Principle

The comet assay, also known as single-cell gel electrophoresis (SCGE), has become an attractive assay in the assessment of DNA damage. Its simplicity, sensitivity, speed and economy make it a prime choice in genotoxicity testing, ecogenotoxicity, human epidemiology studies as well as mechanistic studies of DNA damage and repair. Although this assay is described as a method for DNA damage quantification, the SCGE has evolved to now provide information about all kinds of DNA lesions to be detected. The comet assay was first described by Ostling and Johanson as a method of DNA strand break analysis. Cells with increased DNA damage display increased migration of DNA from the nucleus toward the anode. While this method was groundbreaking, its protocol was modified through the years to improve sensitivity and reproducibility. Singh et. Al. explain that the neutral conditions for lysis and electrophoresis only permit the detection of double-stranded DNA breaks, and do not allow for the detection of single-stranded ones. They also emphasize that most of the DNA damaging agents induce 5- to 2000-fold more single-stranded than double-stranded breaks. For these reasons, they have developed a new comet assay protocol, which allow alkaline conditions to reveal single-strand breaks and to degrade cellular RNA that interfere in the quantification of the stained samples (3). Although single-strand breaks occur more frequently, Olive et. Al. highlight the fact that double-strand breaks are considered to be of greater biological consequence since they can lead to chromosome aberration and loss of genetic material.

Comet Assay Protocol

Day 1 (Cell plating and buffer preparation)

1. Plate 200 000 cells per well – 1ml total volume (2 wells per treatment)
2. Allow cells to attach overnight
3. Prepare the following buffers:

Lysis buffer A (fresh, 1-3 days maximum, 44.5 mL per film with 6 "wells")

146.1 g NaCl
41.6 g Tetra-Sodium EDTA
1.2 g Tris base
10.0 g N-Lauryl Sarcosine
dH_2O
1 L
- adjust to pH 10

Lysis buffer B (50 mL per film with 6 "wells")

146.0 g NaCl
41.6 g Tetra-Sodium EDTA
1.2 g Tris base
dH_2O
1 L
- adjust to pH 10

Neutralization buffer (stored at $+4^0$C) (4 mL per film with 6 "wells")

77 g Ammonium acetate
dH_2O
100 mL

Agarose (in an Erlenmeyer flask)

0.75 g LMP (low melting point, DNA grade) agarose

100 mL PBS
- heat until boiling and translucent
- cover and place at 4 °C

<u>Day 2</u> (treat cells and gel preparation)

1. Adding drugs:
   - Dilute drugs (make 45 min-1 hr time interval between sets of treatments). Concentrations (6): 100, 20, 25, 12.5, 6.25 uM, DMSO control.
   - Aspirate media, add 1mL drug dilution per well
   - Incubate mixture for a specific amount of time (2 or 24 hrs)
2. While the drug is incubating, prepare the gel:
   - Melt the agarose in an Erlenmeyer flask on a hot platform with stir. Leave it on the minimum heating with stir for the entire period of use.
   - Cut the Gelbond film (**<u>in a dark room!</u>**) to fit the electrophoresis apparatus (1 film per 6-well plate, make specific cut of the top right corner for each set of treatment). Put with paper on foil sheet with **hydrophilic** side facing up.
   - Use some agarose to help attach the chambers to the Gelbond film (**<u>in a dark room!</u>**). Pour some agarose into a big dish, dip the chamber (wider base down) into agarose, press the chamber onto the **hydrophilic** side of the film. Repeat 3 times/film -> making 6 "wells". Don't touch agarose with fingers while holding the chamber. Chambers don't need to be washed afterwards.
   - Allow the agarose to cool slightly (5-10 min). Make sure there is a separation between 6 "wells" on the film.
3. Transferring cells into the gel
   - Terminate treatment by removing media and rinsing with PBS (1mL) – start with control and go from lowest dose till the highest.
   - Collect cells through trypsinization (0.5mL of trypsin, wait, add 1mL media (10% FBS)). Transfer cells to labelled 15 mL tubes (collect duplicates into the same 15 mL tube).
   - Pellet cells by centrifugation (1.6 rpm for 5 minutes, 4^{o}C)
   - Aspirate media. Resuspend pellets in 1 mL PBS. Centrifuge (1.6 rpm for 5min, 4^{o}C)
   - Resuspend cells in 1 mL PBS (or in 500ul, if not many cells), put tubes on ice.

   - Turn off the lights in the room. Prepare 3 pipettes (30, 150 and 270 ul), tips and eppendorfs (non-sterile).
   - Vortex gently or pipette up and down cell suspension, transfer 30µl into a labelled 1.5 mL eppendorf tube.
   - Add 270µl of agarose, maximum temperature 37^{o}C (use a thermometer or touch the hand).
   - Take 150 ul pipette, pipette up and down, put 150µl in one of the 6 "wells" on the film (distribute evenly without touching the borders of the "well" with a tip, get rid of bubbles).
   - Repeat for all samples

100 uM	50 uM
25 uM	12.5 uM
6.25 uM	DMSO

   - Put the film (without a paper) with gels into the Petri dish, wrap in foil (protect from light) and leave for about 5 min for polymerization.
4. While the gel is polymerizing, make the lysis buffer in a 50ml tube (1 tube per film). Shake with hand.
   - 44.5 mL of lysis A
   - 5 mL DMSO

- 0.5 mL Triton X-100

20. Pour lysis buffer into large Petri dish (not directly on top of film but around it) with the film (make sure it is submerged).
21. Wrap the Petri dish in foil and place it at 4 °C overnight
22. Return extra agarose to 4 °C.

Day 3 (Unwinding buffer preparation, lysis B transfer, electrophoresis)

1. Discard lysis A buffer into the sink.
2. Wash the gel in the dish with mQ dH_2O (3 times)
3. Pour 50 mL of lysis B into the dish (around the film), wrap in foil and incubate for 60 min. at 37 °C (place the foil sheet on the shelf of incubator to prevent leakage).
4. After 30 min, repeat steps #1-3 for the 2nd film, in another 30 min – the 3rd.
5. Meanwhile, prepare Unwinding/electrophoresis buffer (~320 mL/ film, keep bottle covered with aluminium foil). For 1 L:
   - 12.0 g NaOH
   - 4.2 g Tetra-Sodium EDTA
   - 1.0 g 8-hydroxyquinoline

   Add 700 mL dH_2O
   Add 20 mL DMSO
   - mix using the magnetic stir bar

   Add 280 mL dH_2O
   - check pH (adjust to pH 13, if necessary)
6. Discard lysis B buffer into the sink
7. Wash in distilled water (3 times)
8. Place the gel in the unwinding buffer (~50 mL)
9. Cover in foil and store at room temperature for 30 minutes
10. Collect the unwinding buffer into the organic waste bottle.
11. Set up the electrophoresis apparatus
    20V
    400mA
    20 min.
12. Pour 270 mL of the unwinding buffer into the electrophoresis apparatus
13. Place the film in the electrophoresis apparatus with the top side (that has a specific cut) facing the black (-) electrode.
14. Press run (If the voltage is 19V, run for 25 minutes instead). Cover with a box to protect from light.
15. Dilute the neutralizing buffer (10M Ammonium acetate) (for 1 film: mix 4 mL buffer and 40 mL dH_2O)
16. Place the gel in the neutralizing buffer for 30 minutes at RT.
17. Collect the unwinding buffer into the organic waste bottle. Use fresh buffer for each run.
18. Discard the neutralizing buffer into the sink, place the film in 100% ethanol for 2 hours.
19. Let the gel dry overnight (put the dishes in the box, put the films on the edge of the dish so that the surface underneath the film gets fry). Cover with foil.

Day 4 (staining)

1. **In a dark room** dilute the 10 000X SYBR gold to 1X in ddH_2O (per film: 40 mL H_2O + 4 ul of dye)
2. Pour the dilution of the dye directly on the top of the film.
3. Stain for 10 to 15 minutes in darkness.

4. Discard the dye into the sink.
5. Let the film dry ON in the room or 2-4 hrs under the fume hood in the darkness.
6. Can store at $+4^0$C in darkness (up to 2 weeks).

Day 5 (acquiring images)

1. Turn on microscope power 15 min in advance. Password – "password".

2. LAS -> exposure 1.5s (can increase to 2s); Saturation – 0; Gamma – 1.69 (background); Gain – 4.8x, Input option – greyscale.

3. Turn on shutter – fluorescence ON. Filter – HQF

3. Acquire image. Options->Preferences-> Select the folder where to save.

4. Check Autosave option.

5. Format – bitmap. Take at least 50 pics per concentration. The undamaged nucleus has to be to the left and the tail – to the right from it.

Analysis

1. Comet assay IV. Image bank->Load image->choose image.

2. Click on nucleus. Blue line – beginning of nucleus, green – middle of nucleus; pink – end of tail. For manual adjustment click Edit. Numbers in table – tail moment.

3. Analyze all images for one concentration. Then click File->Save as-> Excel

4. Erase all the results in the software. Move to next concentration.

In Prism

1. n=50 (tail moment). Concentration (X axis)

2. Analyze -> Row means (average)

3. Transform-> K different for each treatment (100)

Potential applications

Applications: in vitro, in vivo and clinical use

One of the primary uses of the comet assay is in genotoxicity testing. It is provides a set of information about safety and toxicology on newly developed pharmaceuticals and chemicals. Since some drugs are prodrugs and therefore become active only after hepatic metabolism, metabolites can be assessed for their genotoxicity potential. The comet assay is not only useful in the assessment of chemical toxicities but it can also allow the study of the protective effect that some phytochemicals have on genes when exposed to some genotoxic insults. Since dietary factors have been estimated to contribute up to 30-40% factor, human nutrition effect on cancer development is an important element to consider. In these studies, the comet assay is a simple cheap a rapid method to assess DNA damage and repair. Since human being as

constantly exposed to the balancing effect on genotoxic agents present in the direct environment and antioxidants, biomonitoring studies allow the monitoring of lifestyle and environmental carcinogenesis. Furthermore, the alkaline comet assay can be used to assess the DNA damage in individuals occupationally exposed to ionizinng radiation. All this biomonitoring can be done with very little invasive test with blood samples, tissue swab or in a more invasive manner, a liver biopsy. Then a standard alkaline comet assay can be performed and results acquired.

Comet assay variants

→*Alkaline and neutral comet assays to discriminate between single- and double-strand break*

The alkaline comet assay separates the two DNA strands around the break by alkaline denaturation to reveal the alkaline-dependent single-strand break but also DSBs, and the neutral SCGE selectively detects double-strand breaks by using a neutral lysis conditions, leaving the alkaline-dependent single-strand breaks undetected.

→*Lesion-specific enzyme to detect the category of DNA damage*

To discriminate between direct damage from the test chemical, intermediates in nucleotide- or base-excision repair, and AP site, lesion-specific enzymes may be introduced. These enzymes target DNA at sites that show a specific base alteration and only cause DNA-strand break at that site.

→*Bromodeosyuridine labeling to determine replicative integrity*

During DNA replication, DNA is naturally forming fragment, which could be detected as DNA strand breaks in comet assay. This assay does not discriminate between S-phase ‘’DNA fragments’’ and non-S-phase or strand-breaks. By introducing bromodeoxyuridine (BudR) as a label for replication, detection with BudR antibody will make comet tails caused by DNA replication distinguishable. This technique can also be used to determine carcinogenesis through replicative integrity.

→*Fluorescent* in situ *hybridization (FISH) in combination with comet assay*

It is used to monitor the repair of particular genes. FISH is a technique used to localize specific DNA sequences within the chromatin and chromosome and identify structural and numerical chromosome changes (32) In the case of comet/FISH, oligonucleotide probes are used to target one strand of a specific gene. These probe are labeled with fluorescent tags to allow the identification of this gene in the comet as whether this DNA sequence is damaged or not

→ *Detection of cross-links*

Since the cross-links keep the DNA covalently attached altogether, this would mean there would be a slower electrophoretic migration of the DNA. This modified comet assay compares the migration of irradiated DNA with a cross-linker versus irradiated non-cross-linked DNA control. It has been shown that the extend of retardation in DNA migration is proportional to the level of interstrand cross-linking. This method can also be used to measure the extent of DNA cross-links repair in patients treated with chemotherapeutics causing these types of damage.

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Appendix - Reagents

Name	Full name	Volume	Producer	Catalogue #	For
NaCl	Sodium chloride	3 kg	Fisher	S271-3	A, B
Tetra-Sodium EDTA, 98%	Ethylenediaminetetraacetic acid tetrasodium salt hydrate	500 g	Aldrich	E26290	A, B, E
Tris base		1 kg	Fisher	BP152-1	A, B
N-Lauryl Sarcosine, ≥94.0 %	N-Lauroylsarcosine sodium salt	500 g	Sigma	L5125	A
Triton X-100					A
DMSO non-sterile	Dimethyl Sulfoxide				A, E
Ammonium acetate, 98%		500 g	Sigma	A1542	N
8-hydroxyquinoline, ≥99.0 %		100 g	Fisher	BP436-100	E
NaOH	Sodium Hydroxide (pellets)	500 g	ACP Chemicals	S3700	E
LMP agarose	Agarose DNA grade (low melting) Electrophoresis grade	25 g	Fisher Biotech	BP165-25	gel
SYBR Gold Nucleic Acid Gel Stain	10,000X Concentrate in DMSO	500 ul	Life technologies	S-11494	dye
Gelbond film, 102mm x 16.5M ROLL	Agarose support medium		Lonza Rockland	53740	film

A – lysis buffer A; B - lysis buffer B; N – neutralization buffer; E – unwinding/electrophoresis buffer.