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Determination of DNA Damage in the Retina Photoreceptors *of Drosophila*

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[Abstract] The retina is sensitive for light damages, because of direct light exposure, especially intense blue and UV light, which increase level of ROS and other toxic phototransduction products in photoreceptor cells. In our previous work (Damulewicz *et al*., 2017a and 2017b), we used 8-oxo-deoxyguanosine (8-OHdG) as a marker for oxidative stress to investigate the role of heme oxygenase in DNA protection against UV light. In this protocol, we showed how to determine the level of DNA damages in the retina using immunohistochemical staining.

Keywords: Oxidative stress, 8-Oxo-deoxyguanosine, UV light, Retina degeneration, DNA damage

[Background] Deoxyribonucleic acid (DNA) is an essential molecule for all living organisms. It contains genetic code with instruction for proteins and other molecules necessary for structure and metabolism of an organism. DNA is composed of two strands of nucleotides. Each nucleotide is built of one of four types of nucleobases (cytosine, guanine, adenine and thymine), deoxyribose and phosphate group. Physical and chemical changes in DNA structure are classified as DNA damages. It includes one or two strands of DNA breaks, missing nucleotide or chemical changes of nucleobases. Damages can be induced by environmental factors, like chemicals or UV light exposure, or in result of metabolic processes, which produce reactive oxygen species, reactive nitrogen species, reactive carbonyl species and alkylating agents (De Bont and van Larebeke, 2004).

One of the most common DNA damage is 8-oxo-deoxyguanosine (8-OHdG), which is a product of DNA oxidation by reactive oxygen species and it serves as a marker of oxidative stress (De Souza-Pinto *et al*., 2001; Swenberg *et al*., 2011; Valavanidis *et al*., 2013). 8-OHdG enhances the risk of transversion mutation, because it has ability to pair with adenine and cytosine bases (Maki and Sekiguchi, 1992). In effect it plays a role in mutagenesis, cancerogenesis and aging (De Bont and van Larebeke, 2004; Kohen and Nyska, 2002). Higher 8-OHdG level was described in breast, renal and gastric tumors (Lee *et al*., 1998; Musarrat *et al*., 1996; Okamoto *et al*., 1994).

There are several methods to determine 8-OHdG level in a specific tissue, like immunohistochemistry (De Carvalho *et al*., 2013), Western blot (Kannan *et al*., 2006), standard HPLC (Herrero and Barja, 1999), liquid chromatography nanoelectrospray-tandem mass spectrometry (Ma *et al*., 2016). Here, we present a detailed protocol for DNA damage determination in the retina of fruit fly, *Drosophila melanogaster* using immunohistochemistry method (Damulewicz *et al*., 2017a and 2017b).

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Materials and Reagents

1. Filter paper (Fisher Scientific, catalog number: 09-790-14D)
2. Cover glasses (Menzel-Glaser)
3. Microscope glasses (Superfrost, Menzel-Glaser)
4. Small plastic holders (*i.e.*, Eppendorf tube lids)
5. Tissue-Tek (SAKURA, catalog number: 4583)
6. *Drosophila melanogaster* (wild-type CantonS, 5-7 days old males)
   Note: Local ethical standards need to be followed.
7. 6% glucose in water (commercial)
8. Protoporphyrin IX (SnPP IX) (Frontier Scientific, catalog number: P562-9)
9. Hemin chloride (Merck, Calbiochem, catalog number: 3741)
10. CO_2
11. Schneider's medium (Sigma-Aldrich, catalog number: S9895)
12. Etoposide (Sigma-Aldrich, catalog number: E1383)
13. 4% paraformaldehyde (PFA) (Sigma-Aldrich, catalog number: P6148)
14. Sucrose (commercial)
15. Liquid nitrogen
16. Normal Goat Serum (NGS) (Sigma-Aldrich, catalog number: G9023)
17. Bovine serum albumin (BSA)
18. Mouse anti-8-hydroxyguanosine primary antibodies (Acris, catalog number: AM03160)
19. HRP/DAB (ABC) kit (Abcam, catalog number: ab64264)
20. Glycerol (MP Biomedicals)
21. Vectashield with DAPI (Vector Laboratories, catalog number: H-1200)
22. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
23. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9541)
24. Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$) (Sigma-Aldrich, catalog number: S3522)
25. Potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich, catalog number: P5655)
26. Hydrochloric acid (HCl) (Sigma-Aldrich, catalog number: H1758)
27. Sodium phosphate dibasic (Na_2HPO_4) (Sigma-Aldrich, catalog number: S7907)
28. Triton X-100 (Sigma-Aldrich, catalog number: T8787)
29. Cornmeal (Glutenex)
30. Agar (Bioshop)
31. Artificial honey (Vortumnus)
32. Yeast (Paneo)
33. Methyl-4-hydroxybenzoate (anti-fungal chemical, Sigma-Aldrich, catalog number: H3647)
34. 90% EtOH
35. Phosphate buffered saline (PBS) (see Recipes)

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36. Phosphate buffer with Triton (PBT) (see Recipes)
37. Standard medium for flies (see Recipes)

Equipment

1. Forceps (Dumont No. 5)
2. UV transilluminator (Vilbert Lourmat, model: UVECX26)
3. White light transilluminator (Vilbert Lourmat, model: TFX53WL)
4. Cryostat (Leica, model: CM1850 UV)
5. Light microscope (Carl Zeiss, model: Axioskop 50)
6. Camera (Canon, model: G10)

Software

1. ImageJ software

Procedure

1. Take 30 males for every group (experimental and control).
   Notes: You can use different control groups, according to your experiment:
   a. *Control for the staining conditions: positive (incubated with etoposide) and negative (without primary antibodies).*
   b. *Control for UV light effect–flies unexposed to light (kept in constant darkness).*
   c. *Control for feeding effect–flies fed with glucose only.*
2. Keep flies for 5 days in constant darkness (DD) on a standard medium.
3. Place flies for 6 h into an empty vial, with no access to food, but with wet filter paper to provide them water *ad libitum*. Keep them in DD conditions.
4. Apply selected chemicals diluted in 6% glucose to a filter paper for 6 h or 6% glucose only for control groups. In this experiment, we used heme oxygenase inhibitor (100 μM protoporphyrin IX–SnPP IX) and activator (100 μM hemin chloride).
   Note: Avoid leaving big droplets of a liquid at the bottom of vial–flies can be trapped in liquid and drown.
5. Use CO_2 to anesthetize one of the control groups.
6. Decapitate one of the control groups and put heads for 4 h in 500 μl Schneider's medium supplemented with 20 μM etoposide at room temperature.
7. Expose experimental flies and second control group (fed with glucose only) to 1 h of UV light pulse (100 lux) and then to 3 h of intensive white light (1,500 lux) (Figure 1).
   Note: Be sure that flies have access to water during the experiment (wet filter paper).

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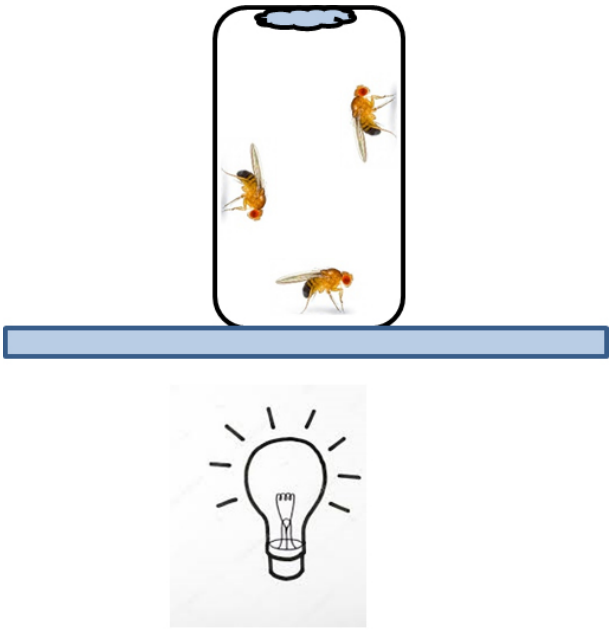

Figure 1. UV/white light exposure. Vial (orientated upside down) with flies is placed on the transilluminator. Filter paper attached to the vial provide water *ad libitum.*

8. Keep one of control group continuously in constant darkness until decapitation.
9. Use CO_2 to anesthetize flies.
10. Decapitate the anesthetized flies (all experimental and control) in a drop of 4% PFA.
11. Fix all heads in 500 µl 4% PFA for 4 h at 4 °C.
12. Remove PFA using a pipette and wash heads 2 times each for 10 min at room temperature (RT) in 500 µl PBS (see Recipes).
13. Cryoprotect material by washing for 10 min in 500 µl of 12.5% sucrose and then overnight in 500 µl of 25% sucrose (Figure 2).

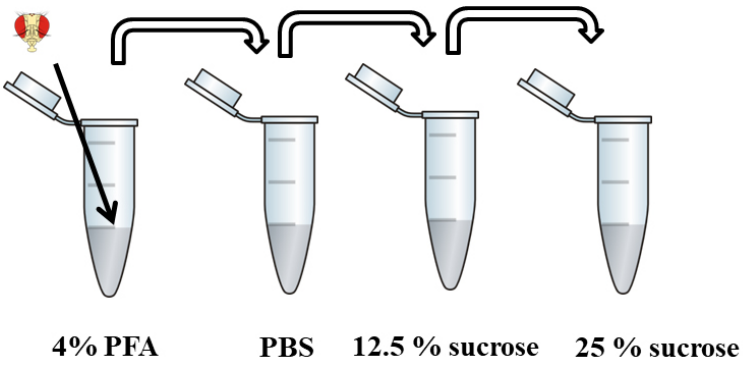

Figure 2. Fixation and cryoprotection of material. Flies heads are fixed in 4% PFA, then washed in PBS and cryoprotected in 12.5% and 25% sucrose.

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14. Put a droplet of Tissue-Tek to small plastic holders, embed heads into Tissue-Tek, put them into the bottom and organize in rows (Figure 3).

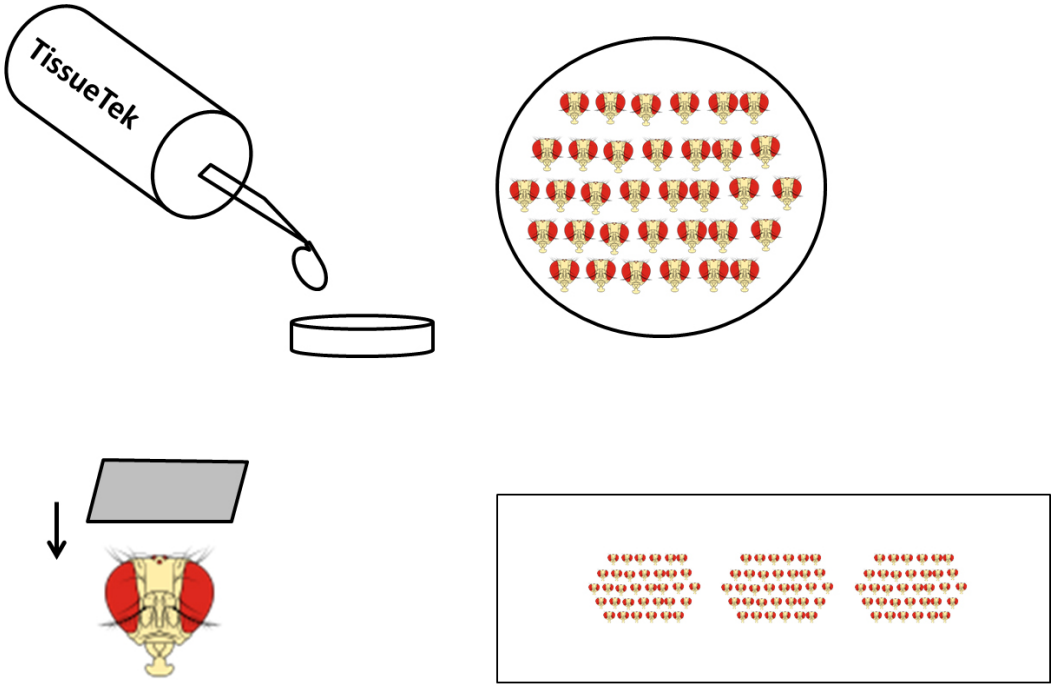

Figure 3. Sample preparation for cryostat. Small plastic holder is filled with TissueTek, then heads are put into the bottom in rows and cut (frontal sections). Sections are collected on microscope glass.

15. Freeze material in liquid nitrogen for few seconds.
16. Remove frozen sample from the holder.
17. Use a cryostat to cut 20 µm thick sections.
18. Dry the cryosections for 30 min at room temperature.
19. Keep the sections frozen for long-term storage at -20 °C or use them immediately for immunodetection at room temperature.
20. Rehydrate material by incubating with 500 µl PBS for 30 min.
    Note: Use PBS, PBT at room temperature.
21. Wash sections for 10 min in 200 µl 0.5% PBT (see Recipes).
22. Wash sections for 5 min in 200 µl 2% PBT.
23. Wash sections 3 times for 5 min each in 200 µl 0.5% PBT.
24. Block unspecific binding sites by the incubation with 200 µl 5% NGS in BSA for 45 min.
25. Apply anti-8-hydroxyguanosine primary antibodies (1:500, in 0.5% BSA) and incubate overnight at 4 °C.
26. Keep one section as a negative control and do not apply primary antibodies.
27. Use HRP/DAB (ABC) detection kit according to the manufacturer's protocol. In this step secondary antibodies are used (they are provided in the kit).

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28. Close sections with glycerol and cover glasses.
29. Take the retina pictures under a light microscope using a microscope camera.
    Note: To visualize cell nuclei in the retina keep one section unstained with anti-8OHdG. Rehydrate material and close with fluorescence medium–Vectashield with DAPI. Use a fluorescent or confocal microscope to take pictures.

Data analysis

1. Analyze DNA damages as Mean Grey Values using ImageJ software. Select area on the top of retina (where nuclei are located, see Figure 5) and measure Mean Grey Value (the range between 0-255). Mean Grey Value is the sum of the grey values of all pixels in the area divided by the number of pixels within selection. Collect three measurements from one picture, use the average as a mean for this picture, collect at least 20 means (different individuals) (Table 1). Copy your data to Excel file and compare means between experimental and control groups.
   Note: The retina photoreceptors nuclei are located in the distal part of the retina (see Figures 4 and 5, nuclei are stained with DAPI), measure Mean Grey Value in this area only. Use the same microscope and camera settings for all groups (experimental and control).

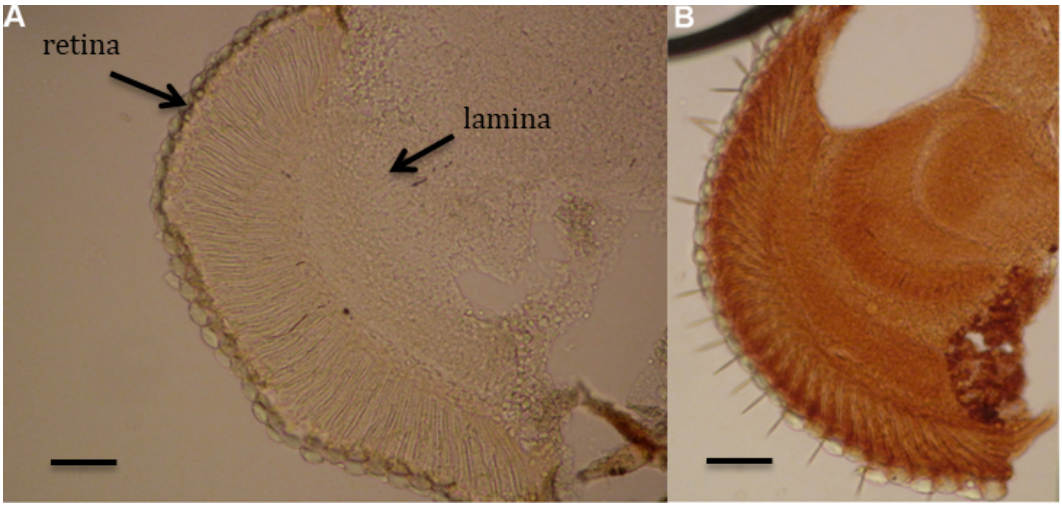

Figure 4. Controls used for DNA damage determination. A. Negative control–without primary antibodies. B. Positive control–etoposide treatment. Scale bars = 20 μm.

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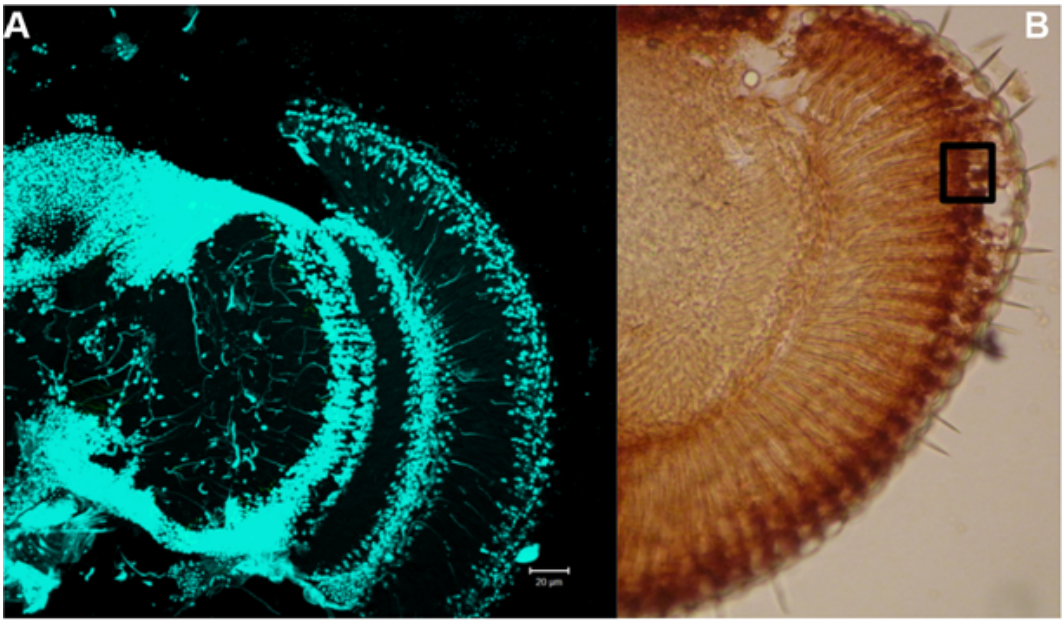

Figure 5. Localization of cell nuclei in the retina. A. DAPI staining–cell nuclei are visualized. B. Retina immunostained with anti-8-hydroxyguanosine, black square shows an analyzed area. Scale bar = 20 μm.

Table 1. Sample triplicate measurements for experimental and control flies

Negative control (without primary antibodies)	**Positive control** (etoposide)	**UV treatment** (fed with glucose only)	**UV treatment** (fed with hemin)	**UV treatment** (fed with SnPP IX)	**No UV treatment** (fed with glucose)
23.08	158.72	98.61	66.51	188.48	55.63
22.77	145.47	97.36	65.12	185.56	58.70
20.64	161.80	102.02	67.60	189.30	61.46

Recipes

1. Phosphate buffer (PBS, 1x)
   8 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_4
   800 ml distilled H_2O
   Adjust pH to 7.4 with HCl
   Adjust volume to 1 L with distilled H_2O
2. Phosphate buffer with Triton (PBT, 1 L)
   0.4363 g $NaH_2PO_4 \cdot H_2O$
   0.9705 g Na_2HPO_4
   7.953 g NaCl
   5 ml Triton X-100 for 0.5% PBT

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Distilled water up to 1 L

Note: PBS and PBT can be stored for a few weeks in the fridge.

3. Standard medium for flies

   120 g cornmeal (Glutenex)

   10 g agar (Bioshop)

   140 ml artificial honey (Vortumnus)

   10 g yeast (Paneo)

   2 L of water

   Boil 30 min

   Add 3 g methyl-4-hydroxybenzoate (anti-fungal chemical, Sigma-Aldrich) in 15 ml 90% EtOH

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