Bleomycin: A Study of DNA Damage and the Cell Cycle

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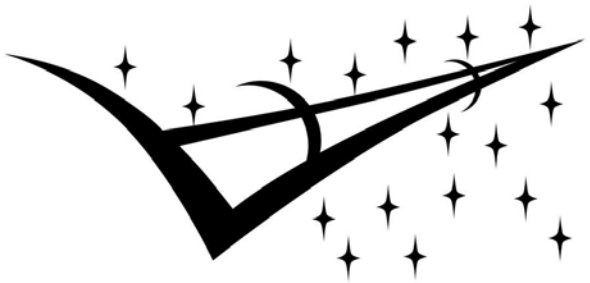
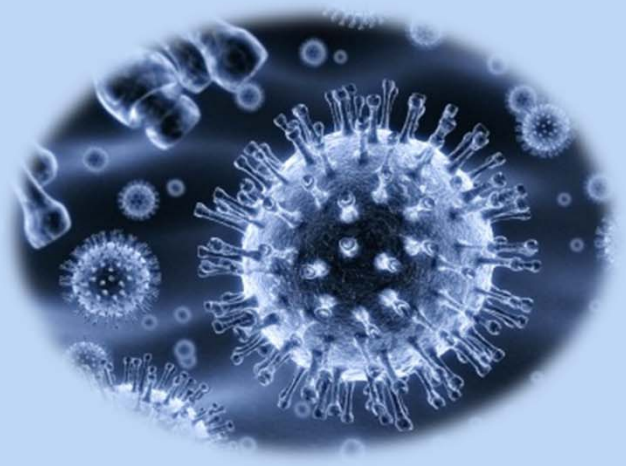
Radiation

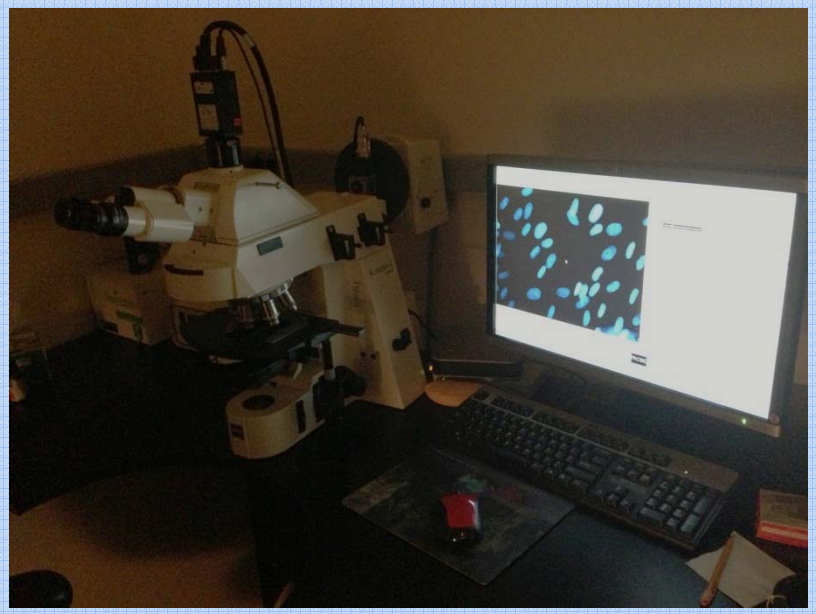
SPACE LIFE SCIENCES
SUMMER INSTITUTE

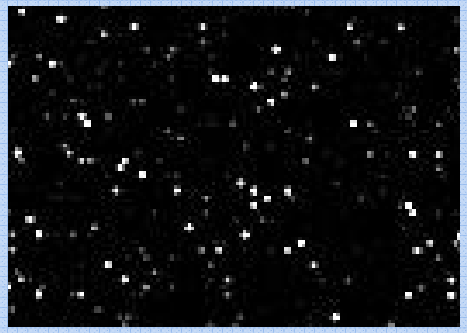


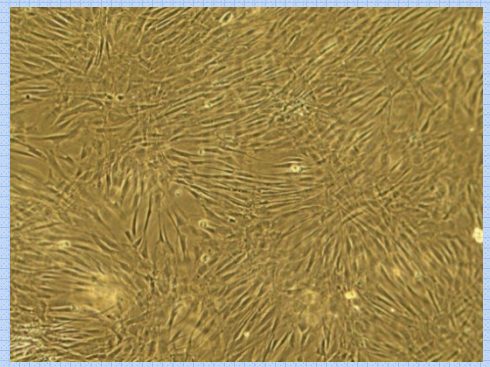


Objectives of Internship

- Enhance proficiency in cell culture (human fibroblast cells)
- Become familiar with assay and analysis techniques
  - Immunofluorescence assays, fluorescence microscopy, DNA microarray analysis
- Apply learned techniques to further bleomycin ground study in preparation for parallel in-flight study
- Learn about space travel and science in space from SLSSI lectures



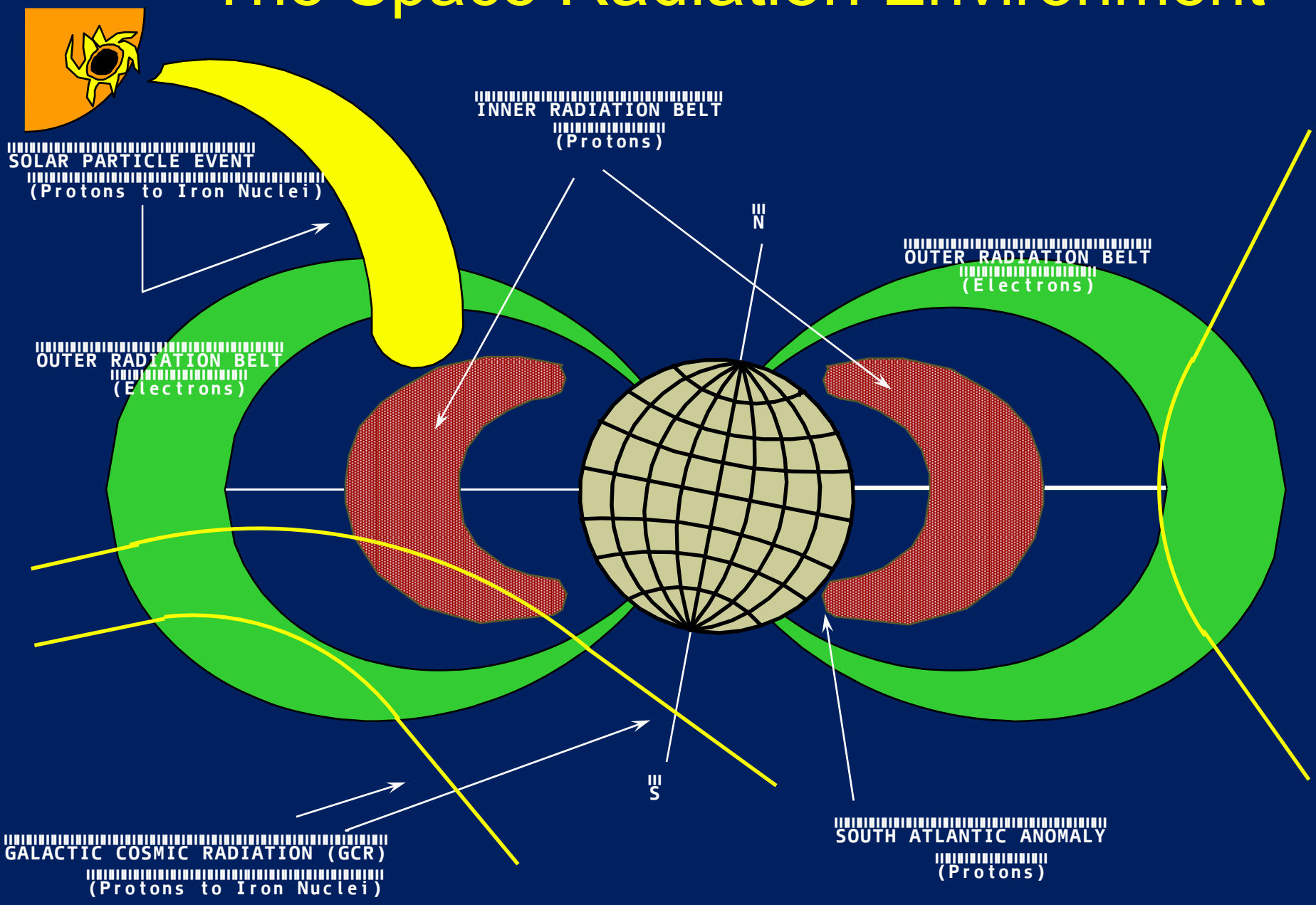




Background

- Space is a environment filled with radiation
  - Trapped protons, Galactic Cosmic Rays (GCRs), Solar Particle Events (SPEs)
- Assess astronaut's radiation risk
  - How cells respond to radiation
  - More important for long-term missions and missions outside of Near Earth Orbit (NEO)
- Question: How is DNA repair altered in microgravity compared to on Earth?
  - A controlled source of radiation is desired
  - Funded by NASA Fundamental Space Biology Program

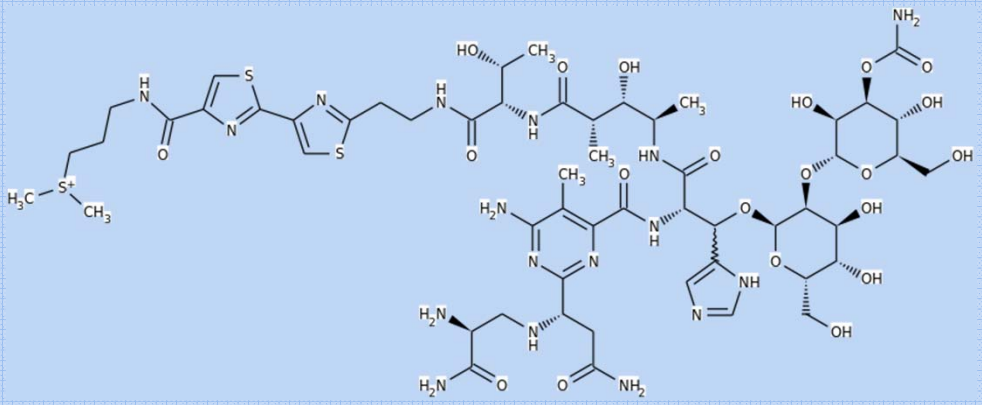
The Space Radiation Environment

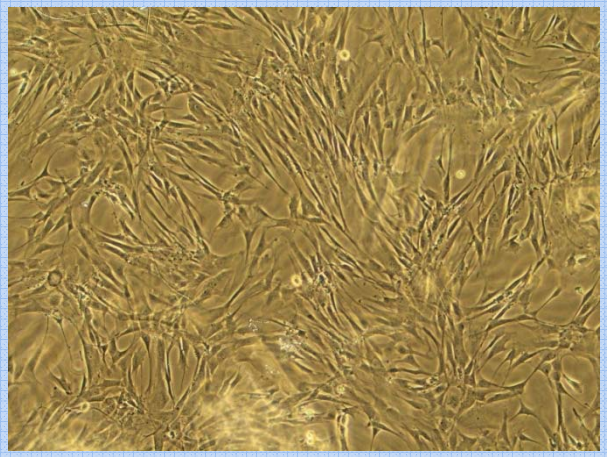


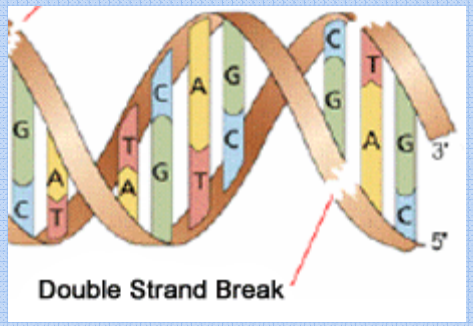
Space radiation : Energetic charged particles, high-LET (linear energy transfer)

Bleomycin

- Chemotherapy antibiotic
- Able to induce DNA double-strand breaks (DSBs) – radiomimetic
- Controlled source of DNA damage

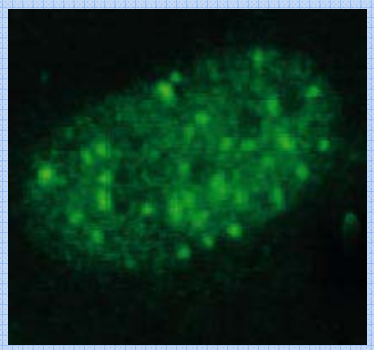
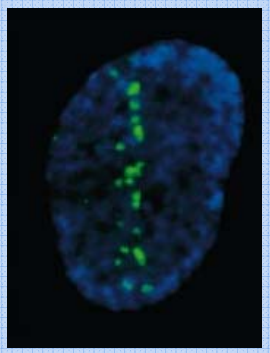
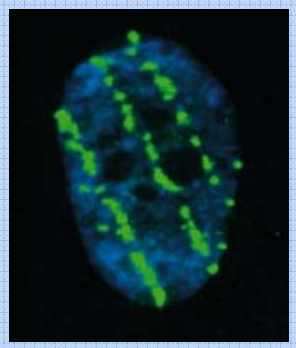
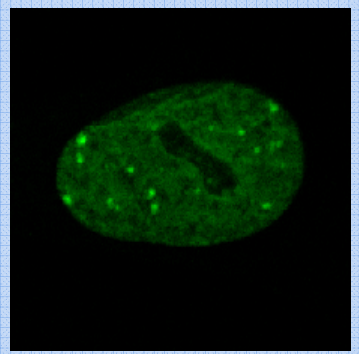






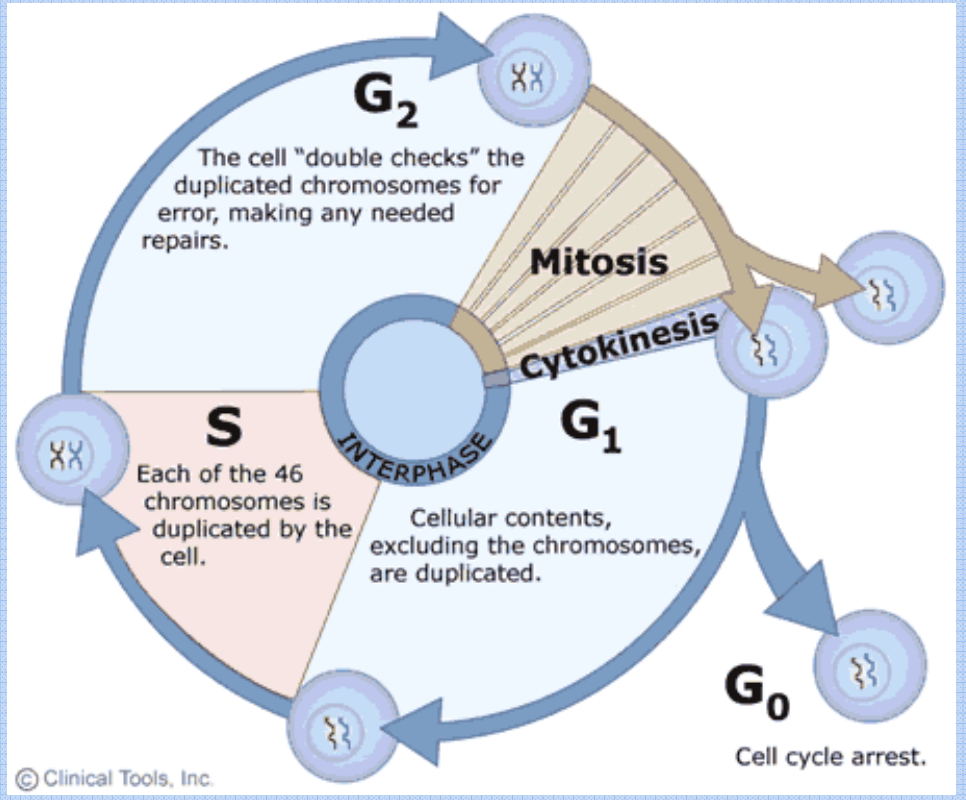
γ-H2AX Assay and 53BP1 Assay

- Quantification of DSBs
  - Creation of foci as a result of DSBs from ionizing radiation
- H2AX – histone component variant
  - Phosphorylation of serine residue indicates DSB
  - Previous study tested high concentrations of bleomycin (0-80 μg/mL) and found little dose response (Venkatachalam et. al, 2008)
- 53BP1(p53-binding protein 1): protein involved in DNA repair and tumor suppression
  - Indirect quantification of DSBs

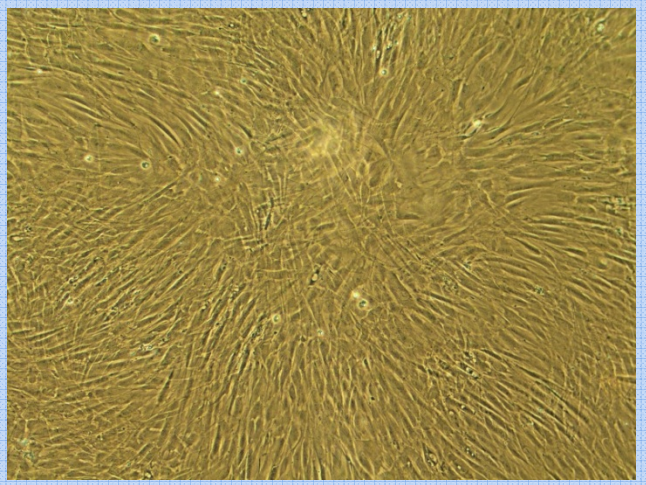
Cell Cycle Markers

- Determine if level of DNA damage correlates to stages of cell cycle
- Cyclin D1 and E: mid-to-late G_1 phase
- Cyclin A: high expression S phase, low expression in early G_2 phase
- Cyclin B1: G_2 phase. Expressed in nucleus in early M phase
- Phosphohistone H3: M phase
- Identify cell cycle stage that is best to study DNA damage and nuclear foci production



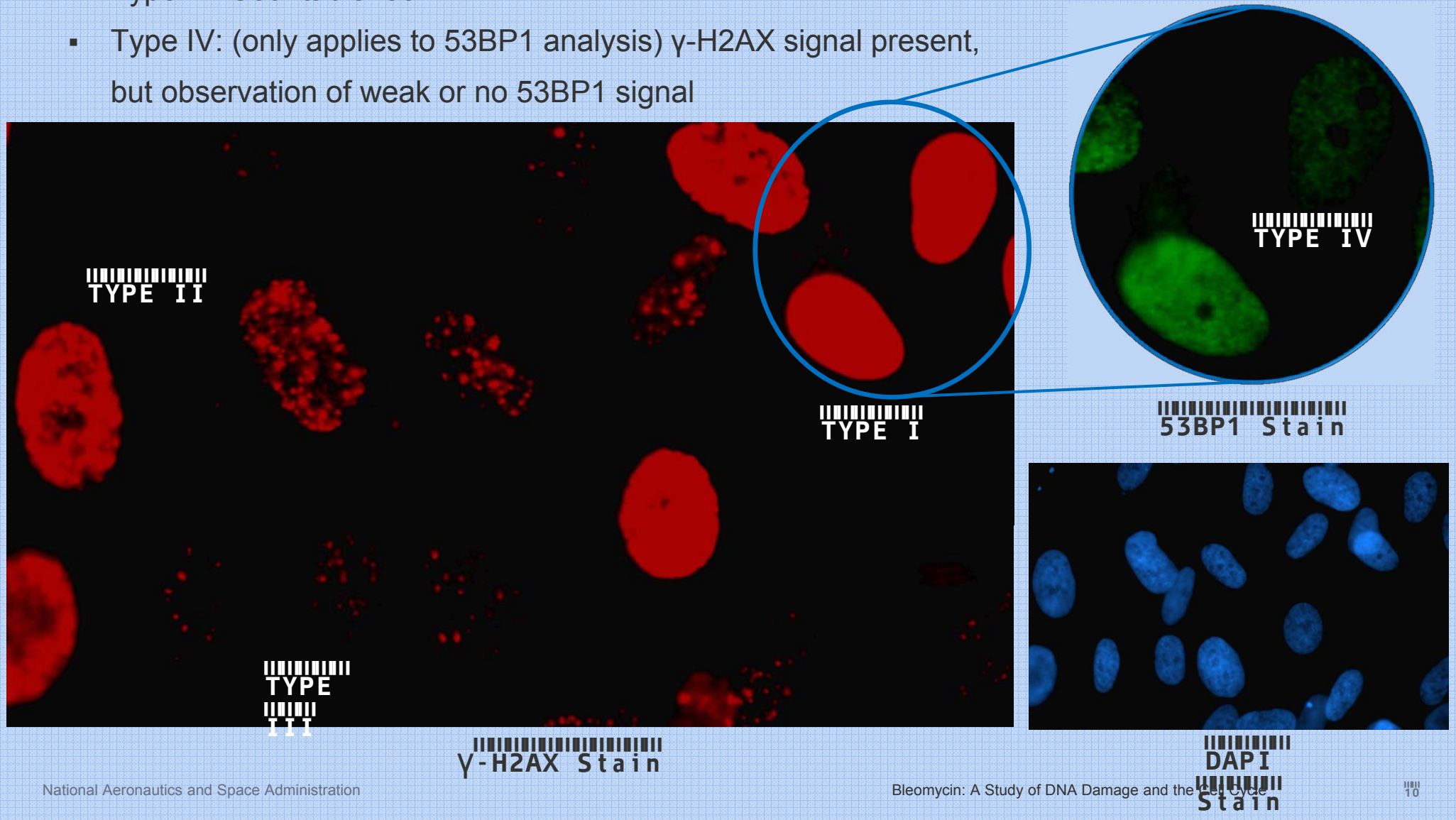
Bleomycin Treatment

- Growth of fibroblast cells in six 8-well tissue culture slides
- Once cells were 90% confluent, bleomycin treatment was performed
  - 0, 0.1, 1, and 10 µg/mL for 3 hours
- Cells fixed with 4% paraformaldehyde
- Immunofluorescence staining:
  - 1: γ-H2AX and 53BP1
  - 2: γ-H2AX and Cyclin A (S phase)
  - 3: γ-H2AX and Cyclin B1 (G_2 phase)
  - 4: γ-H2AX and Phosphohistone H3 (M Phase)
  - 5: γ-H2AX and Cyclin D1 (G_1 phase)
  - 6: 53BP1 and Cyclin D1 (G_1 phase)



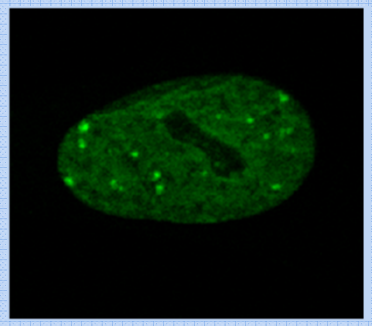
Results: γ-H2AX and 53BP1 Classification

- Classification of cell types:
  - Type I: Bright, pannuclear stain
  - Type II: Uncountable bright foci or near pannuclear stain
  - Type III: Countable foci
  - Type IV: (only applies to 53BP1 analysis) γ-H2AX signal present, but observation of weak or no 53BP1 signal

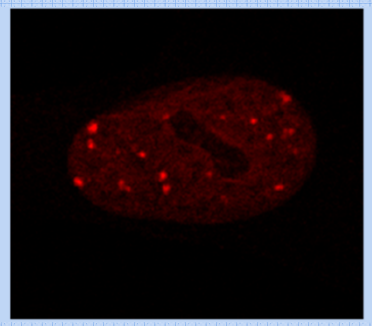


Results: γ-H2AX and 53BP1 Foci Counts

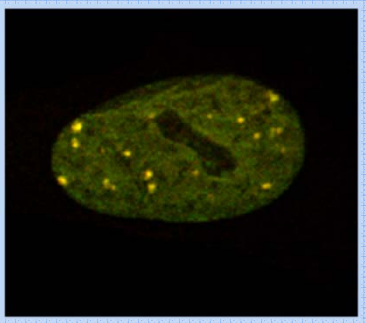
- Distribution of foci counts: unimodal
- One type for all cell with countable foci



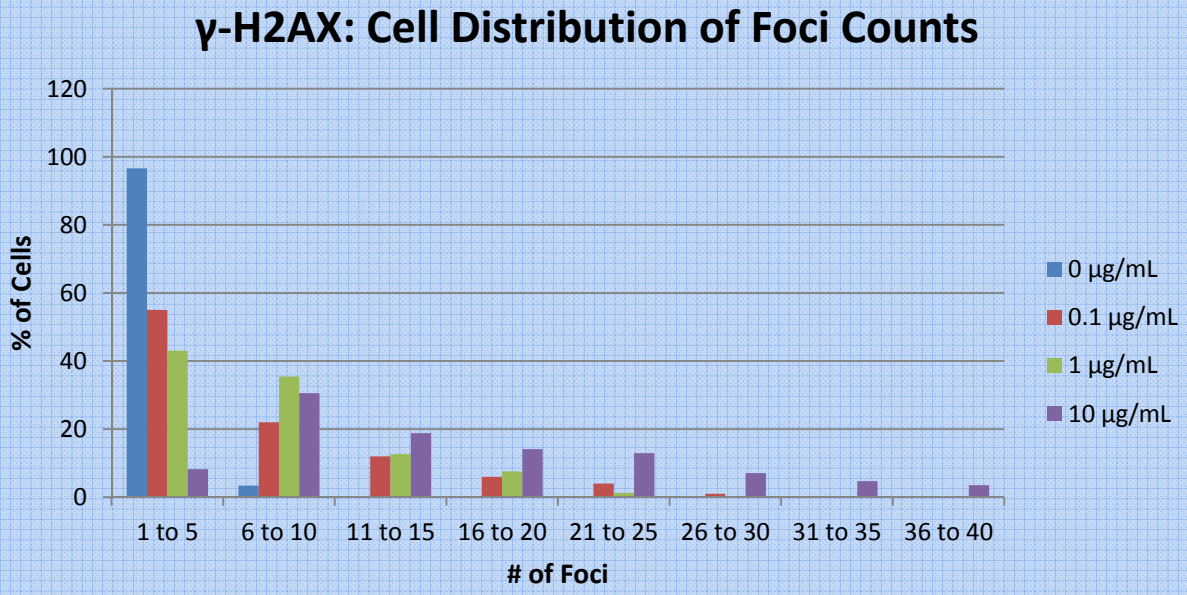
53BP1

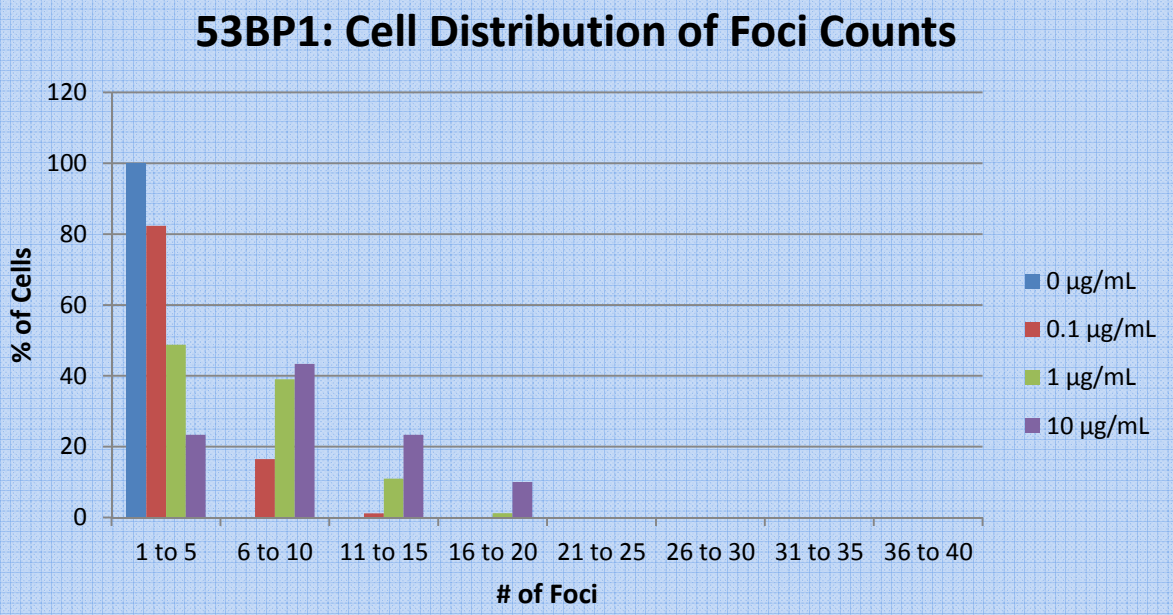


γ-H2AX



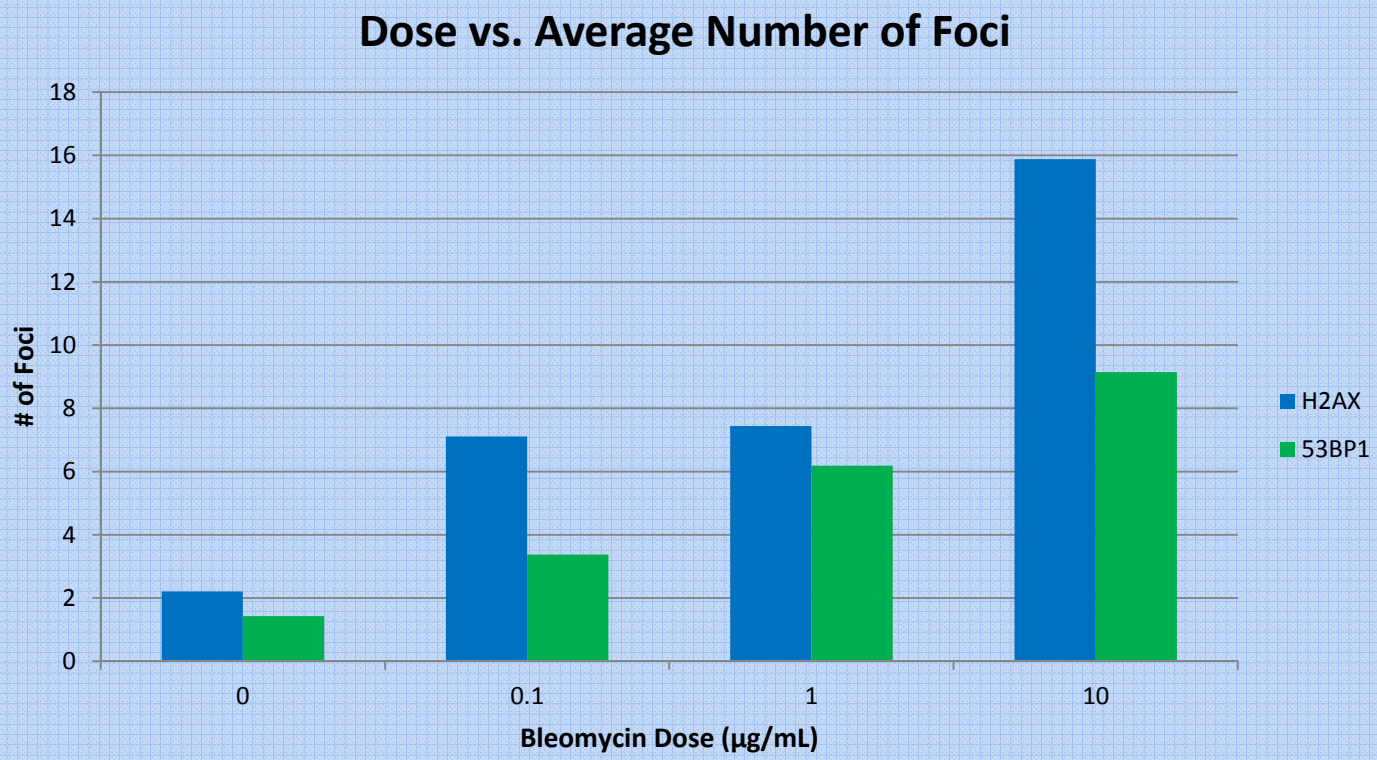
53BP1 and γ-H2AX





Results: γ-H2AX and 53BP1 Foci Counts

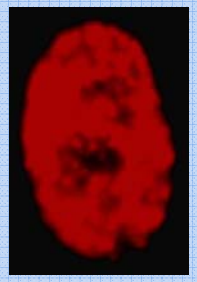
- Slight dose response for 53BP1, but not for γ-H2AX
- All significant changes except between 0.1 and 1 for γ-H2AX



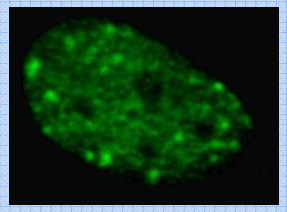
Results: γ-H2AX and 53BP1 Foci Counts

- As dose increases, Type I and II for γ-H2AX increase and Type I for 53BP1 increases

Type I

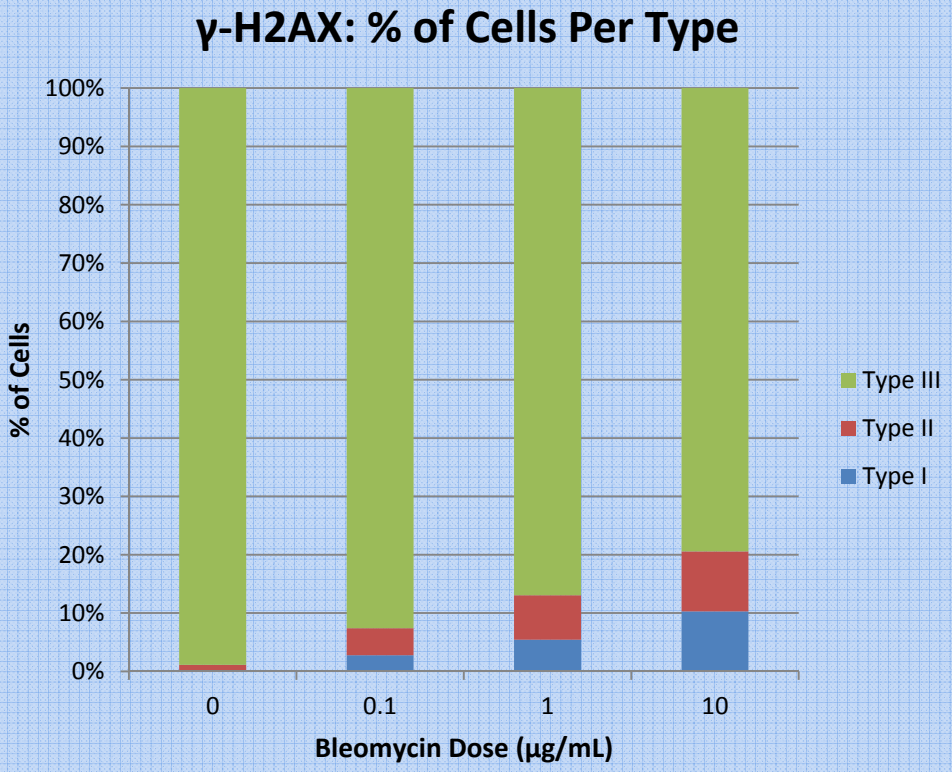


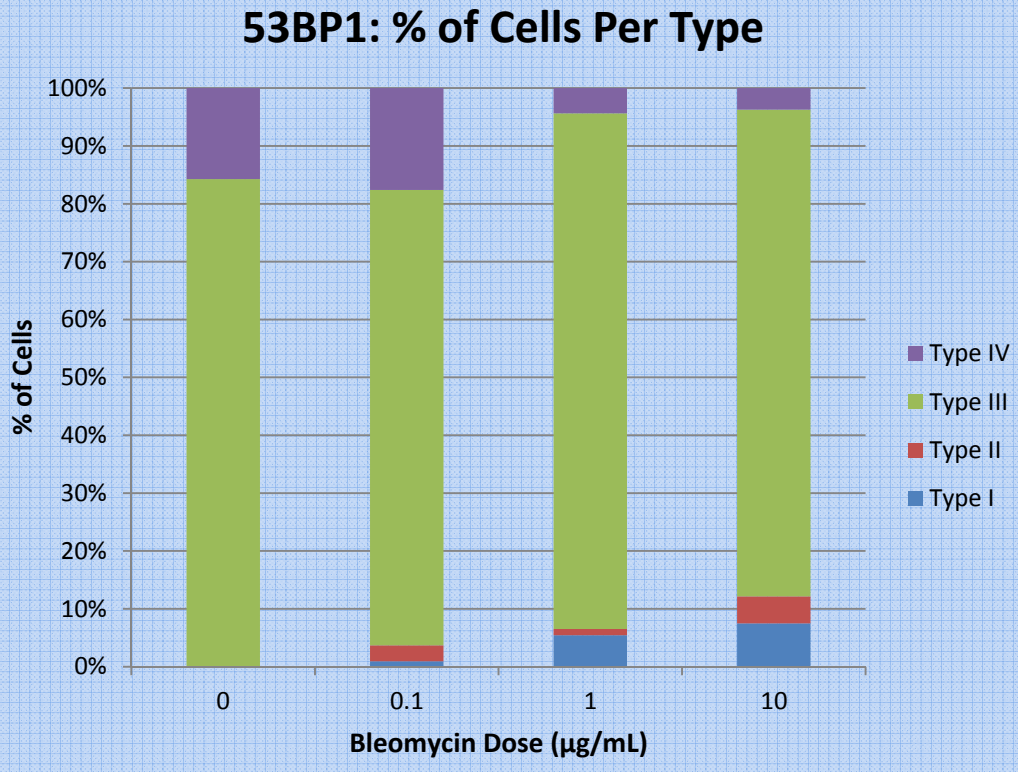
Type II



Type III

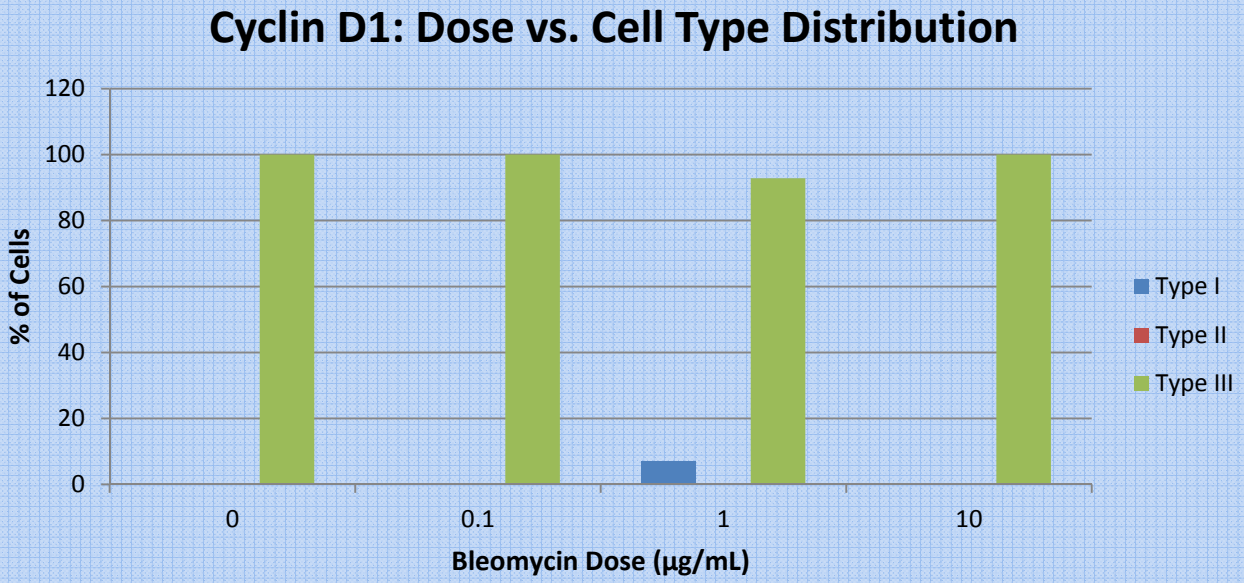
Type IV

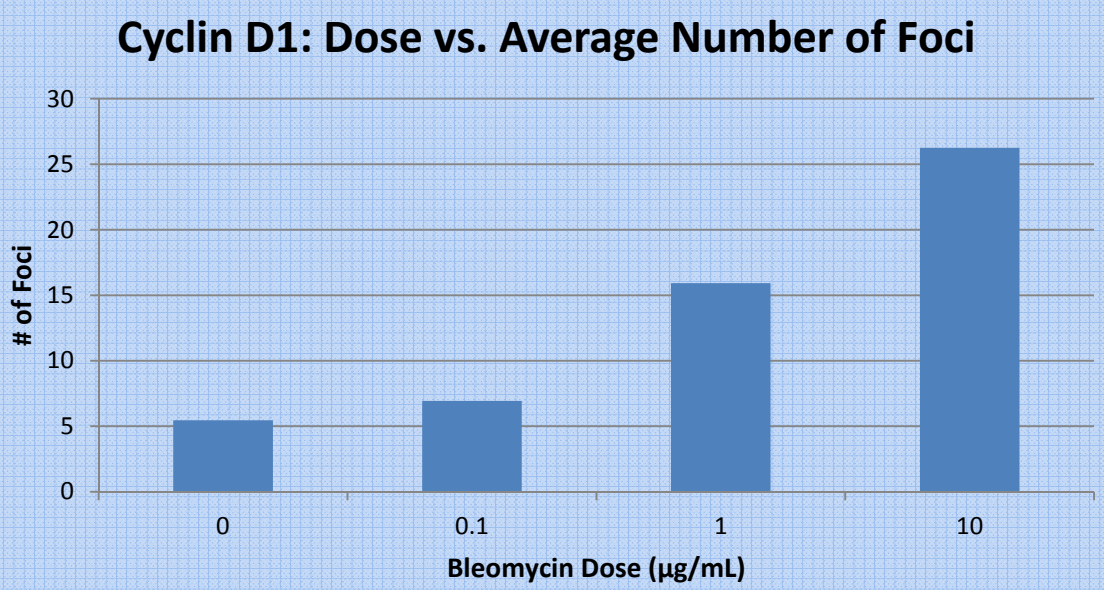


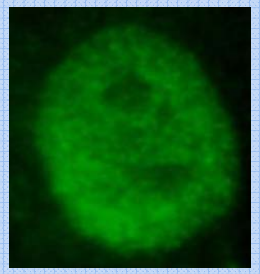


Results: Cell Cycle Study

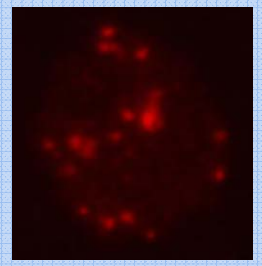
- Cyclin D1 : G_1 phase
- Almost all cells are Type III
- Dose response observed for γ-H2AX foci



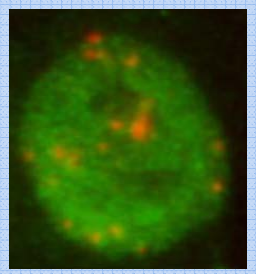




Cyclin D1



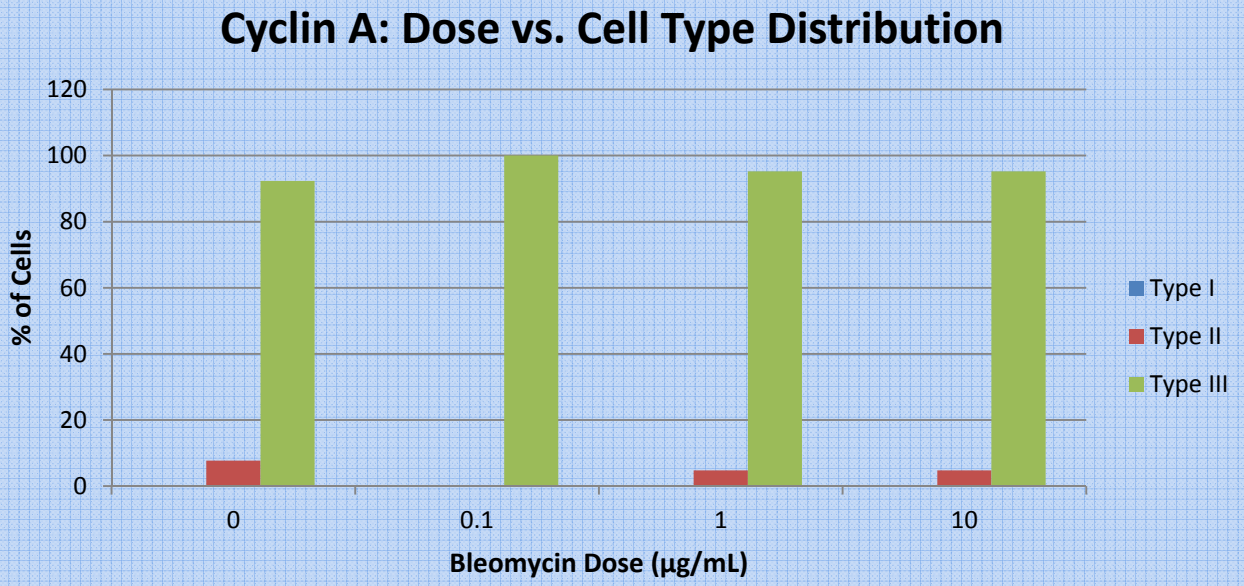
γ-H2AX

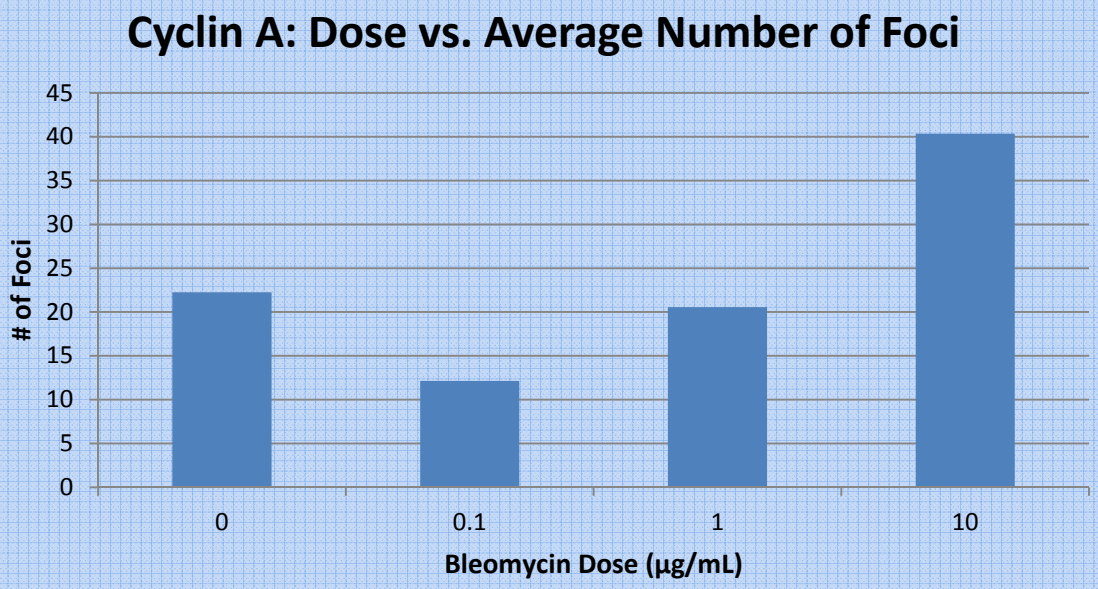


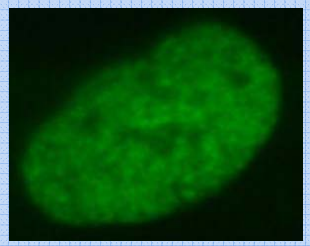
Cyclin D1 and γ-H2AX

Results: Cell Cycle Study

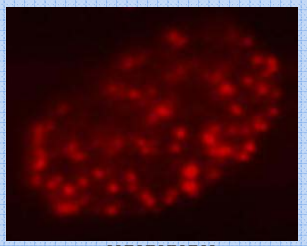
- Cyclin A : S phase
- Most cells are Type III
- No dose response observed, due mainly to the high background in the control



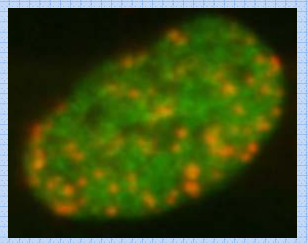




Cyclin A



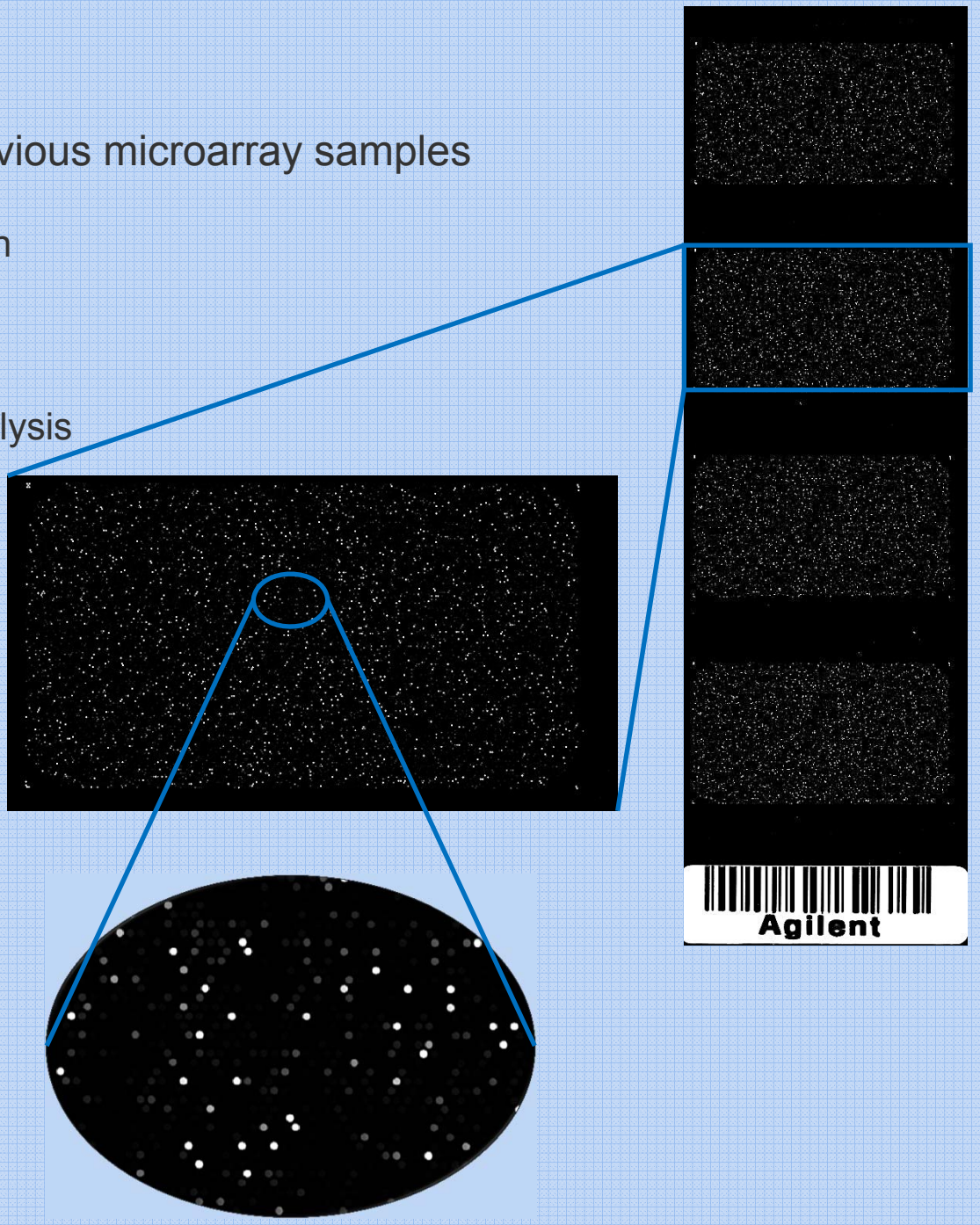
γ-H2AX



Cyclin A and γ-H2AX

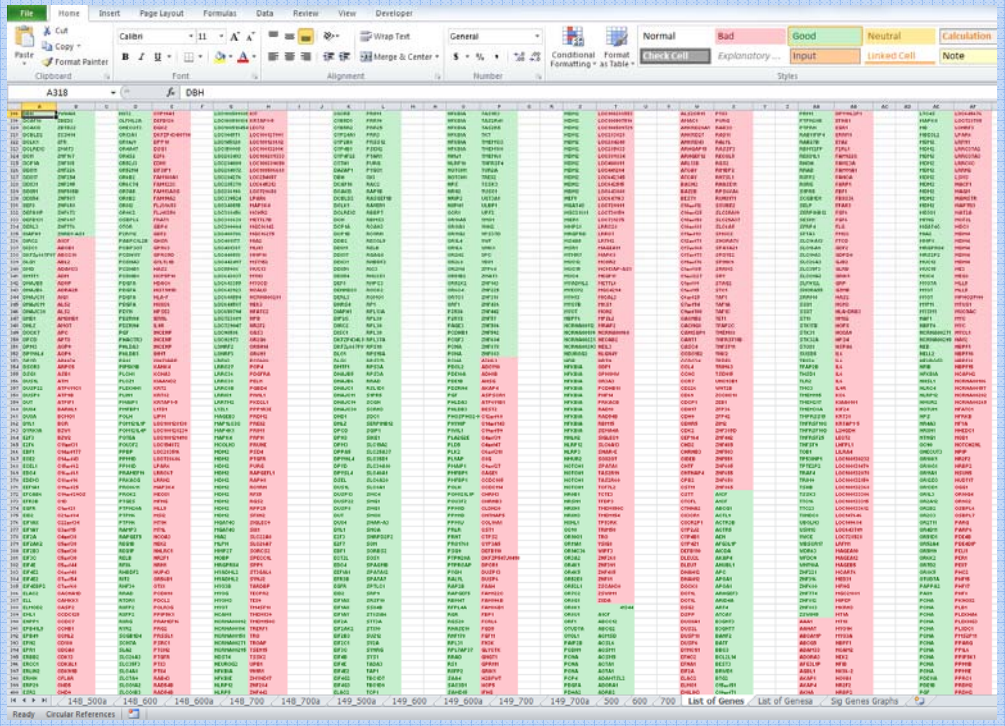
DNA Microarray

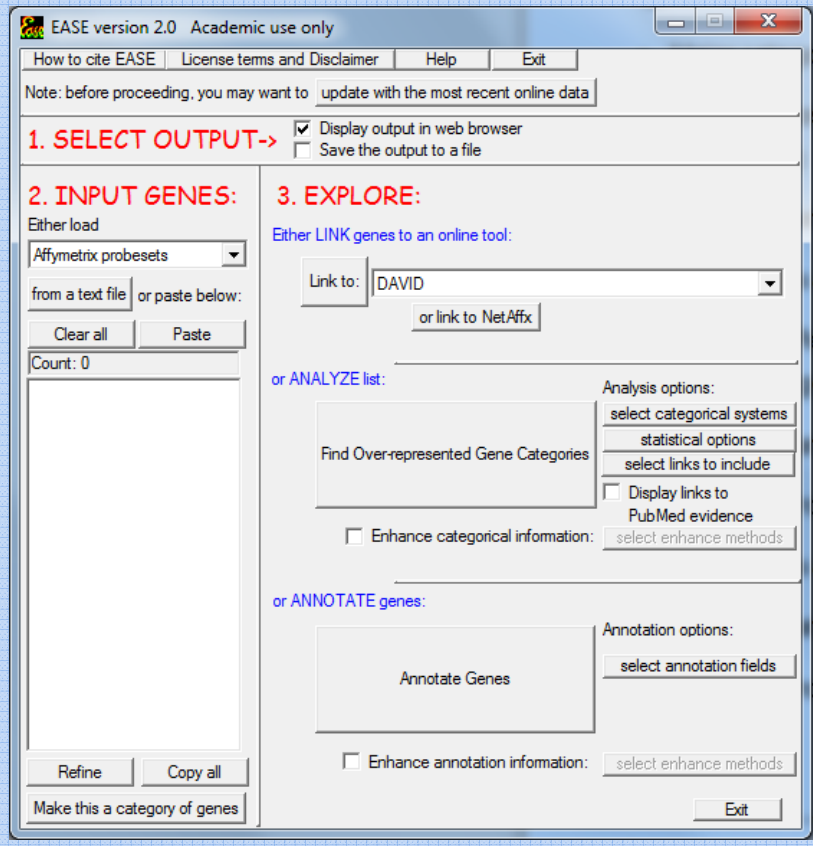
- Analyzed microarray data from previous microarray samples
  - GenePix4000B for data acquisition
  - GenePix Pro 7 for data retrieval
  - Microsoft Excel and EASE for analysis
  - 0, 0.1, 1, and 10 (µg/mL)
  - Up-regulated or down-regulated
- DNA microarray system:
  - Human GE 4x44K v2 Microarray
  - ~44,000 transcripts
  - Target 27,958 Entrez Gene RNAs



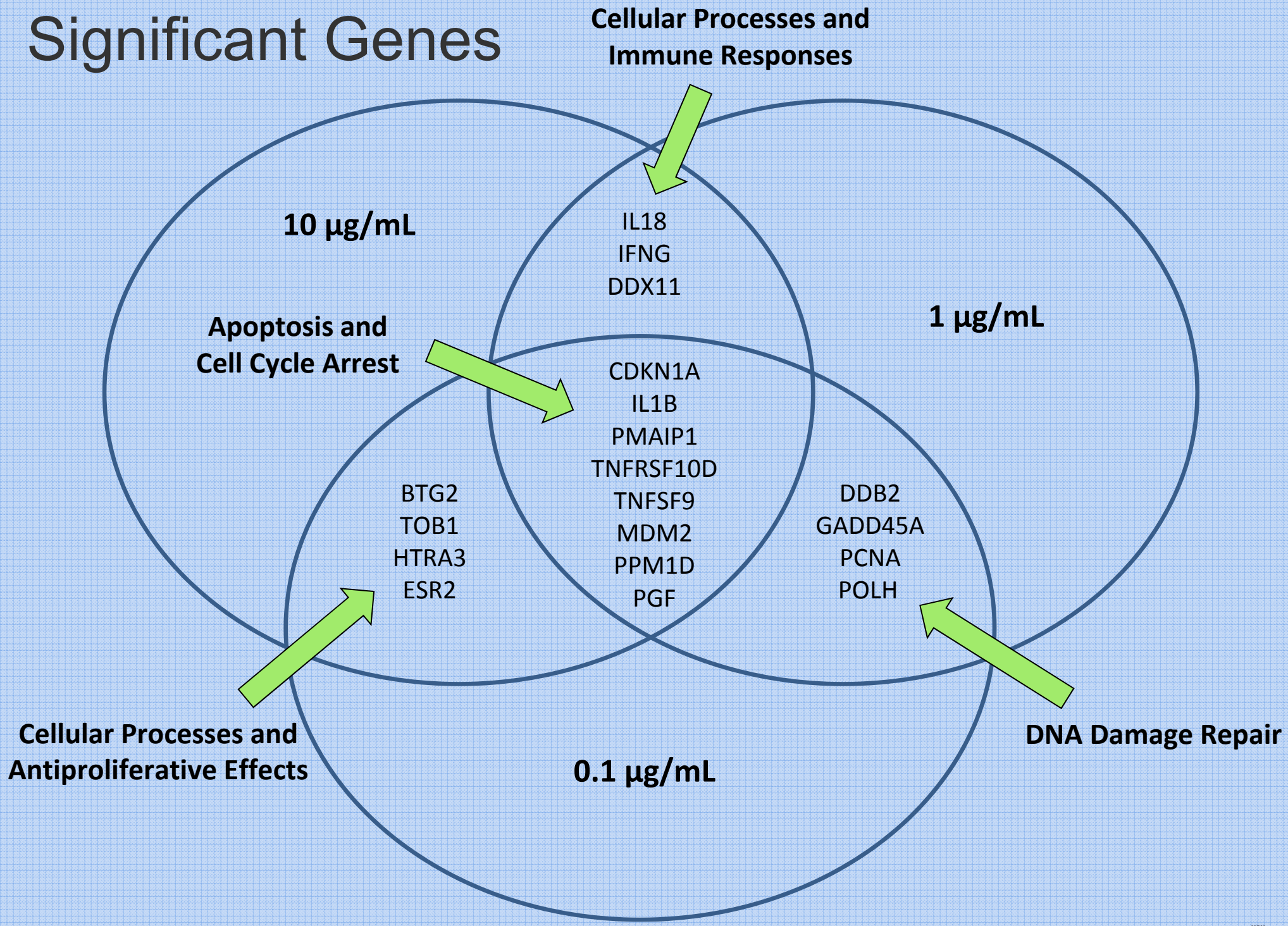
Significant Genes

- Major categories affected (all concentrations):
  - Apoptosis (cell death, programmed cell death, death)
  - Regulation of Cell Proliferation
  - DNA Repair or Response to DNA Damage/Endogenous Stimulus



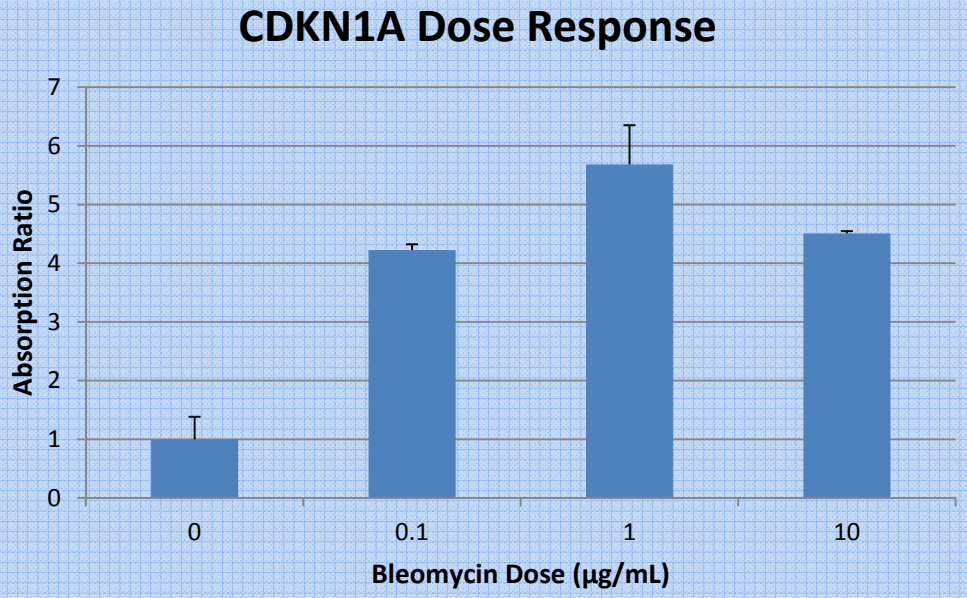


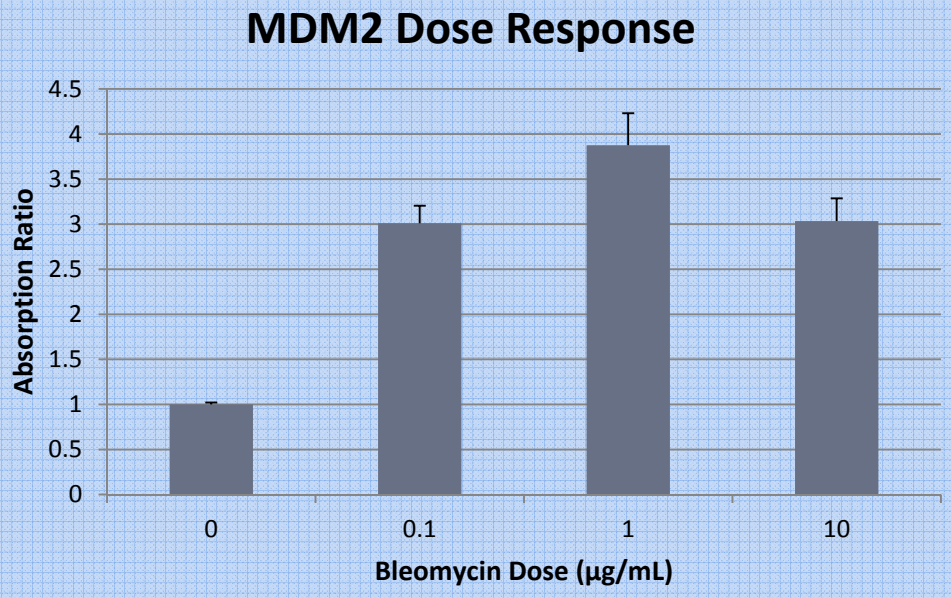
Significant Genes



Significant Genes

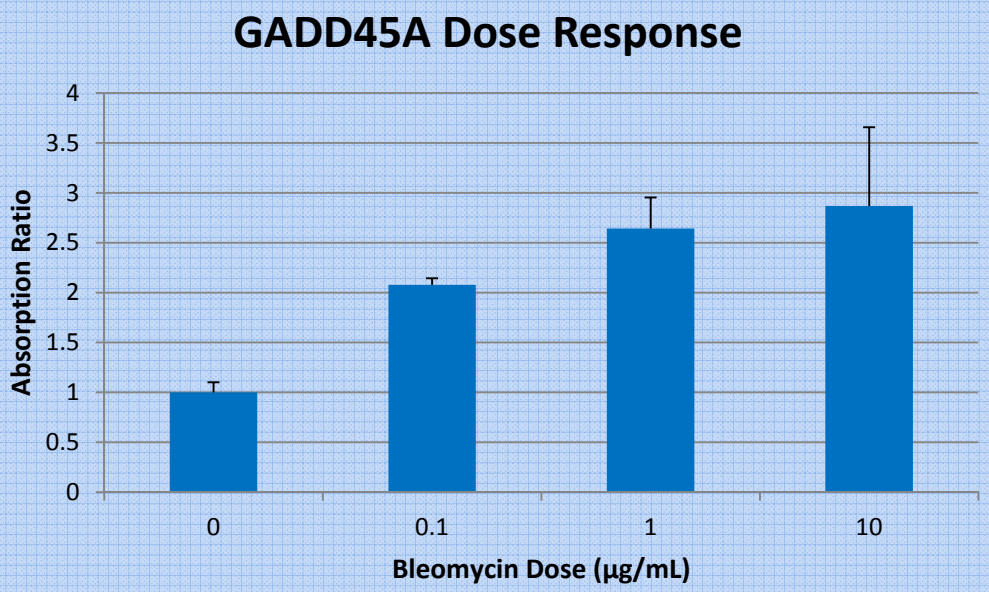
- CDKN1A
  - Regulates G1 checkpoint by inhibiting activity of cyclin-CDK4 and cyclin-CDK2
  - Involved in p53 pathway in response to DNA damage
- MDM2
  - Part of autoregulatory negative feedback loop of the p53 pathway
  - Component in complex which links growth factor and DNA damage response pathways

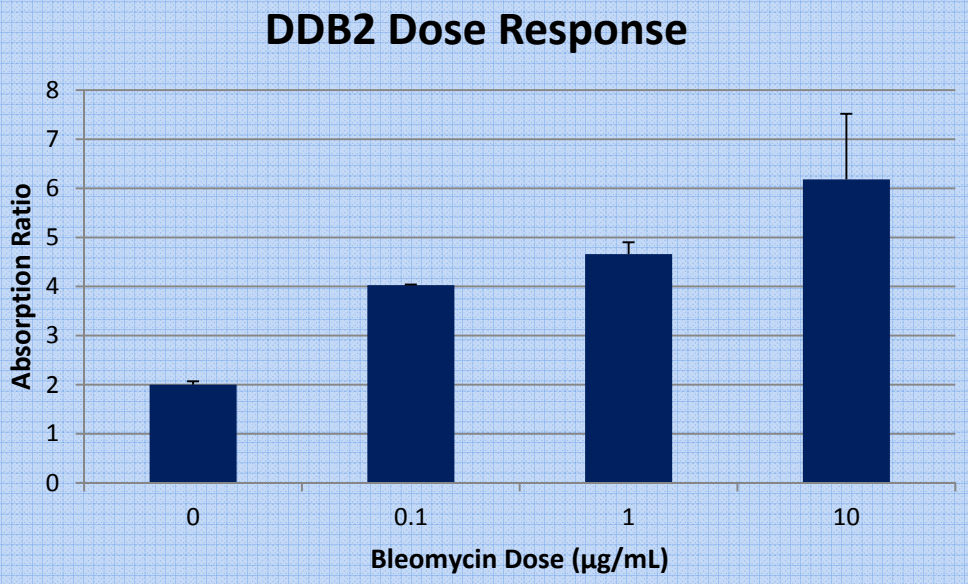




Significant Genes

- GADD45A
  - Increase in transcription usually follows environmental stresses and will lead to activation of the p38/JNK pathway
  - Activation of this gene due to DNA damage is mediated by p53 and other pathways
- DDB2
  - Required for DNA repair (especially UV-induced DNA repair)
  - Ubiquitination of histones H3 and H4 which aids cellular response to DNA damage





Conclusions

- 53BP1 and γ-H2AX are typically used markers for DNA DSBs caused by ionizing radiation, as indicated by colocalization of nuclear foci. However, similar to UV radiation, cells with bleomycin-induced DNA damage generally have more γ-H2AX foci than 53BP1 foci.
- 53BP1 and γ-H2AX have both shown dose dependent characteristics in identifying damange-induced foci. 53BP1 may be a better for DSBs because it showed a dose response more consistently.
- In terms of the cell cycle, cells in G_1 phase tend to have fewer damage-induced foci than cells in S phase.
- In general, an increase in number of genes involved in apoptosis, gene repair, and regulation of cellular processes is seen as the dose of bleomycin increases.
- Based on these data, we established that a concentration of 1 μg/mL should be used for the flight study. In addition, cells in the G_1 phase should be analyzed for comparison to the ground study results.

Future Directions

- Conduct further gene studies based on DNA microarray data including pathway analysis and cluster analysis.
- Test bleomycin treatment for different lengths of time and allow specific time intervals for DNA repair to occur following treatment.
- Complete and analyze remaining cell cycle stains in order to conclude that ground and flight analysis should be completed based on G_1 phase cells.
- Become a practicing physician and aid the space industry in finding countermeasures to mitigate the effects of spaceflight on humans.

Acknowledgements

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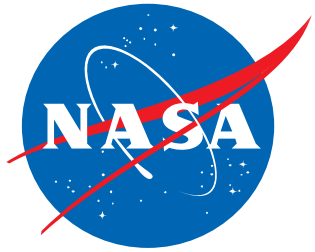
NASA