Comet Assay for Cnidarian Cells

Objective: To determine relative double stranded DNA damage in treatments of a single cell type of cnidarians cells using the Comet assay.

EQUIPMENT AND SUPPLIES

- Comet Assay kit (Trevigen®, or similar)
- Freshly prepared cnidarians cells (single cell type), 1 X 10^5 cells/mL
- Tris(hydroxymethyl)aminomethane (Tris)
- Disodium ethylenediaminetetraacetic acid (EDTA)
- Deionized water
- Ethanol (molecular biology-grade)
- Electrophoresis tank (example uses Trevigen®)
- Comet slides
- Sodium hydroxide (NaOH)
- Dimethylsulfoxide (DMSO)
- SYBR Green I stain, 10,000X (Molecular Probes, Inc., Eugene, OR)
- Refrigerator/cold room
- Ice
- AC Power supply (low voltage, ~20 V)
- Sodium chloride (NaCl)
- Sodium sulfate (Na_2SO_4)
- Potassium chloride (KCl)
- Sodium bicarbonate ($NaHCO_3$)
- Graduated cylinder, glass (1 L)
- Micropipettors and tips (0.1-1000 µL)
- Hemacytometer
- Microscope (100X-400X magnification) with epifluorescence and digital imaging capabilities
- Refractometer
- Ultra-pure water source
- Centrifuge (to 1500 X *g* and accommodates 1.5 mL tubes)
- Small reservoirs for slide immersion (2) (P1000 pipet tip boxes work well)
- Heat block or incubator to 37 ^oC
- Dessicant
- Airtight storage container
- Microwave or hot plate
- 0.5 mL tubes, sterile
- Paper towels
- 50 mL tubes
- Nitrile gloves
- Protective eyewear

- Aluminum foil
- Water bath to 37°C
- Propidium iodide, 20 mM in DMSO (PI)
- Comet assay statistical software (e.g., CometScore™ freeware by TriTek Corp.)
- Filter-sterilized artificial sea water, salinity=35 ppt (FASW)

PREPARATION (One day prior to experiment)

1. Prepare a stock solution of 0.5 M EDTA, pH 8.0 and autoclave.
2. If using the Trevigen® electrophoresis tank, fill the water chamber according to the manufacturer's instructions.
3. Chill the electrophoresis tank in a cold room or refrigerator (4 °C).
4. Make up calcium- and magnesium-free sea water (CMFSW; Strathmann, 1987):
    a. 900 mL MilliQ quality water
    b. 449 mM NaCl
    c. 33 mM Na_2SO_4
    d. 9 mM KCl
    e. 2.5 mM $NaHCO_3$
    f. 1 mM EDTA
    g. Adjust pH to 8.0 with NaOH
    h. Bring volume to 1.0 L with MilliQ water
    i. Verify salinity (35 ppt) with refractometer and adjust with NaCl if needed (may need to increase NaCl to ~520 mM)
    j. Filter sterilize for shelf storage
5. Prepare Tris-EDTA (TE) buffer (10 mM Tris-1 mM EDTA, pH7.5) using the prepared 0.5 M EDTA solution and autoclave.
6. Dilute 10,000 X SYBR Green I to 1X with TE buffer in a 15 mL tube (1 µL in 10 mL TE). Cover tube with foil and store at 4 °C.
7. Make up 70% solution of ethanol and store in airtight container (room temperature).

PROCEDURE

(NOTE: UV light exposure of cnidarian cells should be kept to a minimum.)

1. Prepare fresh Alkaline Electrophoresis Solution (1 L), pH > 13 (200 mM NaOH-1mM EDTA):
    a. Don gloves and protective eyewear (**NOTE: solution is highly caustic!**)
    b. 8 g NaOH
    c. 2 mL 0.5 M EDTA
    d. Distilled water to 1 L and mix well
    e. Remove 50 mL of solution and place in clean 50 mL tube (Alkaline Unwinding Solution). Store at room temperature.

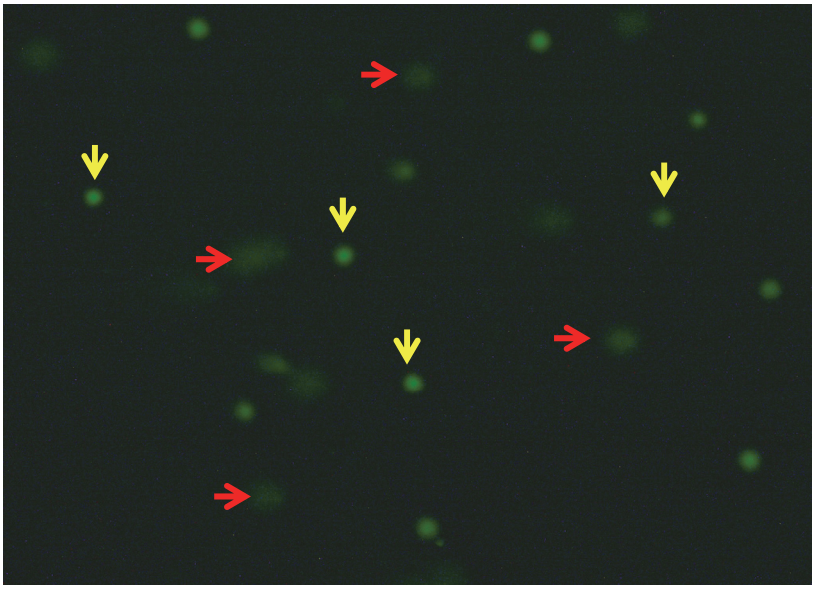
   f. Chill remaining 950 mL at 4 ^{o}C.
2. Warm low melting (LM) Agarose in a microwave until completely liquefied. Place in a 37 ^{o}C water bath until use.
3. Prepare 2mM propidium iodide in filter-sterilized artificial sea water (FASW) from 20 mM stock. Keep at room temperature in dark.
4. Prepare Lysis Solution:
   a. 40 mL Lysis Solution (Comet kit)
   b. 4 mL DMSO
   c. Mix well and chill at 4 ^{o}C
5. Prepare fresh cnidarian cells under dimmed lighting conditions. Lighting should be restricted as much as possible to minimize any unintended DNA damage from that source.
6. Stain an aliquot of cnidarian cells using 2 mM propidium iodide (100:1, cell vol/PI vol) at room temperature for 5 min. Alternatively, trypan blue staining may be used for light microscopy evaluation.
7. Count cells on a hemacytometer using bright field for total cell counts and fluorescent (PI) microscopy for dead cell counts. Determine percent dead cells (should be less than 10 %).
8. At this point cell aliquots can be treated to induce DNA damage. There should be a minimum of 5 X 10^5 cells per treatment replicate.
9. Following treatment, pellet cells by centrifugation (1500 X *g* for 5 min), carefully aspirate supernatant from pellet, and resuspend cells gently in CMFSW by pipetting up and down slowly.
10. Repeat cell wash as in step 9. Resuspend cells gently in CMFSW to an estimated concentration of 5 X 10^5 cells/mL.
11. Verify live/dead cell numbers on hemacytometer as in steps 6-7 above. (**NOTE: coral cells should not remain suspended in CMFSW for extended periods as this could significantly reduce cell viability.**)
12. Pipet 5 µL of each replicate cell treatment into a sterile 0.5 mL tube, one replicate per tube.
13. Add 50 µL LM Agarose (37 ^{o}C) to the first tube and mix gently by pipetting up and down with a P200 µL pipette tip 2-3 times.
14. Immediately aliquot the agarose-cell mixture into one well of a 20-well Comet slide (30 µL /well, **or** 50 µL /well for two-well slides) and spread carefully using the pipet tip. DO NOT TOUCH THE SLIDE WITH THE PIPET TIP!
15. Repeat the steps 13-14 for each replicate.
16. Chill the slide(s) in the dark at 4 ^{o}C (refrigerator) for 15 min.
17. While slide is in the refrigerator, pour chilled Lysis Solution into small reservoir (e.g., clean pipet tip box with lid), cover with aluminum foil and keep on ice.
18. Decant remaining 950 mL of chilled Alkaline Electrophoresis Solution into electrophoresis tank (in cold room or refrigerator).
19. Remove slide from refrigerator and incubate in Lysis Solution, protected from light, for 10 min.
20. Remove slide from Lysis Solution and drain excess buffer on a paper towel.

21. Place slide in a second small reservoir containing room temperature Alkaline Unwinding Solution. Incubate 5 min in dark (covered with foil).
22. Place slide on slide tray in electrophoresis tank and cover with slide tray overlay (part of Trevigen® kit).
23. Apply voltage (21 V) to electrophoresis tank for 5-15 minutes. (**NOTE: electrophoresis time will depend on the integrity of cells and will have to be optimized depending on cnidarian species, cell type and treatment.**)
24. Remove slide from electrophoresis unit and drain on paper towels.
25. Immerse slide in distilled water for 10 min (small reservoir covered with foil).
26. Decant liquid and carefully replace with fresh distilled water. Incubate another 10 min.
27. Decant liquid from reservoir and add 50 ml of 70% ethanol. Incubate 5 min.
28. Dry slide in incubator (37 ^{o}C) for 20 -25 min.
29. Make sure the agarose has completely dried prior to storing the slide in an airtight container (dark) with dessicant.
30. The slide can be stored indefinitely until ready to stain and analyze.
31. Staining: add 100 µL dilute SYBR Green I to each well of slide and incubate slide at 4 ^{o}C for 15 min.
32. Decant liquid onto paper towel and allow slide to air dry at room temperature in the dark (~20 min).
33. Image slide using epifluorescence microscopy (SYBR green I)
34. Statistical analyses should be performed on at least 100 cells per treatment. Free software downloads are available online to assist with data analysis (see LINKs below).

EXPECTED RESULTS

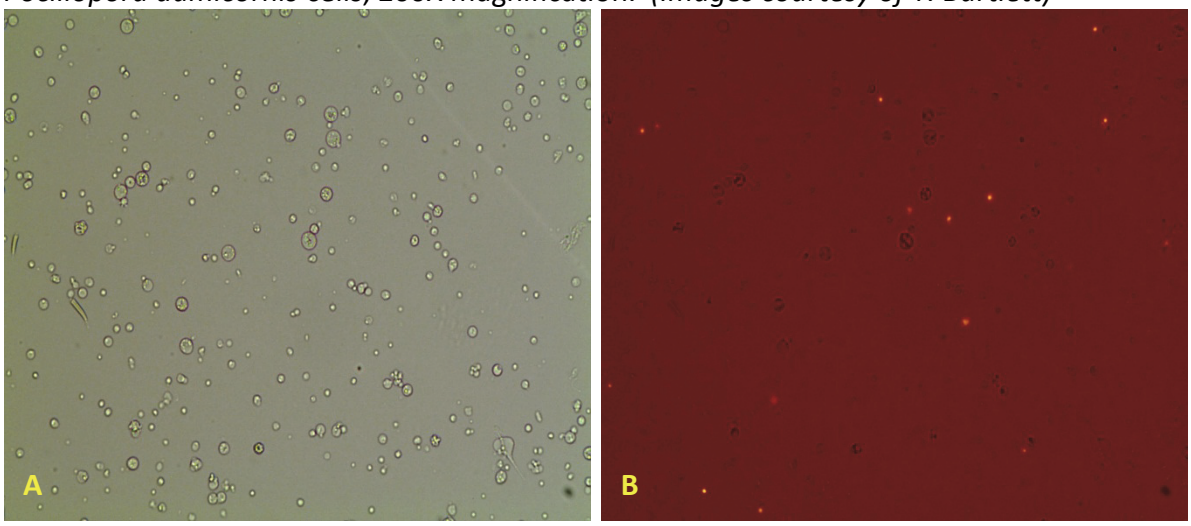
Cnidarian cell isolate quality is highly variable and is the greatest problem to overcome to ensure reproducibility in the Comet Assay. Working with scleractinian coral cells is especially difficult because of the requirement for calcium and magnesium ions, which must be eliminated from the cell buffering solution to inhibit nuclease activity. Additionally, some cells may suffer damage during the isolation process, rendering them unsatisfactory for this particular analysis. Compromised cellular integrity prior to treatment/analysis will result in increased DNA damage that can significantly skew experimental results (Figure 1).

Figure 1. Results of Comet Assay using untreated *Pocillopora damicornis* cells. Both healthy (vertical, yellow arrows) and potentially compromised (horizontal, red arrows) cells are illustrated below (100X magnification).



A propidium iodide staining step is recommended to identify cells with compromised integrity prior to treatment (Figure 2). The bright nuclear stain of this fluorescent dye is easily distinguished from the more diffuse natural fluorescence in some coral cells.

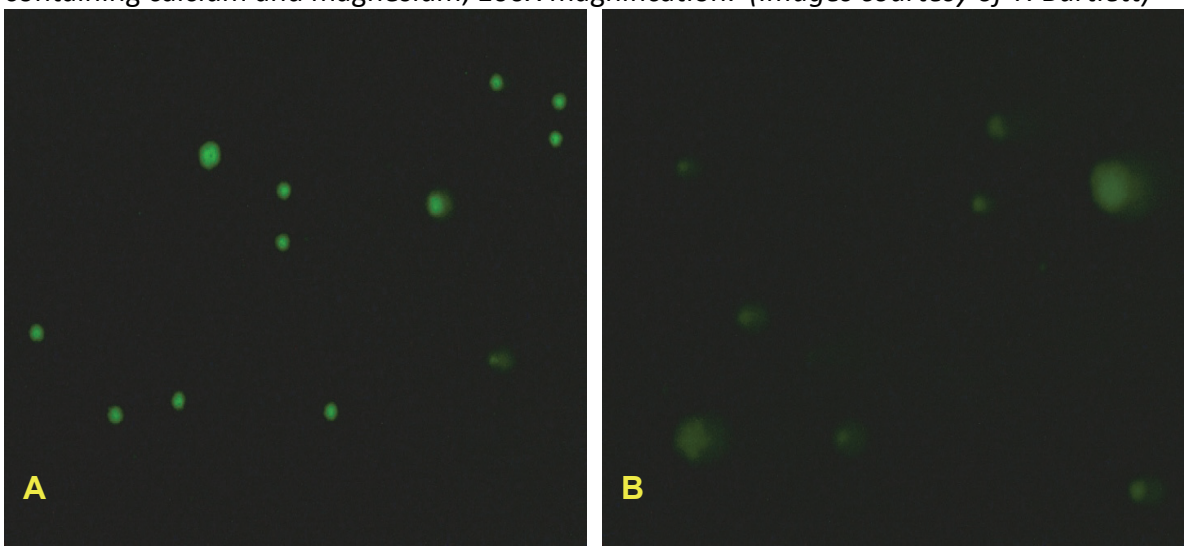
Figure 2. Bright field (A) and epifluorescent (B) images of propidium iodide-stained *Pocillopora damicornis* cells, 200X magnification. *(Images courtesy of T. Bartlett)*



Using natural or artificial seawater for coral cell resuspension prior to immobilization in agarose can have undesirable effects in the Comet Assay. The presence of calcium and magnesium ions can activate endonucleases, which will artificially increase comet tail

length. The use of calcium- and magnesium-free seawater instead of natural or artificial seawater greatly improves assay reliability (Figure 3).

Figure 3. Results of the Comet Assay using untreated *Pocillopora damicornis* cells. Panel A: Coral cells resuspended in calcium- and magnesium-free seawater prior to assay, 100X magnification. Panel B: Coral cells resuspended in artificial seawater containing calcium and magnesium, 100X magnification. *(Images courtesy of T. Bartlett)*



LINKS

http://www.trevigen.com/
http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes.html
http://www.tritekcorp.com/products_cometscore.php

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