Characterization of Radiotolerance Mechanisms in the Tardigrade Species *Hypsibius dujardini*

Ben Cooper[1,2], Sigrid Reinsch[3]

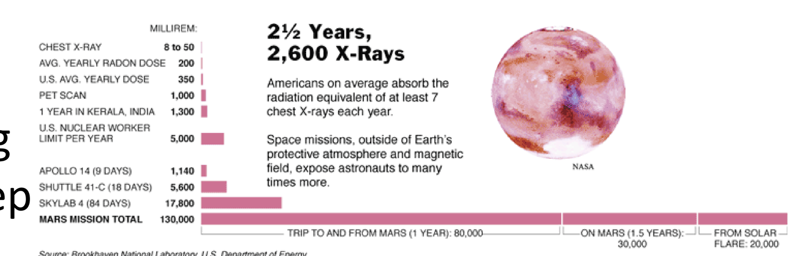
[1]Space Life Sciences Training Program, KBRWyle, NASA Ames Research Center, Moffett Field, CA
[2]Department of Biology, Tufts University, Medford, MA
[3]National Aeronautics and Space Administration, Space Biosciences Division, NASA Ames Research Center, Moffett Field, CA

SUMMARY

Tardigrades are microscopic invertebrates that are uniquely radiotolerant among animals, and while the mechanisms of radiotolerance in some species is becoming understood, such mechanisms in *Hypsibius dujardini*, the most radiotolerant fully aquatic tardigrade, are unknown. We asked 1) Is *H. dujardini* resistant to direct or indirect DNA damage due to ionizing radiation? and 2) Is this resistance through initial DNA protection or efficient repair once damage has occurred? We confirmed *H. dujardini's* extraordinary radiotolerance but encountered challenges in performing molecular techniques, thus identifying a need for standardization of tardigrade experimental protocols.

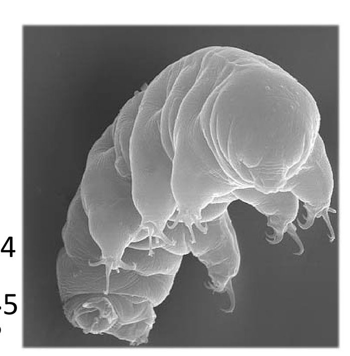
INTRODUCTION

- Protection from radiation is paramount during long-duration deep space exploration



- In transit to and from Mars, astronauts are projected to incur a dose of 13x OSHA's prospective annual limit[1]

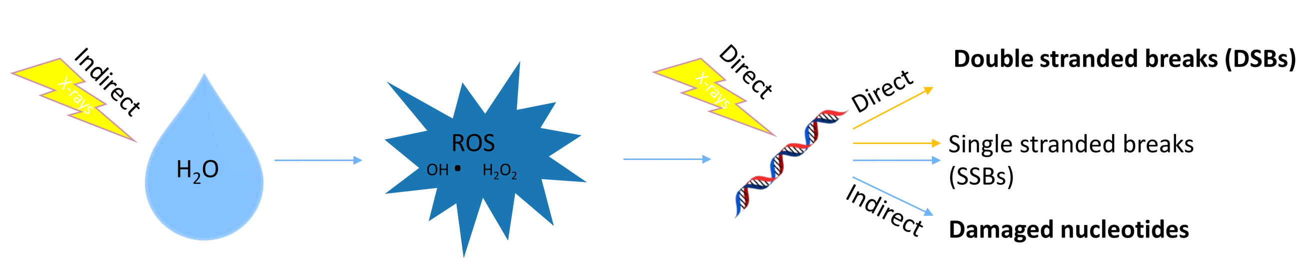
- Tardigrades – experts in extremotolerance
  - Many species; 100-500 µm in length
  - Have survived vacuum of space[2]
  - Unique among eukaryotes in their radiotolerance while in a metabolically active state[3]
- *Hypsibius dujardini*
  - Most radiotolerant fully aquatic tardigrade yet tested[3]
    - $LD_{50/48hr}$ of ~4200 Gy with gamma-rays[4]
    - Humans have $LD_{50/60d}$ of ~4 Gy ionizing[5] radiation



Hypsibius dujardini

- ***How do they do it?***

- Radiation damage occurs through two main pathways[5]:
  - "**Indirect**" generation of reactive oxygen species (ROS) in the aqueous environment → most common, primarily causes single stranded breaks and modified nucleotides[6]
  - "**Direct**" contact with a DNA molecule → less common, primary causes single stranded breaks and double stranded breaks[7]

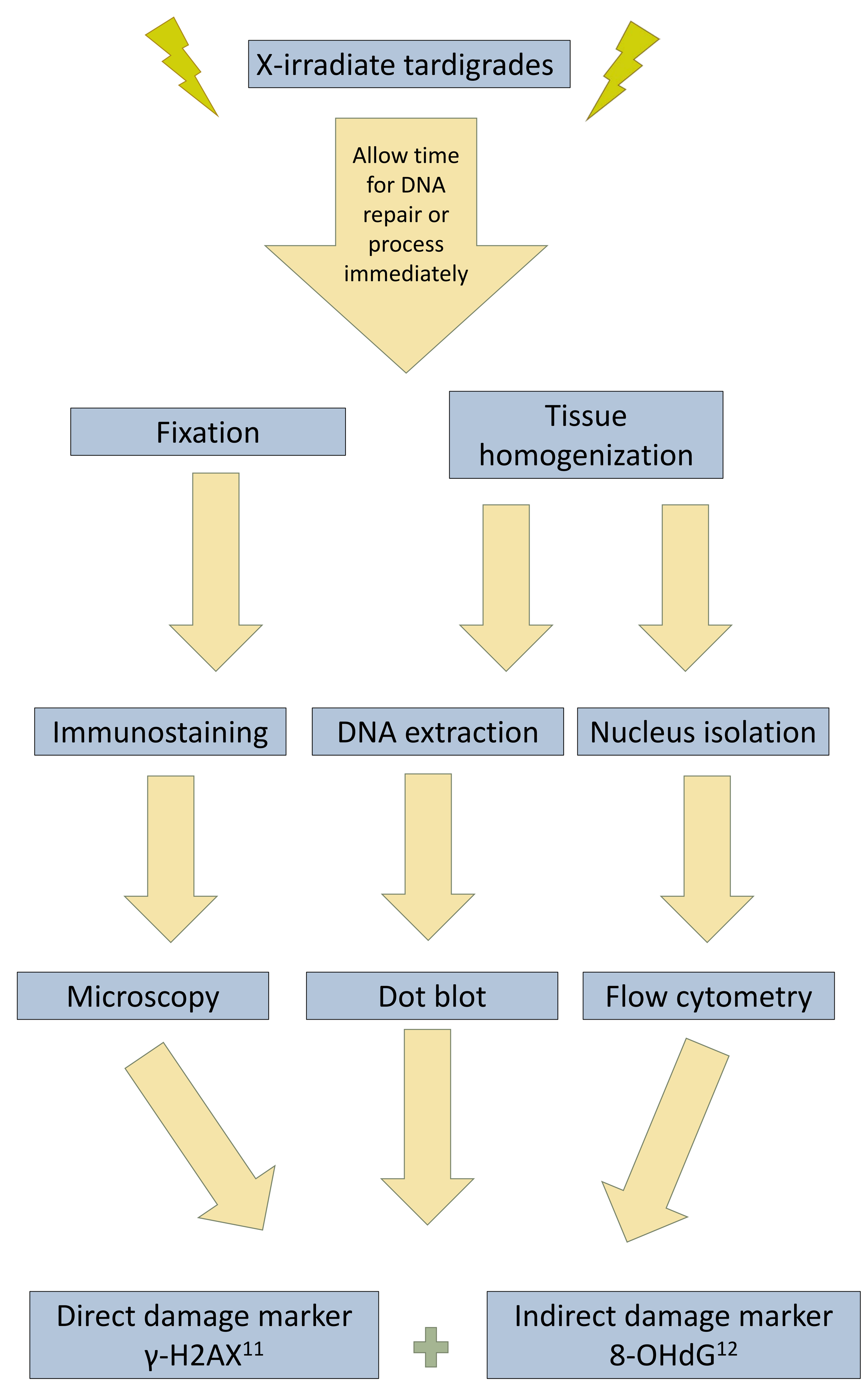


- The tardigrade species *R. varieornatus* recently identified to express a unique protein termed damage suppressor (Dsup)
  - When transfected into human cells, conferred 40% increase in radiotolerance[8]
  - Recently determined that Dsup acts as a "shield" from reactive oxygen species around DNA[9]
- *Hypsibius dujardini*, however, doesn't express Dsup or a homologous protein[10]

HYPOTHESIS

Like *R. varieornatus*, *H. dujardini* is resistant to DNA damage from ionizing radiation through initial protection of damage, either direct, indirect, or both.

METHODS



Potential interpretation

Damage levels immediately after irradiation	Damage levels after time for DNA repair	*Radiotolerance mechanism*
High direct	Low direct	Direct damage repair
High indirect	Low indirect	Indirect damage repair
Low direct	Low direct	Direct damage protection
Low indirect	Low indirect	Indirect damage protection

PRELIMINARY RESULTS

H. Dujardini x-ray survival

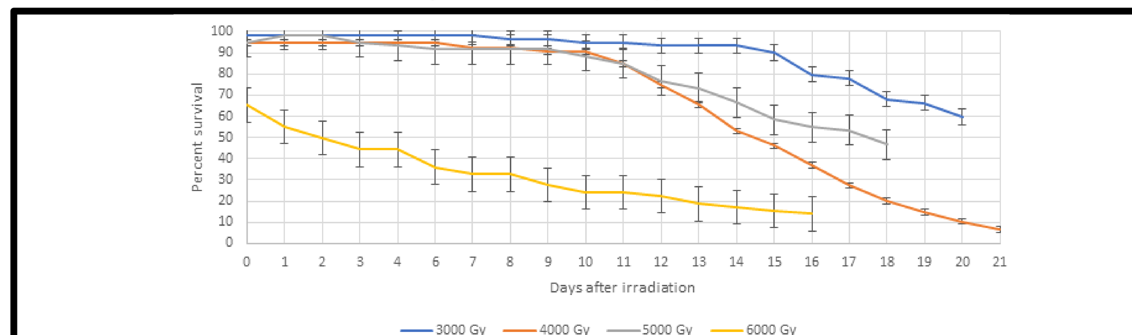


Figure 1. Hypsibius dujardini x-ray survival
H. dujardini were irradiated at doses of 3000, 4000, 5000, or 6000 Gy and then monitored for viability every day. Differing line length between conditions is a result of different lengths of observation for each condition due to logistical restrictions. Data points are averages of triplicate samples of 20 tardigrades.

- $LD_{50/48hr}$ of ~6000 Gy
- Negligible decrease in viability through 10 days with lower doses
- Later decline from all exposure levels

Post-irradiation immunostaining

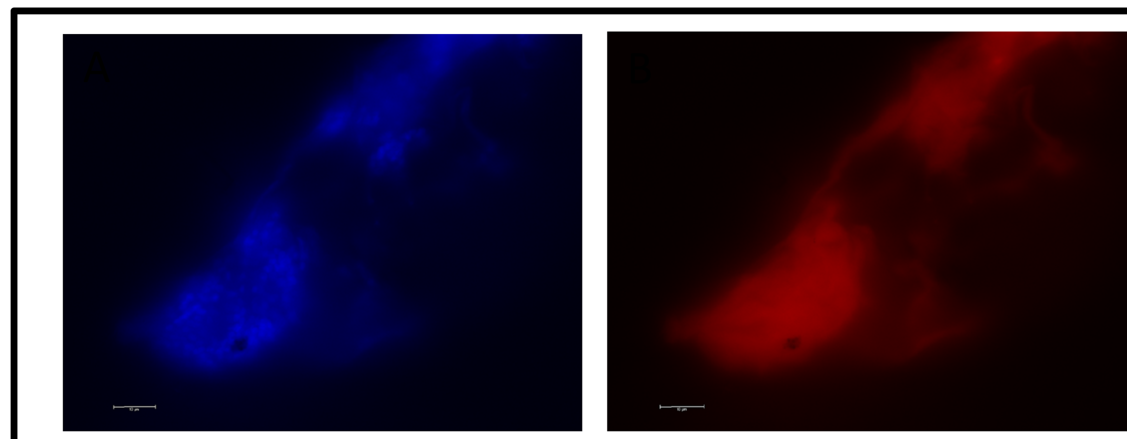


Figure 2. Tardigrade immunostaining (100x magnification)
H. dujardini immunostained for 8-OHdG (**A**) and γ-H2AX (**B**) after 4300 Gy of x-irradiation . Scale bar is 10 microns. Nuclei were measured to be approximately 1-2 µm in diameter using Leica software (**A**).

- High background staining
- Some nuclear localization with 8-OHdG
- Not strong enough magnification for quantitation

Nucleus isolation for flow cytometry

- Potentially isolated some nuclei, measuring similar 1-2 µm diameter
- Insufficient sample recovery for flow cytometry

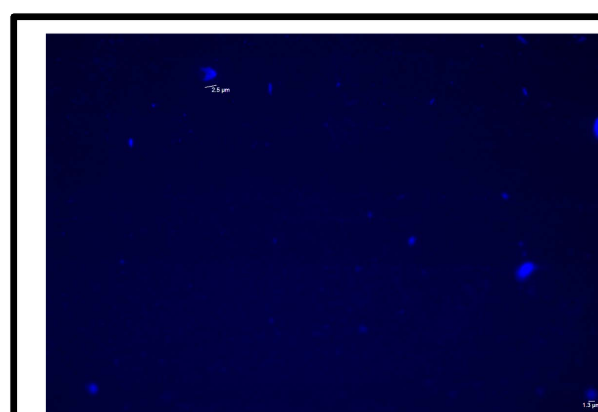


Figure 4. Potential tardigrade nuclear isolate stained with DAPI (100x magnification)
H. dujardini nuclear isolation was attempted and the resulting debris was mounted on a slide and stained with DAPI

Dot blotting for 8-OHdG

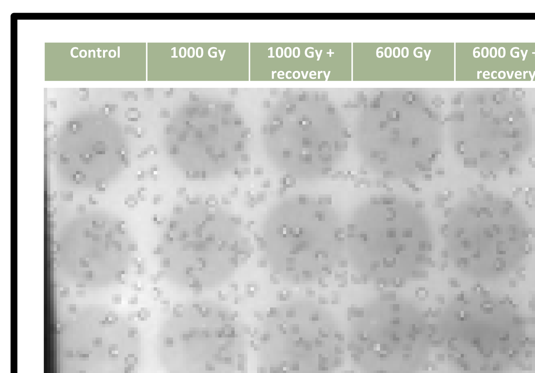


Figure 5. Dot blots for 8-OHdG
H. dujardini DNA was dot blotted for 8-OHdG.

- No notable chemiluminescence
- Potential issues with nitrocellulose membrane and/or antibody used

DISCUSSION

- Corroborated extraordinary *H. dujardini* radiotolerance
- Tardigrades present challenges to techniques like immunofluorescence or cell/nucleus extraction due to their tough exterior cuticle
  - Protocols using cuticle and ECM enzymatic degradation should be explored
- Methods like nucleic acid or protein isolation show promise and appear much easier
  - We were time restricted, but only minor troubleshooting is needed
- It is clear that tardigrades hold biological innovations that can aid in human space exploration, but protocol standardization is necessary for rapid expansion of this model organism

ACKNOWLEDGEMENTS

This research was supported by the Space Life Sciences Training Program (SLSTP) at NASA Ames Research Center and by Wyle Laboratories.

Thank you to my mentor Sigrid Reinsch for teaching me all I know about tardigrades, SLSTP management including Jon Rask and Desi Bridges for general support and care of the program, David Smith and Samantha Waters for help using their X-rad, Jon Galazka and John Hogan for sharing resources, and the GeneLab sample processing team for sharing their lab space with me.

Last but not least, thank you to all of the SLSTP interns and staffer Jordan McKaig and Liz Talburt for creating a great experience!

REFERENCES

1. Zeitlin, C., Hassler, D. M., Cucinotta, F. A., Ehresmann, B., Wimmer-Schweingruber, R. F., Brinza, D. E., … Reitz, G. (2013). Measurements of Energetic Particle Radiation in Transit to Mars on the Mars Science Laboratory. *Science*, *340*(6136), 1080–1084.
2. Jönsson, K. I., Rabbow, E., Schill, R. O., Harms-Ringdahl, M., & Rettberg, P. (2008). Tardigrades survive exposure to space in low Earth orbit. *Current Biology*, *18*(17), R729–R731.
3. Hashimoto, T., & Kunieda, T. (2017). DNA Protection Protein, a Novel Mechanism of Radiation Tolerance: Lessons from Tardigrades. *Life*, *7*(2).
4. Beltrán-Pardo, E., Jönsson, K. I., Harms-Ringdahl, M., Haghdoost, S., & Wojcik, A. (2015). Tolerance to Gamma Radiation in the Tardigrade Hypsibius dujardini from Embryo to Adult Correlate Inversely with Cellular Proliferation. *PLoS ONE*, *10*(7).
5. Hall, E.J.; Giaccia, A.J. *Radiobiology for the Radiologist*, 7th ed.; Lippincott Williams & Wilins: Philadelphia, PA, USA, 2012; pp. 120–121.6. Biaglow, J. E. (1981). The effects of ionizing radiation on mammalian cells. *Journal of Chemical Education*, *58*(2), 144.
7. Povirk, L. F. (2006). Biochemical mechanisms of chromosomal translocations resulting from DNA double-strand breaks. *DNA Repair*, *5*(9–10), 1199–1212.
8. Hashimoto, T., Horikawa, D. D., Saito, Y., Kuwahara, H., Kozuka-Hata, H., Shin-I, T., … Kunieda, T. (2016). Extremotolerant tardigrade genome and improved radiotolerance of human cultured cells by tardigrade-unique protein. *Nature Communications*, *7*, 12808
9. Chavez, C., Cruz-Becerra, G., Fei, J., Kassavetis, G. A., & Kadonaga, J. T. (2019). The tardigrade damage suppressor protein binds to nucleosomes and protects DNA from hydroxyl radicals. *ELife*, *8*, e47682. 10. Yoshida, Y., Koutsovoulos, G., Laetsch, D. R., Stevens, L., Kumar, S., Horikawa, D. D., … Arakawa, K. (2017). Comparative genomics of the tardigrades Hypsibius dujardini and Ramazzottius varieornatus. *PLOS Biology*, *15*(7), e2002266
11. Kinner, A., Wu, W., Staudt, C., & Iliakis, G. (2008). γ-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic Acids Research*, *36*(17), 5678–5694.
12. Valavanidis, A., Vlachogianni, T., & Fiotakis, C. (2009). 8-hydroxy-2′ -deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. *Journal of Environmental Science and Health. Part C, Environmental Carcinogenesis & Ecotoxicology Reviews*, *27*(2), 120–139