Standardizing electrophoresis conditions: How to eliminate a major source of error in the comet assay

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There is still too much variation in comet assay results

Our ambition is to contribute to reducing such variations

Approach in this project: Analysis of critical parameters, and revision of protocols if needed.
The comet assay: biology, chemistry and physics

• The degree of DNA damage formed in a cell depends on biology and chemistry.
• The conversion of this DNA damage into comet tails is determined by the rate of migration of DNA during electrophoresis.
• Electrophoresis is first of all a physical process.
Analysing the physics of comet tail formation through electrophoresis

In principle,

- Total DNA-migration = Tail%DNA
  = f(dragging forces; retarding forces)

Here,

- Dragging forces = Constant x U[V/cm] x time
- Retarding forces = Constant x Viscosity
  (temperature; matrix (agarose concentration))
The conditions known to affect DNA migrations

1. Electric potential (Voltage gradient, V/cm)
2. Time
3. The matrix (agarose concentration affects the pore size)
4. Temperature
5. Electrophoresis solution (if the pH is low)
Some points to note

• What is the significance of the electric current?
  – Answer: None (but it depends on the volume of the liquid).

• What is the inter-relationship between the applied voltage and the current?
  – Answer: It’s a constant (= related to the electric conductance of the liquid in the tank)

• How can you alter the current?
  – By adding more liquid
  – By changing the conductance of the liquid
  – By using another tank, with different dimensions
The current is determined by the volume of the electrophoresis liquid (the voltage is kept constant).
The current is determined by the voltage (when the volume is kept constant)

- The voltage has to be above a certain level (i.e., the potential of the electrode, which for platinum is close to 2 volts). Above 2 V, the current increases almost linearly.
96 minigel format (4 microliter per gel)


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The **96 minigel format** confronted us with a potential problem in comet electrophoresis.

We reported substantial variations between technical replicates in neighbouring gels processed in the same electrophoresis (Gutzkow et al., *Mutagenesis* 2013).
Our understanding of the principles of electrophoresis should allow us to explain the cause of these variations

- Hypothesis: There are larger variations in local voltage \textit{without} than \textit{with} circulation of the electrophoresis solution
Local voltage measured using a multi-electrode

Electrophoresis tank with platform, with unit containing 19 thin platinum electrodes. They are covered with plastic so that **only the lower 1 mm part is exposed to the solution.** Their distance to the platform surface may be adjusted.
In reality the unit looks like this…
Tank with multi-electrode
Voltages measured in all electrodes, every 10 seconds

- A device (Agilent 34972A, with the 34901A Multiplexer) scans all electrodes during 1 second, at a preset interval (every 10 seconds).
- Accurate determinations of time-dependent local voltages are thus made.
- One channel is used to record the temperature in the solution.
Electrodes placed in a thin agarose gel
Circulation rate 3
Measurements during 25 minutes.
Left axis: Voltage, Right axis: Temp (deg)

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No Circulation
Measurements for 25 minutes.
Left axis: Voltage, Right axis: Temp (deg)
Are these curves so different?
Yes indeed, when you transform the measurements into local voltage (V/cm) and its variation with time!

**No circulation, uncorrected measurements**

**With circulation rate 3, uncorrected measurements**

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Some variation in the spacing of the electrodes
Results are corrected to account for varying spacing of the electrodes.

No circulation corrected measurements

With circulation rate 3 corrected measurements
Calculations

• Voltage recordings used to calculate time-integrated «dragging force»
  – (Voltage per cm x minutes)

• At each electrode location.
Expected comet tail
(=time-integrated electric potential)

No circulation
Mean: 5.01
CV (%) = 12.21

With circulation rate 3
Mean: 4.96
CV (%) = 0.25
Is there an optimal rate of circulation?

Mean time-integrated electric potential

CV (%) time-integrated electric potential

Circulation: ml per minute

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Comet assay can be easy and cheap

- Refrigerator
- External pump
- Circulation of electrophoresis solution through heat exchanger in ice/water
A new concept
A new concept

Electro4ease
Large gels (e.g. on glass slides) are most likely subjected to the same error as small ones!

![Diagram of a large gel on a glass slide]

Do you score comets over all the surface of a large gel (often 10,000 comets), in a systematic way?
- If the answer is yes, then your only problem is probably that your comet tails within one sample vary more than necessary
- If the answer is no, then you introduce significant errors
- In both cases, you may not be aware of the problem!

Do you use small gels (2 – 10 microliter of agarose, 100 – 400 comets)?
- You probably score comets over the whole surface, and you often score all of them
- You probably observe rather large variations between parallel samples (technical replicates).

A comet assay gel may cover a substantial portion of a glass slide (2.5 x 7.5 cm). The gel surface hence spans several of the electrode positions for which we have measured large voltage variations.

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In addition to possible experimental variations in your cell work…

- Are you prepared for errors introduced in your results, by the electrophoresis alone, of probably

  $\pm 25\%$
Collaborators

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