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Chapter 4

Electrophoresis in the Comet Assay

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76880

Abstract

The comet assay is a sensitive technique to measure lesions in DNA, based on electrophoretic separation of DNA from cells embedded in agarose. Movement of DNA fragments is determined by the potential (V/cm), the time, and the viscosity of the medium (agarose). There is historically considerable confusion as to other factors, that is, current, liquid depths, circulation of the liquid, and temperature. Lack of standardization of electrophoresis including suboptimal power supplies and electrophoresis tanks causes considerable variations within and between laboratories. Ring trials have not been able to clearly identify the cause(s) of variation. Comparison of comet data from cohorts of human blood lymphocytes is used in the COST project hCOMET to identify early biomarkers of the disease. This calls for standardization of analysis. We performed measurements of electric potentials in a tank using multiple electrodes. Variations (time/position) were reduced by circulating electrophoresis liquid at 10% (volume) per min; this also stabilized the temperature. Circulation was accompanied by only slightly reduced variation in DNA damage among 384 irradiated cell samples electrophoresed concomitantly. In conclusion, comparing data between laboratories and cohorts must give emphasis to electrophoresis conditions. Results should be specified with respect to voltage (V/cm), time, and agarose concentration. We expect that suitable correction factors for these parameters may reduce inter-laboratory variations in comet data, allowing more precise comparison of results from different human cohorts.

Keywords: comet assay, electrophoresis, electric potential, agarose, power supply

1. Introduction

Efficient methods for quantifying DNA damage in cells of various types have been available for more than 30 years. Methods have suffered from low sensitivity, complicated protocols, or

they were used only for a limited spectrum of cell types. The assay mostly used during the last three decades was first published by the Swedes Östling and Johanson in 1984 [1]; other versions followed, from N.P. Singh and R. Tice in 1988 [2], and Peggy Olive and co-workers [3]. These protocols are in principle quite similar but vary in their use of neutral or alkaline electrophoresis conditions and also in some other steps of the procedures, such as lysis cell conditions. The methods were initially given different names: microelectrophoresis [1], single cell gel electrophoresis [2], and Comet Assay [3]. The latter name—which we will mostly use in this chapter—stems from the comet-like image seen in micrographs of damaged cellular DNA as it stretches out after electrophoresis.

In the comet assay, cellular DNA embedded in thin layers of agarose (researchers choose different concentrations) are electrophoresed while placed in a horizontal electrophoresis tank. At this stage the cells are dead and devoid of most of their proteins—whether they originate from a live organism: human blood or a specific organ, an animal, a fish or a sea star, or a plant (there is in fact hardly no limitation)—or from cells or organ cultures treated with radiation, or chemicals in vitro.

The comet assay has often been used simply to show the formation or presence of DNA damage in cells in a relative scale, without any reference to a standardized output or a calibration. However, in various applications, there is a need for standardization and calibration of comet assay data. Examples are when results from different experiments, cell types, and laboratories are to be compared, or when different human cohorts are combined in international collaborative studies. In the EU COST action hCOMET, the main aims are to create a unified database of comet assay data relating to human health and disease that will be the base for pooled analysis on DNA damage and repair in humans; the ultimate purpose is to investigate the significance of comet assay results as prognostic markers of disease. Such comparisons do not make sense if the sensitivity of the assays used is very different.

Attempts have indeed been made to establish standardized protocols, and/or identifying the experimental steps that are most significant in determining the sensitivity and reproducibility of the comet assay. Validation studies and ring trials of various types have been described [4–9], involving, for example, distribution of treated cells to different laboratories followed by local comet assay analysis, or local treatment, and analysis of cells with chemical mutagens according to specific protocols. In spite of these attempts, no key factor explaining the observed rather large inter-laboratory differences has yet been identified [8]. It is concluded that there is hence still a need for further validation of the assay [5].

The separation of DNA fragments according to their size and structure—either single- or double-stranded—is in all versions of the comet assay based on electrophoresis at low-voltage (across the tank approximately 25 V) and short duration (20–30 mins). Cell isolation and manipulation, including long-time storage through freezing, are of uttermost importance for the final output measured by the assay; this concerns, in particular, the background level of DNA damage in "untreated" cells. We shall not deal with such issues here, but rather focus on technical aspects of the electrophoresis, including some of its physics and chemistry.

http://dx.doi.org/10.5772/intechopen.76880

2. Comet assay electrophoresis in principle

Analysis of charged molecules by means of an electric potential (or electric field) is a technique that has been known, understood and applied in experimental biology and biochemistry for decades. Electrophoresis separates charged molecules according to their mobility. The driving force is the electric potential; the mobility depends on the charge, size and shape of the molecules, and the movement is inhibited by viscous forces (depending on the medium including its pore size). Cellular DNA (in a diploid human cell) has a total size of approximately 2 $*10^{12}$ Da, which in an intact cell is organized in 23 pairs of chromosomes. Under alkaline conditions, DNA from a lysed cell exists in stretches of single helixes, which may be of all sizes, either linear and free or more or less entangled with each other. When these negatively charged molecules are broken—either into discrete fragments or into a distribution of differently sized molecules—they are separated in size when subjected to an electric potential in a medium through which they have some freedom to move.

The charge per unit length of DNA is determined by its base composition, which is quite constant on a large scale. The movement (velocity per time) may be adjusted via the electric potential (V/cm) and the viscosity (related, primarily, to the agarose concentration), so that the separation of a distribution of differently sized DNA molecules may be optimized. This optimization may vary depending on the specific application of the assay. For most purposes, however, molecular sizes expected to be measurable in the comet assay would be in the range $2*10^8$–10^{10} Da (i.e., from a few hundred to ten thousand breaks per cell). The DNA distribution after electrophoresis is determined with fluorescent micrographic imaging. Using various algorithms, characteristic parameters have been worked out to characterize the population of differently sized molecules, to give the medium, the average and the distribution of the molecular weight or size (denoted scoring).

3. Early protocols for electrophoresis in the comet assay

Publications during the last 30 years specify experimental protocols often by referring to the early comet assay protocols. Quite often, however, the conditions are still undefined or confusing. This relates, in particular, to a lack of precise definition of the local electric potential; at the same time, there is a frequent—and surprising—specification of the current used (mostly 300 mA). We have attempted to understand the basis for the apparent misconceptions; it seems to reflect history rather than science. The electrophoresis step was described and specified somewhat differently in the three early publications [1–3]. Östling and Johanson [1] separated cellular DNA under neutral (non-alkaline) conditions, "… and electric potential of about 5 V/cm is applied for 5 minutes …". The tank size, the current or the volume of liquid are not specified, which is in fact quite OK since the magnitude of the local electric potential is defined. Singh and

co-workers, the first to describe an alkaline version of the assay, specify that electrophoresis is carried out for 20 mins at 25 V, at 300 mA, and a depth of liquid of 0.25 cm [2]. Since the tank dimensions are not known, the local electric potential is undefined. In a follow-up review paper, the electrophoresis is at 12 V and 100 mA, with the circulation of the electrophoresis solution at 100 ml/min of a total volume of 1 liter [10]; again, this implies an undefined electric potential. In a later publication [11], the same group describes electrophoresis at 12 V (0.4 V/cm), 250 mA, and a duration of 20 mins.

In guidelines from 2000 [12], R.T. Tice (who was also co-author of the 1988 protocol with N. P. Singh [2]) states that "… due to the large variability in the size of commercially available electrophoresis units, it is more accurate and useful to present the voltage in V/cm". Concerning voltage, 0.7–1.0 V/cm and a duration of 5–40 mins are specified, and circulation (whereas current, liquid volume, and depth are not mentioned in these guidelines) [12]. In the comet assay later described by Peggy Olive [3, 13], electrophoresis was run at 1 V/cm for 20 min (nothing said about volume and current). In a follow-up protocol by Peggy Olive in 2006 [14], electrophoresis is conducted at 0.6 V/cm for 25 min; "The current should be about 40 mA if using 20 V. The distance in centimeters is measured between the negative and positive electrophoresis in the electrophoresis chamber." There is a problem associated with this approach, which we will discuss later.

Among the early comet assay protocols, the most cited ones (by March 2018) are [2] (6522 citations) and [12] (2906 citations); citations for Östling & Johansson [1] are not available. Judging from these bibliographic data, and since the electrophoresis conditions in [2] are undefined with respect to electric potential but recommending a specific current (300 mA), it seems that much of the confusion in the literature stems from the uncritical reference to [2]. A more frequent use of the guidelines [12] might have avoided some of the unexpected comet assay results published during the last 20–30 years.

The current plays no direct role in electrophoretic mobility, but may indirectly affect the local distribution of the electric potential in a tank. The organisation for economic co-operation and development (OECD) In vivo comet assay Test Guidelines protocol 489 [15] from 2014, correctly states that the "… level of DNA migration is linearly associated with the duration of electrophoresis, and also with the electric potential (V/cm)." However, there is still the recommendation of a "starting current of 300 mA (—), the depth should be adjusted to achieve these conditions, and the current at the start and the end of the electrophoresis should be recorded". It is worth noticing, however, that attempts to control the current often lead to very shallow liquid levels. In [16] the level of the buffer is described as "… about 2–3 mm above the agarose on the slide"; in [2] the depth was 0.25 cm. It is often underlined in the protocols that the electrophoresis tank must be in the level, so that the depth above the gels is equal over the platform surface. However, keeping a large tank level in each run, within an error of, for example, 1 mm is hardly achievable. The implication is that the resistance of the liquid on the platform will vary relatively much, leading to potentially large differences in local electric potential. Specifying a much higher depth, for example, 6–10 mm solves this problem, although a larger current would put demands on the power supply. We will get back to this problem in the recommendations at the end of this text.

4. Results

4.1. Electrophoresis conditions and DNA damage levels

We have previously studied experimentally [17] the conditions which determine the level of migration measured in the comet assay. Cell cultures of two types were treated with genotoxicants and then assayed for DNA damage using different physical conditions during electrophoresis. Linear regression of the experimental data in [17] shows highly linear relationships for V/cm and time (**Table 1**) and an inverse relationship for agarose. Fitting straight lines to the figures in [17] results in the regression data (for human blood mononuclear cells) in **Table 1**.

There was no effect of altering the electrophoresis current (between 210 and 400 mA), except for a slight downward trend which is explained as an indirect effect on the electric potential [17].

4.2. Position- and time-dependent variations in local electric potential

Since the local electric potential determines the mobility of DNA fragments in the comet assay, it is essential that the electric potential is defined at all positions of the electrophoresis platform. This may be particularly important if the comet gels are small in the surface. We have described a high-throughput comet assay with 96 minigels, each of 4 μl, placed in an array of Gelbond plastic membranes as substrate [18, 19]. The minigels give results which are highly similar to results obtained with standard glass slides. We also evaluated this revised comet assay with respect to variation in DNA damage levels measured within one electrphoresis. Based on preliminary studies [18], DNA damage measured in parallel samples depended significantly on the circulation of the liquid during electrophoresis. We now report on a series of measurements of local electric potentials, with the purpose of identifying possible time-dependent differences in electrophoresis between neighboring samples. A multi-electrode gauge was made (**Figure 1**) consisting of 20 evenly spaced (5 mm) platinum electrodes, each covered with a thin plastic tube except for 1 mm protruding free end.

This multi-electrode gauge was placed in an electrophoresis tank with the free electrode ends immersed in a thin layer of agarose of the same concentration (0.675%) as used to embed cells in the comet assay (**Figure 2**) [20]. The electrode ends were a fraction of a millimeter above the

Factor and unit (and range)	*Linear regression parameters*	*Goodness-of-fit (R^2)*	*Comment*
Voltage (V/cm) (0.16–1.48)	34.1 (*V/cm)	0.964	Linear, through zero
Time (min) (5–40)	1.42 (*min)	0.950	Linear, through zero
Agarose (%) (0.4–0.95)	−30.29 (*% + 52.7)	0.955	Inverse

Table 1. Linear regression of data from [17], with goodness-of-fit. The regression curves for electric potential (V/cm) and time (min) were both forced through zero.

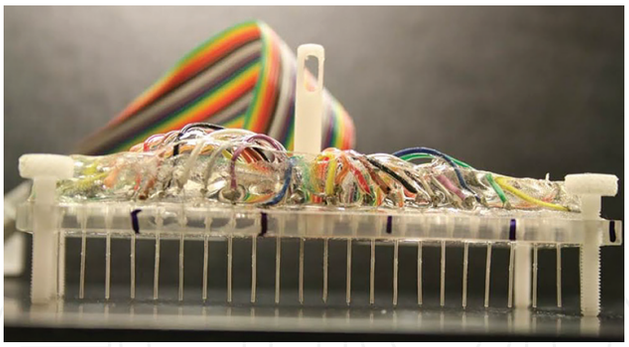

Figure 1. Multi-electrode gauge connected to a multiplexing digital voltmeter (Agilent 34972A with Multiplexer 34901A) allowing the potential at up to 20 electrodes to be sequentially scanned at intervals.

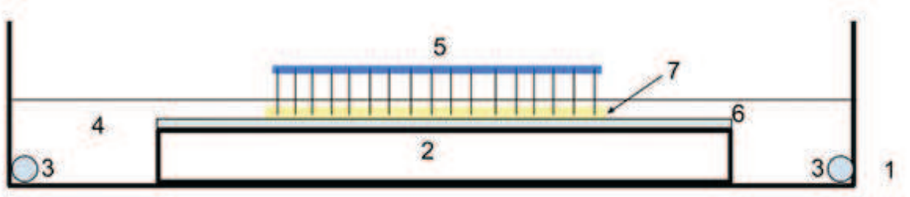

Figure 2. A schematic diagram illustrating the electrophoresis tank (1) with the platform (2) and the electrodes (3). During electrophoresis the tank was filled with liquid (4) and an electrode gauge (5) was connected to an electrode plate (6) and placed across the center of the platform with its electrodes covered in agarose (7) when performing the measurements of the electric potentials.

GelBond films. The dimensions of the tank are listed in **Table 2**. The electric potential of each minielectrode could be scanned and recorded automatically during 1 s, for every 10 s, using a multiplexer and a digital voltmeter with input resistance in the mega-ohm range. This also allowed continuous measurements of the temperature in the solution.

Systematic studies of the role of circulation were carried out, using external circulation at 21, 58, 109, 201, and 285 ml/min. With a total volume of 1640 ml, this compares to 1, 4, 7, 12, and 17% exchange of volume per min, respectively. Apparently, there were significant differences in electrode potentials at neighboring positions and also time-dependent changes (**Figures 3** and **4**, upper panels). With circulation, however, these variations were much reduced (**Figures 3** and **4**, lower panels; figures show graphs for 109 ml/min circulation). In **Figure 4**, the data from **Figure 3** have been converted into local electric potentials.

Description	*Length (mm)*	*Width (mm)*	*Height (mm)*
Tank	290	262	70
Platform	180	262	26
Side wells × 2	110	262	26
Electrode plate	180	262	9

Table 2. Physical dimensions of the electrophoresis tank and the electrode plate.

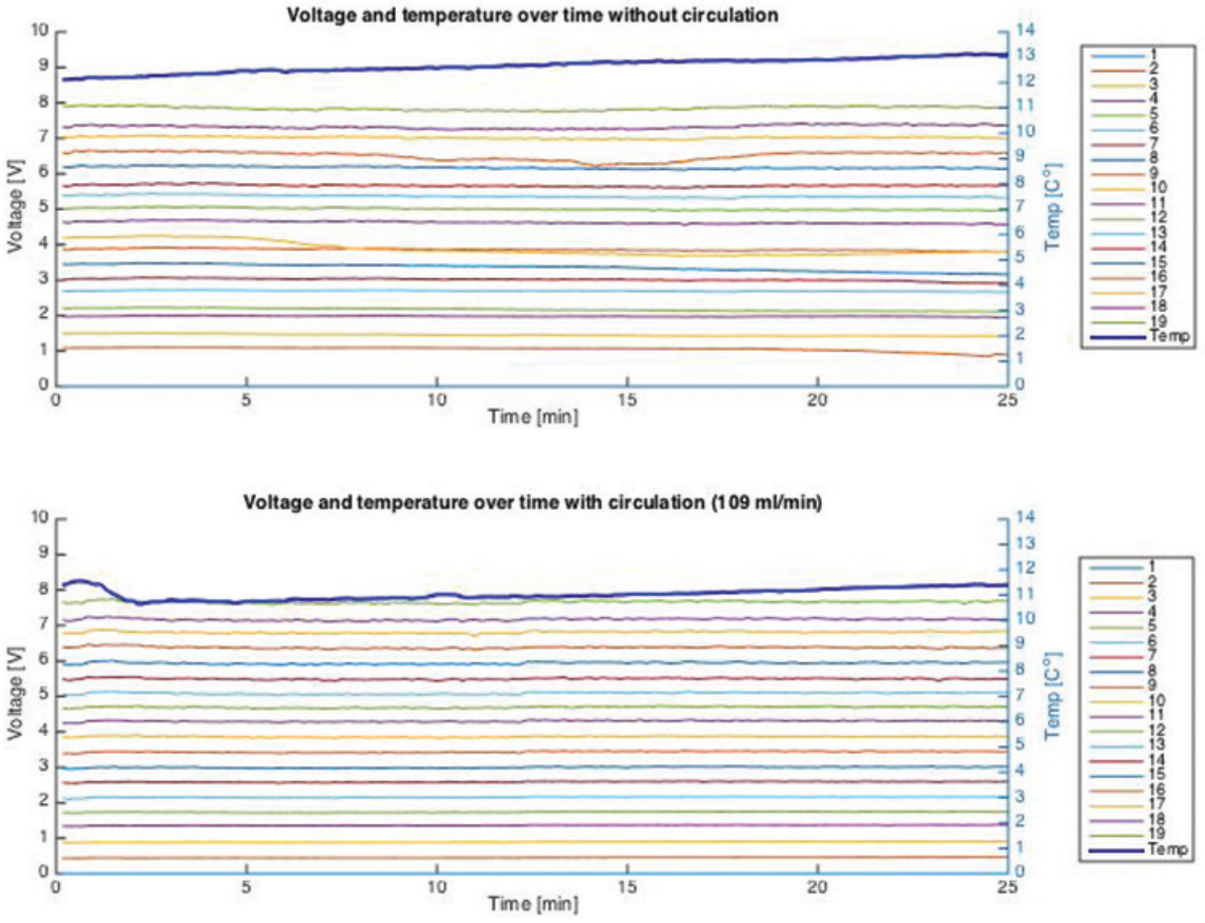

Figure 3. Measured voltage across the platform during electrophoresis. The relative voltage at each electrode and temperature over time during an electrophoresis time of 25 mins from one representative experiment with (109 ml/min) and without circulation. The number of each electrode and the temperature are indicated to the right of the figure. The blue thick lines in both plots represent the temperature. The figures are from one representative experiment [20].

The calculated total migration of DNA in each electrophoresis is derived from the time-integrated electric potential (dimension V/cm*min). **Figure 5** presents these data for electrophoresis during 25 min, with and without circulation. Corrections due to some differences in the spacing between the electrodes were included in the calculations.

These experiments were repeated several times; data averaged for all electrodes are presented in **Figure 6**. In total, there was a consistent and clearly reduced inter-experimental variation, when circulation was used (109 ml/min).

4.3. Position-dependent variations in DNA damage measured in samples exposed to irradiation

The electric potential measurements were paralleled by analysis of DNA damage in cell samples from a batch of human blood mononuclear cells exposed to a fixed dose of ionizing radiation (X-rays) on ice. This dose (8 Gy) was established from a dose-response curve (**Figure 7**). Materials

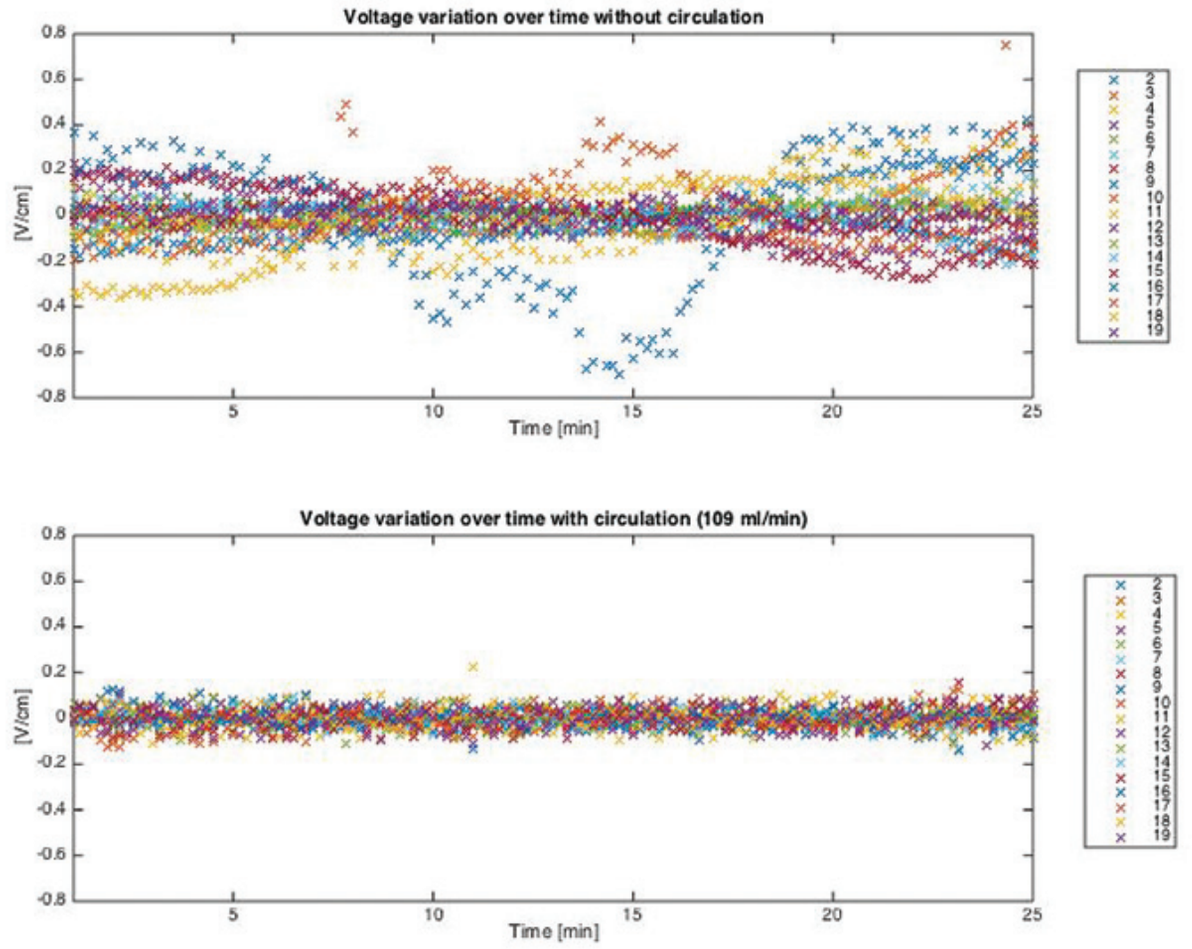

Figure 4. Stabilization of the voltage at each electrode position with circulation. Corrected voltage variations per cm (calculated from **Figure 3**) during the electrophoresis time of 25 mins for each of the electrodes, with 109 ml/min or without circulation (Figure from [20]). One representative experiment. The number of each electrode is indicated to the right of the figure. In this specific experiment, electrode #16 showed particularly large variations without circulation, whereas other electrodes were affected in other experiments.

and methods used in the experiments were the same as in [18], based on our system of 96 minigels on GelBond films. For scoring of comets, a fully automated system (Imstar Pathfinder™, Paris, France) was used, for two reasons; (1) unsupervised scoring avoids errors introduced by operator interactions; (2) there were very large numbers of samples to be scored (384 samples per electrophoresis). To ensure that automated scoring gives comet results comparable to the standard semi-automated scoring (Comet IV, Perceptive Instruments Ltd., Bury St Edmunds, UK) we used both systems to generate the data in **Figure 7**; they gave highly similar rates of median DNA damage (denoted as Tail%DNA, i.e. the fluorescence of the comet tail divided by the total fluorescence of the comet), versus the dose of radiation. At 15 Gy the Tail%DNA is saturated and the dose response is no longer linear; this data was therefore omitted in the linear regression analysis. The dose 8 Gy which was used in cell exposures is in the linear part of the curve and is well below saturation.

A large number of comet electrophoresis experiments was carried out with irradiated (8 Gy) mononuclear cells. We studied differences in means between experiments, with and without

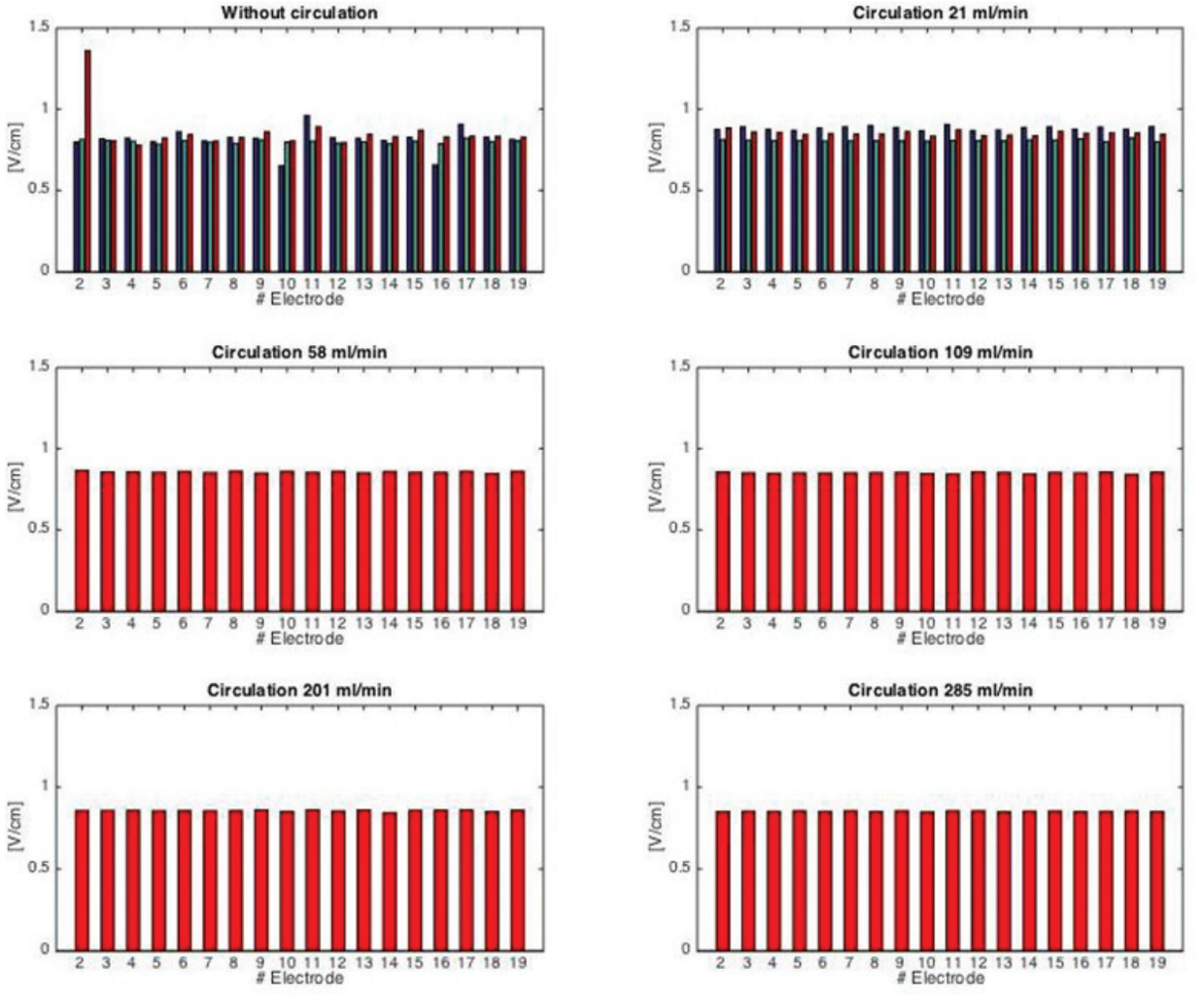

Figure 5. Voltage gradient at each electrode position during electrophoresis with circulation at different flow rates. The corrected electric potentials were time-integrated during an electrophoresis time of 25 mins, and are presented (V/cm) during one (average) mins. Data are from three experiments without circulation or with circulation at 21 ml/min (upper panel); the blue, green, and red bars correspond to each of the three experiments. For higher flow rates (58, 109, 201, and 285 ml/min), the data (lower panels) represent one representative experiment. Figure from [20].

circulation, and differences between samples belonging to different rows or columns in the array (8 columns × 12 rows) on each of four films in one electrophoresis.

The experiments included electrophoresis of either one film placed in the center of the platform, or 4 films totaling 384 samples covering the whole platform [18] (**Figure 8**).

The median (Tail%DNA) of all scored comets in each sample was used as the basis for further statistical analyses. In some calculations, the values were weighted, based on the number of comets scored in each sample. Normality of distribution of DNA damage in each cell sample was tested using a Lilliefors test, in MATLAB R2014b (significance level 5%). Differences between groups (rates of circulation, sample positions in rows and columns) were tested with one-way ANOVA. A structure of statistics obtained from the one-way ANOVA was used to perform the multiple comparison tests, which determined whether any group mean was significantly different; two group means were significantly different if their intervals were disjoint. A Wilcoxon

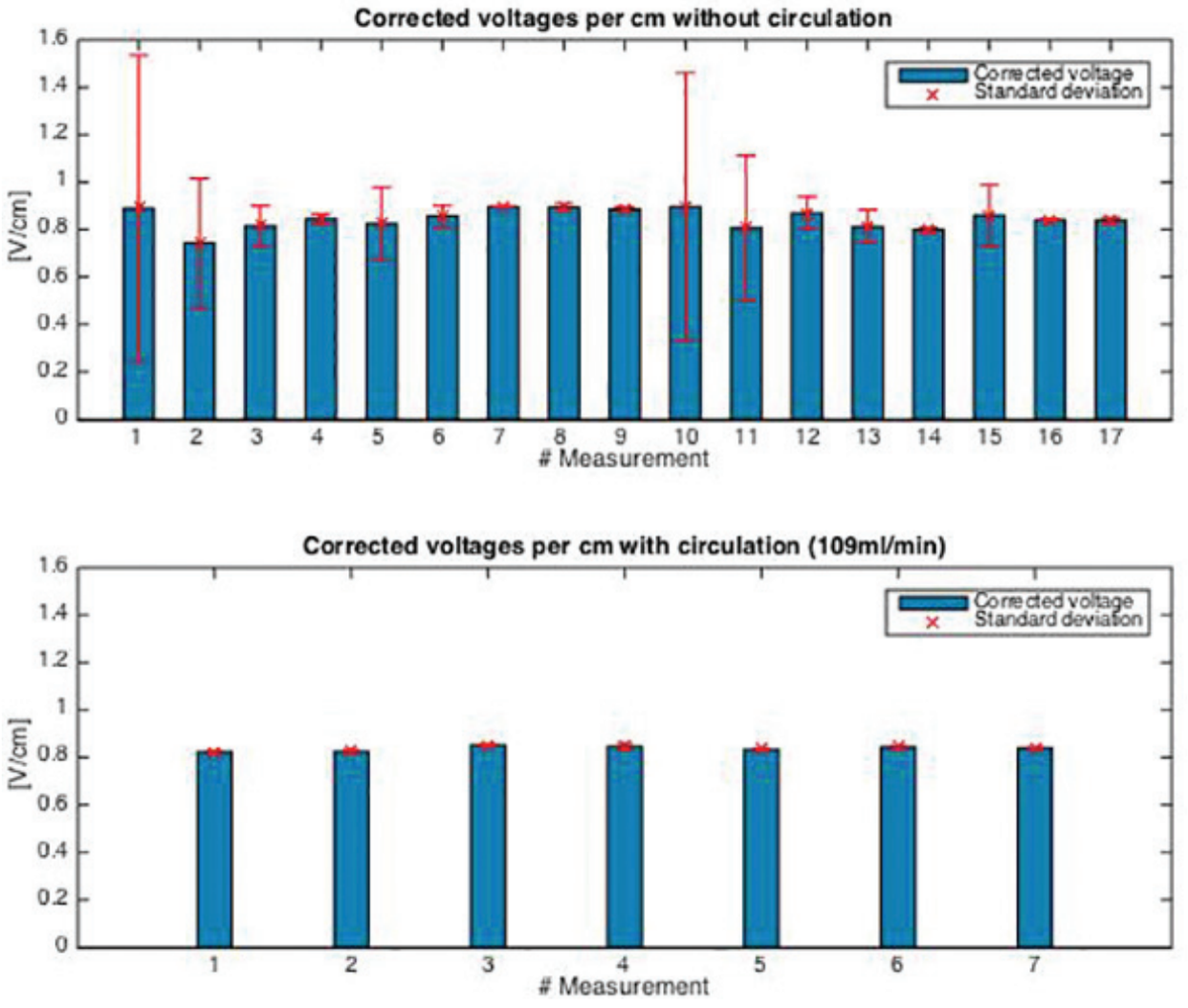

Figure 6. Average electric potentials measured in several experiments. Time-integrated corrected voltage per cm averaged for all electrodes during an electrophoresis time of 25 mins for each measurement with (109 ml/min, lower panel) and without (upper panel) circulation. The standard deviations are shown as red error bars and illustrate the degree of variation in voltage per cm for each measurement. Figure from [20].

rank sum test—not requiring the normal distribution of samples—was used when the Tail% DNA values were shown not to be normally distributed by the Lilliefors test.

We observed small differences in Tail%DNA levels in experiments using different rates of circulation. This is illustrated in **Table 3** and in **Figure 9**, both based on weighted data (to avoid that low numbers of scored comets should be given too much weight).

5. Discussion and conclusions

The ultimate aim of this study [20] was to establish electrophoresis conditions that are optimal for the comet assay, hopefully contributing to reducing the unexplainable variations, which are often found both within and between experiments. A further aim is to establish correction

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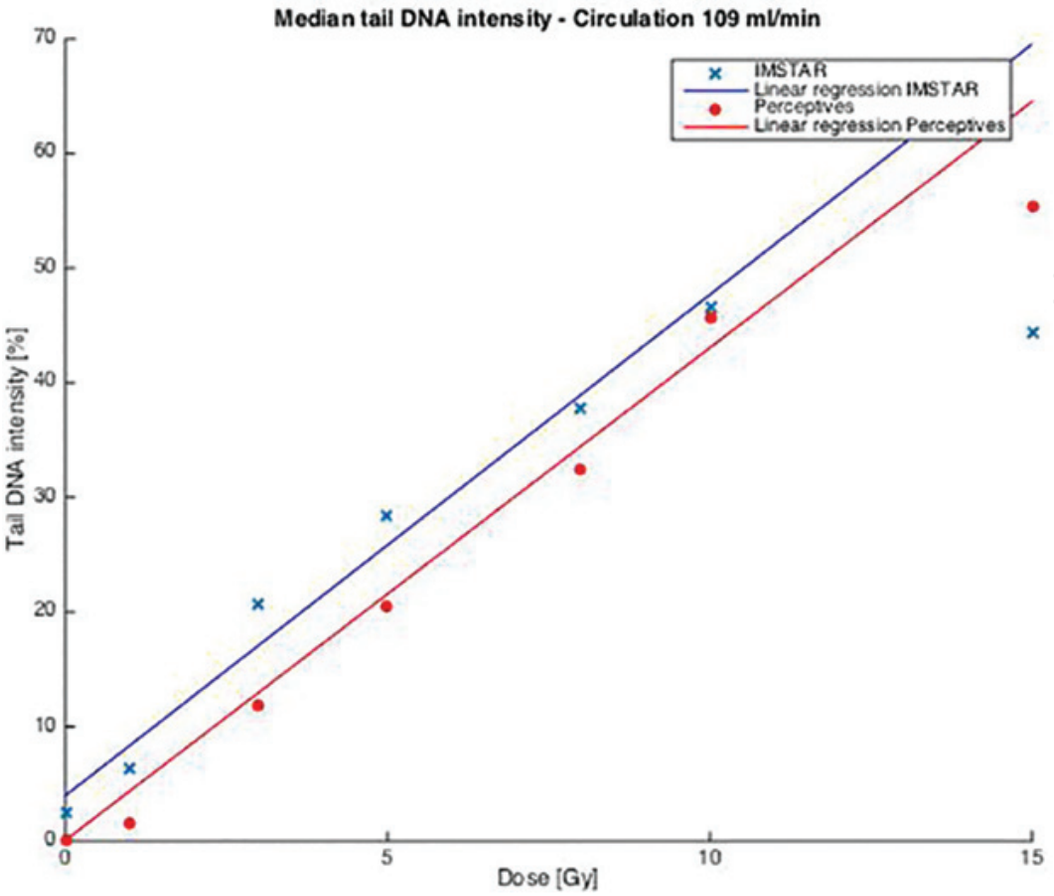

Figure 7. Dose-response curves showing DNA damage versus radiation dose. One experiment; scoring of DNA damage in 96 samples with the semi-automated (Perceptives) and the automated scoring (IMSTAR Pathfinder) systems. Irradiation with X-rays (0–15 Gy) and circulation (109 ml/min) during electrophoresis. The Tail%DNA relative to the head is given as the median value of at least 50 comets in each sample. Linear regression lines are shown, with linear relations $y = 3.99 + 4.37x$ and $y = 0.1 + 4.30x$, and coefficients of variation (CV) $R^2 = 0.72$ and $R^2 = 0.96$, for IMSTAR and Perceptives, respectively. The experimental points at dose 15 Gy were omitted from the linear regression.

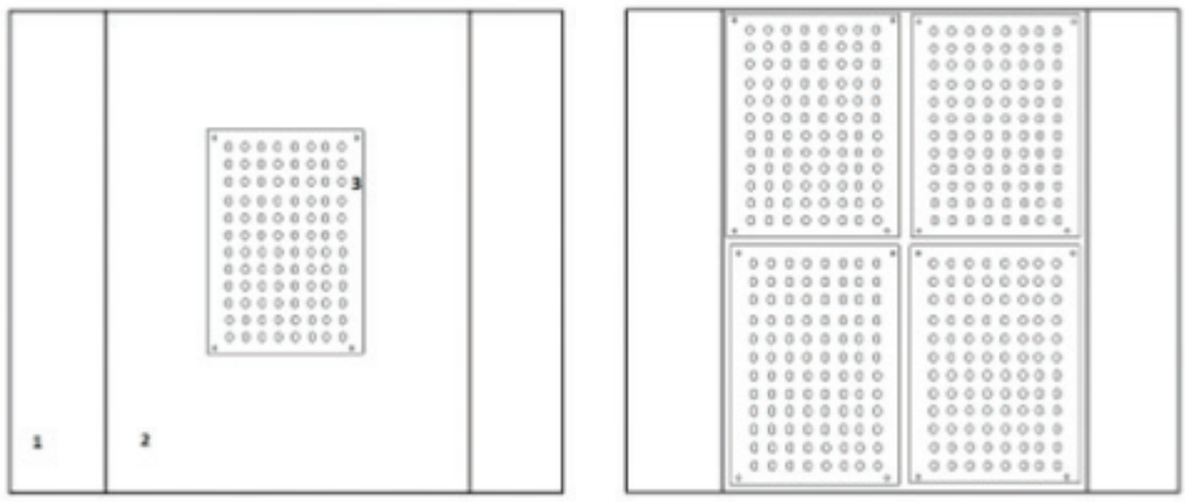

Figure 8. Illustration of positioning of Gelbond films with one film (left; 96 minigels), or four films (right; 384 minigels) on a platform in an electrophoresis tank. Figure from [20].

Flow rate (ml/min)	Mean tail DNA intensity (%)	Mean STD (%)	Mean CV (%)
0	39.53	4.15	10.50
21	42.75	4.31	10.07
58	34.83	3.89	11.18
109	34.75	3.28	9.44
153	36.27	2.28	8.14

Table 3. Mean Tail%DNA in electrophoresed samples of cells irradiated with X-rays (8 Gy). Standard deviations of parallel samples and coefficients of variation are also listed. Data are from three experiments with or without circulation at different flow rates during electrophoresis. Data are weighted on the basis of the number of comets scored in each sample.

factors, which may be used to translate data from laboratories using different electrophoresis conditions.

A systematic investigation of comet assay electrophoresis had not previously been carried out. Since the electric potential is known to be the major physical force causing charged molecules to move during electrophoresis, we recorded the electric potential at multiple sites on the platform of a standard horizontal tank used for comet assay electrophoresis. In a previous study, we had observed that variations in DNA damage levels among parallel samples were considerably reduced when the liquid was circulated during electrophoresis [18].

We made detailed measurements of electric potentials using a gauge containing 20 platinum electrodes coupled to a voltage recording device with input resistance in the mega-ohm range. Using this setup we could confirm that the measured local electric potential was highly affected by circulating the electrophoresis liquid; that is, time- and position- dependent variations seemed to be reduced by circulation. For a standard electrophoresis time of 25 mins, the time-integrated variation (Coefficient of Variation, CV) of the electric potential at electrode positions was more than 8% without circulation; this was reduced to less than 1% with circulation at more than 58 ml/min, i.e. 5% volume change per min (**Table 4**). We initially hypothesized this reduction to

Flow rate	Mean corrected electrode voltage	Mean CV
ml/min	V/cm	%
0	0.82	8.28
21	0.85	1.17
58	0.86	0.53
109	0.83	0.65
201	0.84	0.70
285	0.83	0.50

Table 4. Electric potentials and their variation at electrode positions as a function of circulation flow rate. The corrected electrode voltage per cm averaged over 25 mins is shown, with CV averaged for all electrodes. Data from three experiments with and without circulation at different flow rates.

be due to a better stabilization of local temperature that could alter the conductivity; a further mechanism could be a build-up of local concentration gradients of electrophoresis liquid, which are likely to be reduced by an increased flow. A positive feedback loop could then possibly take place in the absence of circulation.

The logical extension of these observations was that the variations should be paralleled by variations in the level of DNA damage. We therefore examined populations of cells exposed to a genotoxic treatment (X-rays) under different electrophoresis conditions. The mean Tail% DNA of all samples in our minigel 4 × 96 array was remarkably constant, as concluded from three independent experiments (**Figure 9**). There was, however, a slight—but statistically not significant—tendency to a lower CV at the two highest rates of circulation (**Table 3**). This was unexpected in view of the markedly reduced variations in local electric potential with circulation. This is also unlike our preliminary findings [18].

A statistical analysis of Tail%DNA of samples in the row and column positions in the 4 × 96 array also did not reveal systematic differences that could be attributed to electric potential variations. However, for each film, there were clear indications of lower levels of median DNA damage in samples, which were applied late in each pipetting (data not shown). We interpret this as due to some rapid repair of radiation damage, during the short period when cell samples are heated to 37°C for mixing with agarose.

It is possible that variations in electric potentials are related to properties of the measuring gauge. The electric potential is dependent on the concentration gradient in a solution. Small differences in the concentration of reactants and products involved in the redox reaction

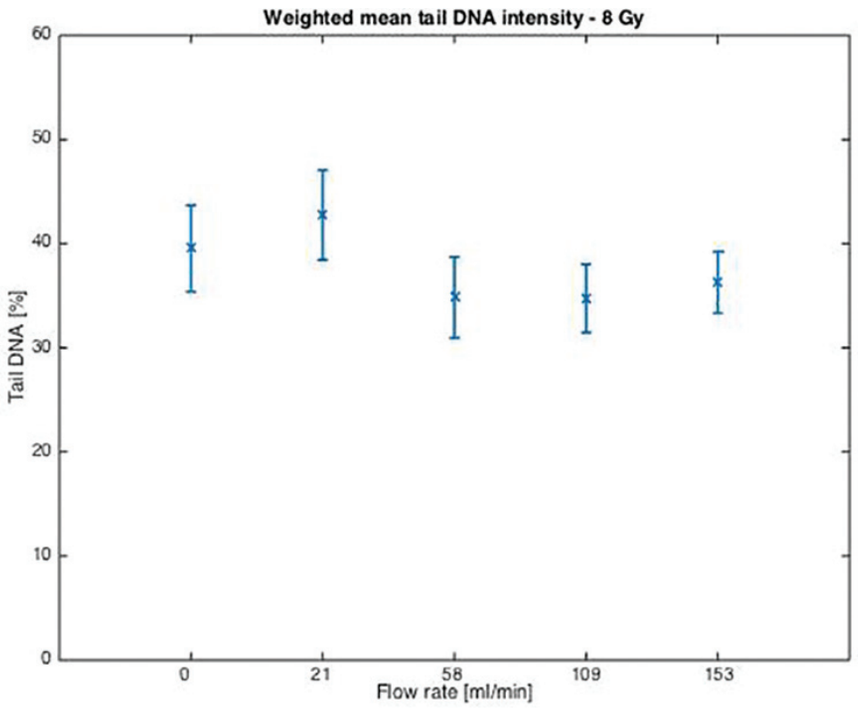

Figure 9. Mean Tail%DNA in electrophoresed samples of cells irradiated with X-rays (8 Gy). DNA damage levels of data from three experiments are shown (+/− STD; error bars) as a function of circulation flow rate. Figure from [20].

occurring at each electrode surface during measurements may cause variation in the recorded electric potential. Circulating the electrophoresis solution may contribute to reducing the build-up of concentration differences, resulting in turn in less variation in the measured electric potential. A reference electrode should be used in order to determine the potential differences across the platform with higher precision. The reference electrode must have a well-defined and reproducible potential, that is, both reactants and products must be present with the kinetics of the reactions sufficiently fast for the species to be present at their equilibrium concentrations. Taken together, the measurements using the multi-electrode gauge were probably more representative for the local electric potential in the presence of circulation. Without circulation, however, these measurements are less reliable.

In conclusion, it seems that circulation of the liquid during electrophoresis has minor effects on the recorded level DNA damage. Even so, there are several arguments for using circulation in comet assay electrophoresis. Uncontrolled changes in temperature, not analyzed in this project but studied by Sirota and co-workers [21], may lead to heterogeneities in DNA damage expression. External peristaltic pumps combined with a spiral of stainless steel tubing placed in ice/water represent an efficient method to stabilize the temperature during electrophoresis, which also becomes more important with higher currents (more heat dissipation). Furthermore, standardization of data relies on the accurate determination of the electric potential. This should be determined using a suitable gauge; from our experiments, it appears that circulation stabilizes the measurement of the potential. It is stated in [16] that "A

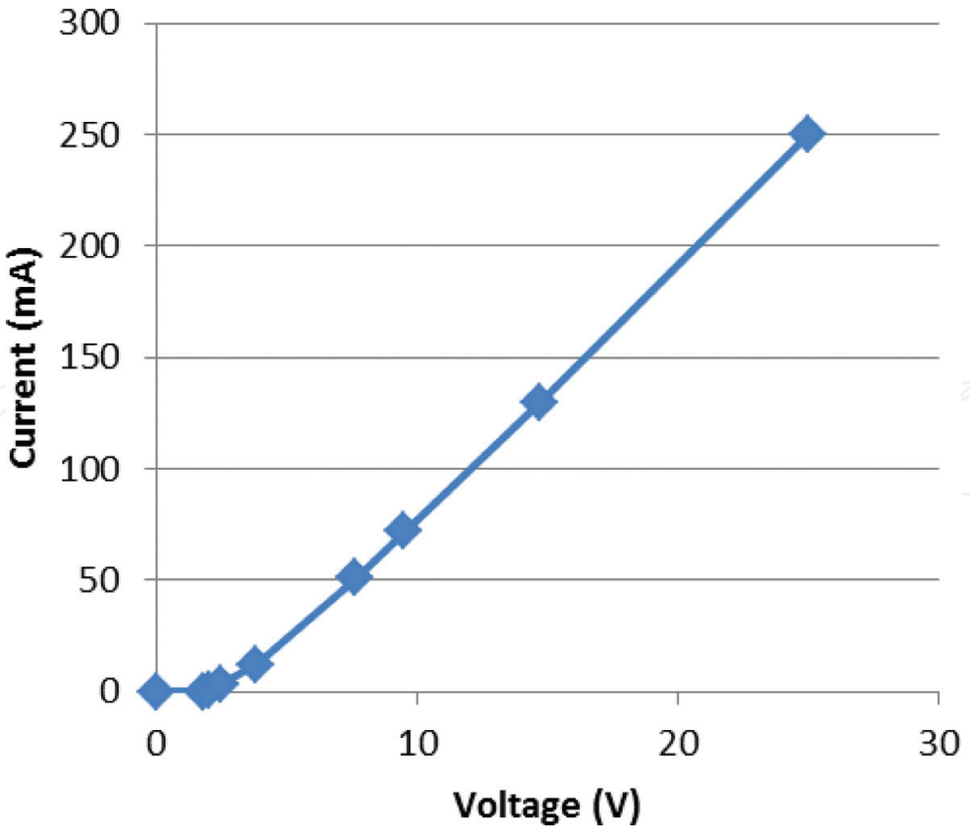

Figure 10. Current in an electrophoresis tank measured as a function of the applied voltage.

typical voltage for an electrophoresis chamber is determined by measuring the perpendicular distance in cm between the positive and negative electrodes in the electrophoresis chamber, and multiplying this distance by 0.6." This simplified method to determine the electric potential (V/cm) may introduce errors and should not be used. Firstly, there is a voltage drop at each electrode (the electrode potential at standard state for platinum equals 1.18 V [22]). This electrode potential is evident from the measurement of the current at increasing electrode voltage (from the power supply) in a tank; see **Figure 10**. There is no current at voltages lower than 2.5 V; the curve extrapolates at zero current to 2.7 V, which is close to the electrode potentials at both electrodes (2.38 V). Furthermore, the side wells have higher conductivity (per length) than the platform. In a small tank, the electrode potential may introduce serious errors.

Care should also be taken concerning the resistance of the cathode and anode electrodes. There is a voltage drop along these electrodes, even with platinum (specific resistance $1.05 * 10^{-7}$ohm *m (at 20°C)). Depending on the electrode cross-section and the current, this could result in as much as 5–10% lower voltage at the far end of the tank (i.e. opposite to where the connecting leads are). This error can be reduced by using thicker (very expensive) platinum, but a cheaper solution is to connect both ends of each electrode to the power supply (4 leads). An alternative approach is to measure and/or calculate the electric potential (V/cm) along both sides of the platform and introduce correction factors to account for the difference in each position.

In summary, based on our experiments and the evaluations above, we suggest a number of simple recommendations:

6. Recommendations for comet assay electrophoresis

- When introducing a new tank: measure and record the voltage and the current of the electrophoresis tank, when containing different volumes of liquid.
- Circulate the electrophoresis liquid during electrophoresis using an external pump (e.g., there are cheap peristaltic pumps made for aquariums). The advantages are (1) stable conditions allowing more precise measurement of the electric potential; (2) more stable temperature during electrophoresis; and (3) (probably) reduced variations in the local electric potential.
- Use sufficient volume of electrophoresis liquid to cover the agarose samples with at least 5 mm.
- Ensure that a power supply is used which can supply the output current at the constant voltage and with sufficient volume of liquid (two car batteries are a very cheap and useful alternative).
- Use electrodes of sufficient size (cross section), to avoid a voltage drop due to high ohmic resistance. The overall resistance is reduced if both ends of each electrode are connected to the output cable from the power supply.

- Measure the local potential on the platform, preferably using a gauge with platinum electrodes at defined positions. Measure in the presence of circulation.
- Take great care in preparing the agarose used for embedding gel samples (avoid evaporation during heating).
- Calculate the Electric Potential * Time (EPT) value (dimension: V/cm*min) for your setup. Use the EPT value in your publications and when comparing your results with those from other experiments and laboratories.

Acknowledgements

The authors are grateful to the COST Action CA15132, "hCOMET," for support. This work was financed by the Research Council of Norway through its Centers of Excellence funding scheme, project number 223268/F50 CERAD Centre for Environmental Radioactivity (CoE CERAD).

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