBS (Phosphate buffered saline-pH 7.4), where they were cut open using needles. Approx 25 µl PBS containing storage cells from 6 animals were transferred to 0.5 ml microcentrifuge tubes which contained 225 µl 0.75% low melting-point (LMP) agarose (Invitrogen). The tubes were kept at 37°C on a heating block until use. Relatively high amounts of cells were lost during the transfer to the microcentrifuge tubes, possibly by adherence to the petri dish and the plastic pipette tip.

**Comet assay**

The method was to some extent a modification of the original description by Singh *et al.* (1988) and at the same time modified from Palmqvist *et al.* (2003) and Neumann *et al.* (2009).

Of the resulting (LMP) agarose mixture, containing PBS and storage cells, in the microcentrifuge tubes, 200 µl was cast on the hydrophilic side of 85×100 mm Gelbond film (Cambrex Bio Science) using Lab-Tek II chamber slides (Nunc) as molds. The chamber slides were fixed on the Gelbond films with 1% normal melting-point (NMP) agarose (Invitrogen). The chamber slides were carefully removed after drying of the microgels for 10 min at about 5°C. For each group (0 hours, 24 hours and positive controls), six microgels were made.

**Lysis of cells**

Afterwards, the Gelbond films were immersed in cold lysis (4°C) buffer containing 4 M NaCl (AppliChem), 10 mM Tris-Base (Sigma-Aldrich), 100 mM EDTA (Merck), 10% DMSO (Sigma-Aldrich) and 1% Triton X-100 (Sigma-Aldrich), pH 10, and incubated for two hours at about 5°C.

**Electrophoresis**

Following lysis, the Gelbond films were washed with millipore water (3×5 min) before being incubated in cooled electrophoresis buffer (0.3 M NaOH (Merck), 1 mM EDTA, pH 13.2) in a horizontal electrophoresis tank (Bio-Rad Sub-cell GT) placed on ice for 20 min to allow unwinding of DNA. Electrophoresis was then conducted for 20 min at 20 V, 310 mA using a power supply 500/400 (Pharmacia Fine Chemicals). After electrophoresis the films were neutralized by immersion in neutralization buffer (0.4 M Tris (Merck), pH 7.5) (3×5 min) and then washed with millipore water (1×5 min).

The newly washed films were then covered in 96% ethanol for 90 min and subsequently allowed to air-dry in dark overnight.

**Staining**

The microgels were stained with SYBR Gold (Molecular Probes) for 10 min while placed on an orbital shaker (KS125 Basic, IKA). The films are washed with millipore water (1×5 min)
and cut into 65 × 25 mm pieces, placed on microscope slides and covered with 65×25 mm cover glass (Menzel-Gläser). The steps described above are as far as possible performed under dark conditions. Care should be taken not to alter the orientation of the gels during electrophoresis and slide preparation.

For a more detailed method see Appendix 1.

**Positive controls**

Tardigrades for positive controls were collected from the same culture as the 0 hours animals. After fixing the storage cells in the LMP agarose on the Gelbond film, the entire film was exposed to pure UV-C radiation (λ = 253.7 nm) for 30 sec. This was done to generate DNA damage in the storage cells. The UV-light used for the positive controls was provided using a 15 watt TUV G15-8 (Philips) bulb. After the exposure to UV-light the positive controls followed the same procedure as the Gelbond films with 24 hours and 0 hours storage cells. Making positive controls with the use of 35% hydrogen peroxide was initially tried. Exposure to hydrogen peroxide made the tardigrades burst from within and concurrently bubbles were observed. Even so, extraction of cells was attempted, but no comets could be seen on the microgels.

**Scoring of comets**

The microgels were examined with a fluorescence microscope (Dialux, Leica) with 625 × magnification and a green excitation filter (BP 546/20, IDP). The microgels were blind scored, and it was made sure that the comets were only scored once. Owing to the relatively low concentration of cells, every comet found was captured and analysed with the software Comet Assay III image-analysis system (Perspective Instruments). The % DNA in the tail, as calculated by the software, was further used for statistical analysis. In total, two hundred comets were counted and scored for each test group.

**Statistical treatment of data**

Data is presented as median value ± standard deviation of % DNA in tail. This end point was chosen because it is easily comparable across studies and laboratories (Burlinson et al. 2007). As reviewed by Lovell & Omori (2008), there are some statistical problems associated with the use of comet assay. Our data did not confine to a normal distribution, and could not be transformed adequately. For this reason, only non-parametric methods were used. A Kruskal-Wallis test was used to compare overall differences of the median values of each test group. A non-parametric multiple range test was used for post hoc pair wise analysis (Zar 1998). The statistical significance level was set to p<0.05.
Results

Half of the gels were stored for 24 hours before staining and scoring, but no pattern arose from this procedure, and it is safe to assume that it had no effect on the outcome of the assay. The group that showed the highest amount of DNA in the tail was the 24 hours sample (32.00 ± 20.621), followed by the 0 hours group (25.69 ± 20.66) and the positive control (22.25 ± 15.04) (figure 1). The groups are overall significantly different (Kruskall-Wallis test, p<0.05), but only the 24 hours and positive control groups differ significantly (multiple range test, p<0.001). The remaining groups (0 hours & positive control and 0 hours & 24 hours) are statistically inseparable (multiple range test, p>0.05).

Figure 1: Box plots showing the median and 50% fractile of %DNA in tail in three test groups. 24 hours: (32.00 ± 20.621), 0 hours: (25.69 ± 20.66) and the positive control: (22.25 ± 15.04)

The comets from each group did not look noticeably different in the microscope. The positive control group showed a slight tendency towards the “hedgehog look” (Lovell & Omori 2008) (figure 2c). The 0 hours and 24 hours groups did not seem to look significantly different from each other (figure 2a and 2b, respectively).
Figure 2: Fluorescence microscope captures of comets made from storage cells of R. coronifer at various time intervals after rehydration. (A) 0 hours; (B) 24 hours and (C) positive control. Comets represent the median values of each test group.
Discussion

As this project can be considered as a pilot study for further work involving *R. coronifer* and comet assay, our discussion has been extended to favor the proposal of an improved method and possible workings of repair and protection mechanisms in tardigrades.

Results and method

Our results show, that the group of animals that have been hydrated for 24 hours, is the one with the most DNA damage. The positive control group is the one with the least amount of DNA damage and there is no difference between the 0 hours and positive control or the 0 hours and 24 hours groups. The method has not been extensively tested or optimized for this specific organism, but there were not any obvious technical changes that would have yielded a better result. Our hypothesis states that animals with an active metabolism will repair their DNA, and that the amount of damage should decrease proportionally with the time after rehydration. Keeping in mind that survival for the first 24 hours was close to 100%, we would expect that DNA damage had been drastically reduced. This was not the case.

In HeLa cells, it was shown that UV-radiation can only be used to induce a certain amount of DNA damage (Xu *et al.* 2008), which is detectable by comet assay. The mechanism behind this phenomenon might be the same in tardigrades. Because there is no significant difference between DNA damage in the positive controls and the 0 hours group, it might be that both groups of animals were saturated with the kind of DNA damage, that can also be induced by UV light. However, the damage is most likely not caused by UV radiation, but can be a result of ten months in anhydrobiosis.

The group of animals that were hydrated for 24 hours, was the one with the most DNA damage. Neumann *et al.* (2009) show that the % DNA in tail increased until 90 min after rehydration, whereupon it declines. They presume that the increase in DNA damage is based on the work of the NER system, which cuts out faulty nucleotides and insert new ones. In other words, when *M. tardigradum* has been in cryptobiosis for two days, the NER system is active for 90 minutes before the repaired damages are detected. The *R. coronifer* used for this study had been in anhydrobiosis for ten months and has consequently accumulated more damage, which leads to the suggestion that the detection of repaired DNA damages might be delayed. Thus, 24 hours after rehydration an increase in % DNA in tail may be observed.

The initial attempt at using hydrogen peroxide for positive control treatment did not yield any comets. It did however gave rise to an interesting observation. Put into hydrogen peroxide, the animals burst with bubbles, of what was presumeably oxygen. The reaction was relatively intense, considering the size of the animals. The catalatic reaction that splits hydrogen peroxide into water and oxygen can be simplified substantially as: 2H₂O₂ --> 2H₂O + O₂. The observed reaction in the laboratory could mean, that these animals contain large amounts of catalatic enzymes to protect themselves against oxidative stress. See Kirkman & Gaetani (2007) for detailed description of catalase. They may rely heavily on these enzymes to protect their cells during anhydrobiosis. Whether this apparent catalase activity is connected to anhydrobiosis, or is a general trade, has not been investigated as of yet.
A fundamental problem with the combination of this method and the test organisms, is the lack of a negative control. Since all animals had been in anhydrobiosis for ten months, no test group will be without treatment, so to speak. To get a more precise understanding of the way these animals protect, or repair, their DNA, a much larger experimental set up can be proposed. To observe the activity of the suggested repair mechanism, the starting point for any experiment, must be on animals with a certain amount of DNA damage. Animals that have been in anhydrobiosis for ten months serve this purpose perfectly. The core method of rehydrating animals and testing them for damage using comet assay also seems to work well for this purpose. However, the time frame of the experiment needs to be expanded substantially. Our results show that no damage has been repaired during the first 24 hours of rehydration. This does not necessarily mean the system does not work, but it could mean that more time is required to see any significant changes in % DNA in tail. The problem of negative controls is a result of the time spent in anhydrobiosis, as well as the specific species of tardigrade. R. coronifer is yet to be succesfully cultured, which is a problem if offsprings should be used as a possible negative control group. The offsprings of M. tardigradum show very little or no DNA damage although the parental generation is heavily damaged (Neumann et al. 2009). Should R. coronifer be succesfully cultured, then a population like this could be used to investigate the amount of radiation, or chemical treatment, needed to create a significant difference between a negative and positive control group. Furthermore, the suggested UV-damage saturation mechanism could also be tested using negative control population as test animals. To study the role of catalase in cryptobiosis, a measure of catalatic activity in both recently rehydrated animals and the negative control group, would have to be performed spectroscopically (Upadhya & Nagajyothi, 2000).

An experiment, including some of the above improvements, might have served the purpose of proving our hypothesis to a greater extent.

Repair or protection

The prokaroyte, Deinococcus radiodurans, capable of coping with excessive DNA damage, has been investigated. D. radiodurans is known as the most DNA damage resistant organism to be identified (Battista 1998). Mattimore & Battista (1996) showed that D. radiodurans’ resistance to ionizing radiation is a fortunate side effect of the ability to survive prolonged desiccation. The reason behind D. radiodurans' resistance is believed to be due to a rapid and accurate repair of the DNA damage (Battista et al. 1999).

Bacteria with resistance to radiation are dispersed in a phylogenetic tree suggesting that the mechanisms responsible for the resistance have evovled independently (Blasius et al. 2008). Additionally, the fact that other cryptobiotic organisms, such as tardigrades, exhibiting desiccation tolerance contributes to the idea, that this ability has been acquired independently in these organisms too. Also, it might give an indication of how R. coronifer survives and repairs its DNA after exiting anhydrobiosis by looking at the way D. radiodurans copes with the DNA damage. Because of the connection between D. radiodurans' resistance to ionizing radiation and dessication and its very effective DNA repair system, Neuman et al. (2009) also suggest that tardigrades have a comparable DNA repair system. But at the present time, there is no
molecular details about the DNA repair systems in tardigrades (Neuman et al. 2009). Therefore a further investigation on the molecular level of the repair mechanisms in R. coronifer as well as in other tardigrades could shed light on the ability of the organism to survive prolonged periods in anhydrobiosis.

Although an efficient repair system might exist in R. coronifer, or in tardigrades in general, it is yet unknown how big a role this system plays for anhydrobiosis in comparison with mechanisms for protection of cell components.

There are clearly different ways to achieve successful anhydrobiosis and several adaptations are without much question, required. Nevertheless, the understanding of these adaptations that permit these organisms to survive anhydrobiosis is still remarkably superficial (Crowe et al. 2002).

It seems that the accumulation of disaccharides prior to anhydrobiosis is nearly universal in organisms displaying this ability (Crowe et al. 2002; Guo et al. 2000; Schill et al. 2009). A feature of trehalose is that it can form high melting point glass (vitrify) at low water contents, by which it has the ability to preserve biological membranes and macromolecules at very high temperatures and at extreme levels of desiccation (Crowe & Crowe 2000; Clegg 2001; Ma et al. 2005). Trehalose is thought to be necessary for successful anhydrobiosis in animals, but does not seem sufficient for anhydrobiosis by itself (Crowe et al. 2002; Schill et al. 2009). In continuation of this, Oliver et al. (2002) point out that certain types of adventitious enzymatic reactions are possible at low water contents, which along with free radicals can cause damage. And disaccharides, as trehalose, are not necessarily preventing these damages, consequently other compounds might also be necessary for protecting an organism during anhydrobiosis.

Considering the important involvement of heat shock proteins (Hsp) in protection of proteins against damage from several environmental stresses, it seems very likely that Hsp are also involved in the ability of organisms to reversibly desiccate (Clegg 2001). Additionally, potential disaccharide-Hsp interactions have been investigated. These interactions may indicate that no adaptation alone can be singled out, but the diverse adaptations should be considered as a whole. Studies on the very resistant encysted embryos from the crustacean Artemia franciscana report that trehalose and a small Hsp, called p26, act synergetically in improving survival following desiccation and rehydration (Collins & Clegg 2004; Ma et al. 2005; Viner & Clegg 2001). Both components are necessary for optimal survival, and without trehalose there is no detectable protective effect of p26 (Collin & Clegg 2004; Ma et al. 2005).

Ma et al. (2005) propose that trehalose provides protection during desiccation, whereas p26 protects and repairs damage during and especially after rehydration. In this context, Liang et al. (1997) suggest that the function of p26 may depend upon its ability to function as a molecular chaperone and, that p26 has an ability to limit damage in stressed cells by preventing complete denaturation and aggregation of proteins (Liang et al. 1997). Further studies on embryos from Artemia have shown that about half of the p26 in the cytoplasm is translocated into the nucleus during anoxia and other imposed stresses, and this translocation is reversed when the stress is removed (Clegg et al. 1994; Clegg et al. 1995; Clegg et al. 1999). This could lead to the deduction that p26 is involved in the protection of DNA during imposed stresses.

Besides p26, it appears that trehalose and Hsp104 complements each other synergistically in the heat shock response ofSaccharomyces cerevisiae (Elliott et al. 1996; Iwahashi et al. 1998; Singer & Lindquist 1998). Singer & Lindquist (1998) reported that trehalose limit
aggregation during heat shock of proteins by maintaining them in a nonnative form until they could be refolded by Hsp104.

According to a bias made by Clegg (2001), anhydrobiotic organisms prevent desiccation damage, chiefly by participation of water substitutes, such as trehalose, and vitrification, rather than relying heavily on repair by molecular chaperones. But in event of denaturation during desiccation, a stress response will be launched when hydrated cells resume metabolism (Clegg 2001).

This is consistent with findings of Jönsson & Schill (2007), which shows a reduced level of Hsp70 in R. coronifer in desiccated state, while the results show an increase during rehydration. On the basis of these results it can be deduced that the Hsp70 class may be involved in repair processes after anhydration rather than in biochemical stabilization during desiccation.

In summary, there was found no evidence for an efficient repair system in R. coronifer. Even so, the hypothesis is not disproved and cannot be rejected. Actually, several studies seem to contribute to the suggestion that an effective repair system is involved in the ability of anhydrobiotes to exhibit successful anhydrobiosis and following revival. At the same time, it seems likely that a complex combination of adaptations is responsible for the desiccation tolerance of R. coronifer. Evidently, more investigations must be done to clear up questions concerning anhydrobiosis in R. coronifer.

Acknowledgements

We thank Jette Rank and Klara Jensen for help with fluorescence microscopy.
Thanks also go to Annemette Palmqvist for a detailed insight of the lab procedure.
The valuable contribution by Morten Foldager on statistical assistance is also very much appreciated.
We would like to thank Louise Madsen for help in the laboratory.
At last but not least we would like to acknowledge the help and guidance provided by our supervisor Hans Ramløv.

References


Appendix 1 - detailed comet assay procedure

Extraction of cells

Solutions

1×Phosphate Buffer (PBS): To make 1 liter 20×PBS 160.0 g NaCl (58.44 g/mol), 4.0 g KCl (74.56 g/mol), 28.8 g Na₂HPO₄ 2H₂O (177.99 g/mol) and 4.8 g KH₂PO₄ (136.1 g/mol) are dissolved in 1000 ml millipore water and adjusted to pH 6.5. To make 500 ml 1×PBS, dilute 25 ml of 20×PBS in 475 ml millipore water and adjust to pH 7.4 using HCl. Solution is autoclaved and kept at room temperature.

0.75% low melting point (LMP) agarose: 0.075 g of LMP agarose is mixed with 10 ml of 1×PBS and shortly heated in a microwave oven until the agarose has melted. Keep the agarose at 37°C for it to remain liquid.

Procedure

For each sample, 200 µl LMP agarose is transferred to a micro centrifuge tube. These tubes are kept on a heating block at 37°C until use.

A drop of approx 30 µl 1×PBS is placed onto a petri dish. The adequate number of rehydrated animals is transferred to the drop using an Irving sling. Using two needles under a microscope, the animals are cut open to release the cells. Using a pipette, approx 25 µl of PBS containing cells is removed while avoiding dead animals. These 25 µl are transferred to a micro centrifuge tube with LMP agarose. When mixing the solution using a pipette the cells tend to adhere to the pipette tip.

Fixation of cells in agarose

Solutions

1% normal melting point (NMP) agarose: 0.1 g NMP agarose is mixed with 10 ml of 1×PBS and shortly heated in a microwave oven until the agarose has melted.

Procedure

To fix the Lab-Tek II chambers to the Gelbond film, the chambers are dipped into the liquid 1% NMP agarose and placed on the hydrophilic side and allowed to solidify at 5°C for ½ to 1 hour (this can be done the day before). Three chambers per Gelbond film is optimal.

Approx 200 µl of LMP agarose containing cells from one micro centrifuge tube is distributed evenly into a chamber well. The Gelbond films are placed at 5°C in dark for 10 min to allow the LMP agarose to solidify. By cutting the edges of the Gelbond films can be marked.

Lysis of the cells
Solution

*Lysis buffer* (4 M NaCl, 10 mM Tris-Base, 100 mM EDTA, 10% DMSO, 1% Triton X-100, pH 10): To prepare 1 liter lysis buffer 262.4 g NaCl, 1.36 g Tris-Base (121 g/mol) are dissolved in 500 ml 225 mM EDTA while stirring and millipore water is added to obtain a volume of 1000 ml. pH is adjusted to 10 using 5 M NaOH or NaOH pellets. Stored in dark at room temperature (but can be stored in refrigerator for convenience). Right before use, 11.25 ml DMSO and 1.125 ml Triton X-100 is added per 100 ml lysis buffer.

Procedure

The Lab-Tek II chambers are carefully removed from the Gelbond films which are immersed in cooled (approx 5°C) lysis buffer for 2 hours in dark at approx 5°C. Make sure that the Gelbond films do not overlap. After lysis, the Gelbond films are washed 3×5 min in millipore water.

Alkanline treatment and electrophoresis

Solution

*Electrophoresis buffer* (0.3 M NaOH, 1 mM EDTA, pH 13.2): To prepare 1500 ml (depending on the size of the electrophoresis tank) electrophoresis buffer, 45 ml 10 M NaOH and 7.5 ml 0.2 M EDTA is diluted in 1447.5 ml millipore water. pH of the electrophoresis buffer is 13.2 and needs no adjustments.

Procedure

The electrophoresis buffer should be chilled and freshly prepared for every electrophoresis. The cold electrophoresis buffer is poured into the electrophoresis tank, which is placed on ice and in dark. Before the Gelbond films are placed in the electrophoresis tank, the power supply is adjusted to 20 V and approx 300 mA. Turn the power supply off and place the newly washed Gelbond films horizontally in the tank, so that they are covered by the electrophoresis buffer. The Gelbond films are incubated in the electrophoresis buffer for 20 min before electrophoresis is conducted. Subsequently, the power is turned on and the electrophoresis is conducted for 20 min. Make sure to keep track of the direction of the electric current. Again the Gelbond films must not overlap.

Neutralization

Solution

*Neutralization buffer* (0.4 M Tris-HCl, pH 7.5)

Procedure

Gelbond films are removed from the electrophoresis tank and washed 3×5 min with neutralization buffer and 1×5 min with millipore water. After neutralization, the Gelbond films are placed in 96% ethanol for 90 min in dark. The Gelbond films are air-dried in a darkened box overnight. Dried Gelbond films can be kept for about a week before staining.

Staining
**Solution**
TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5-8.0)
SYBR Gold

**Procedure**
To obtain a 1:20000 ratio of staining solution, 2.5 µl SYBR Gold is dissolved in 50 ml TE-buffer. The Gelbond films are immersed in 50 ml staining solution each on an orbital shaker for 10 min. The Gelbond films have to be stained separately. The Gelbond films are washed 1×5 min with millipore water after staining. The micro gels are cut from the Gelbond films and placed on microscope slides and covered with cover glass.

Comets are scored using a fluorescence microscope (Dialux, Leica) with 625 × magnification and a green excitation filter (BP 546/20, IDP).