Quantifying Protein Mobility in Living Drosophila Embryos Using Fluorescence Recovery After Photobleaching (FRAP)

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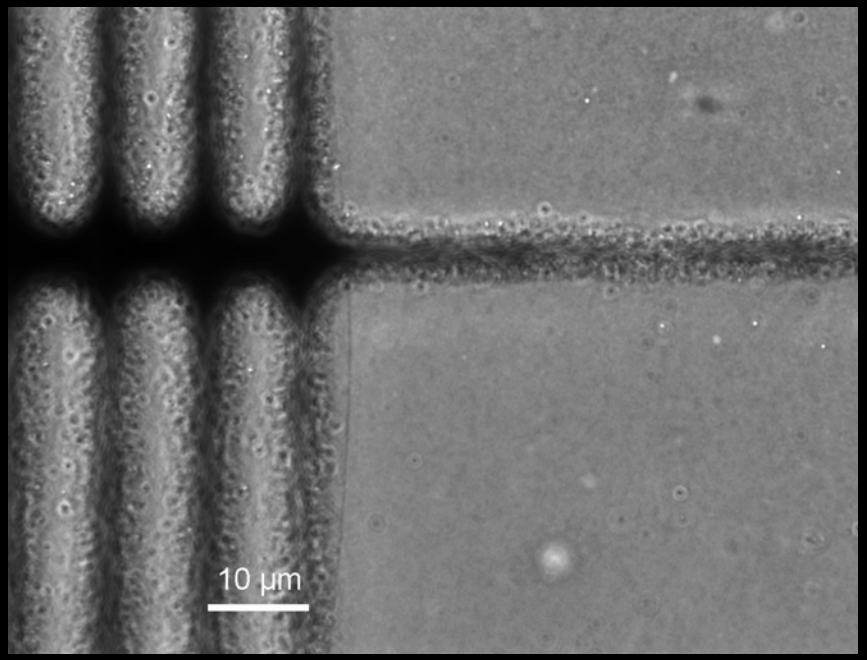
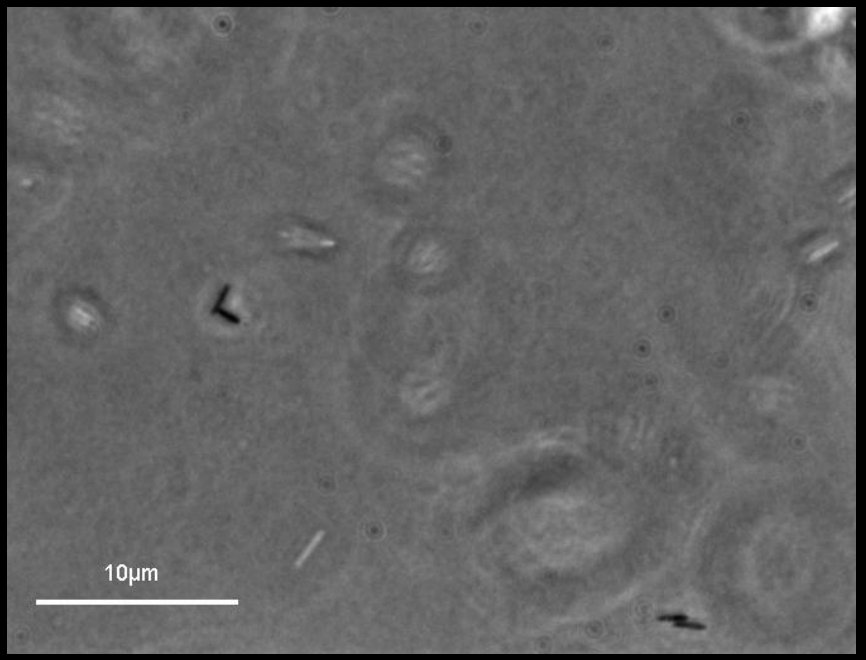
Overview

- Days 1 and 2
- Drosophila Introduction
- Protocol
- Results
- Conclusions
- Future Propositions
- References
- Acknowledgements

Fluorescence Microscopy



Calibration



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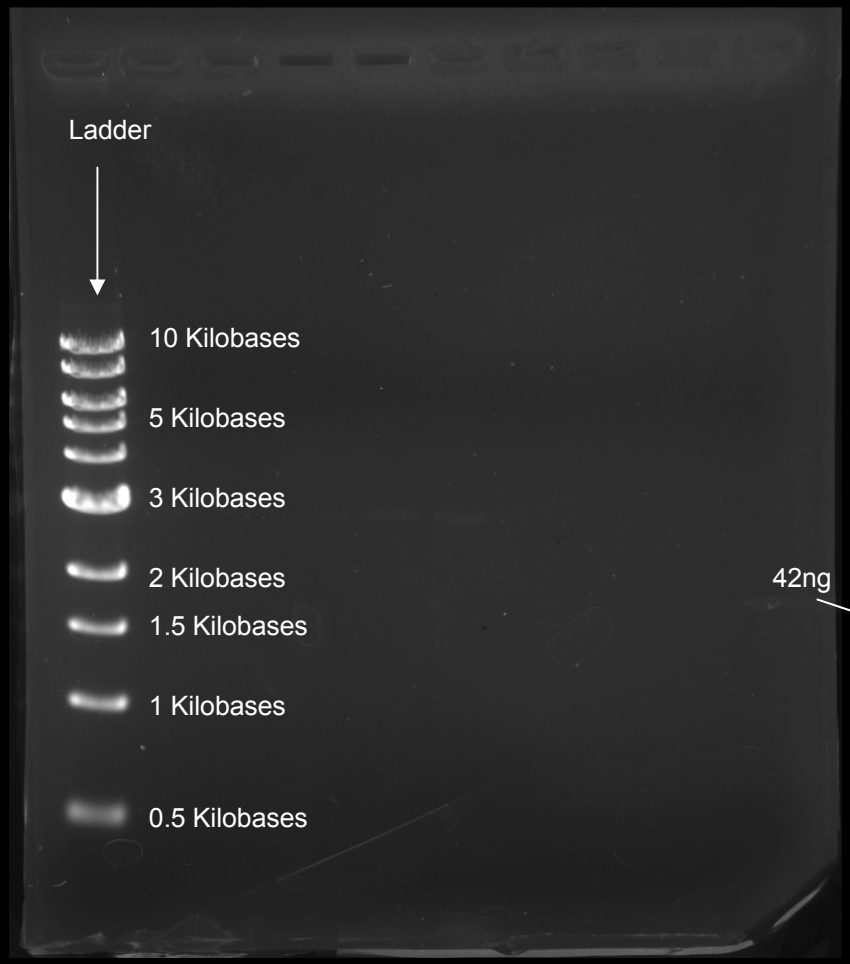
Gel Electrophoresis



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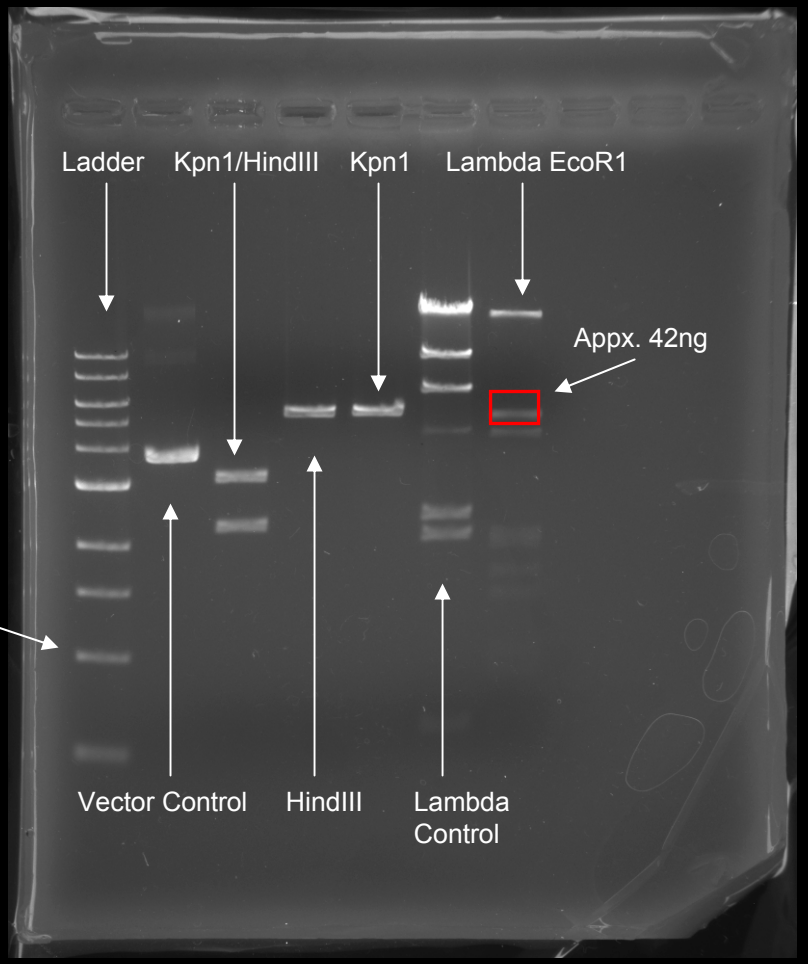


Ladder Info from:
http://www.neb.com/nebecomm/products/productN3232.asp

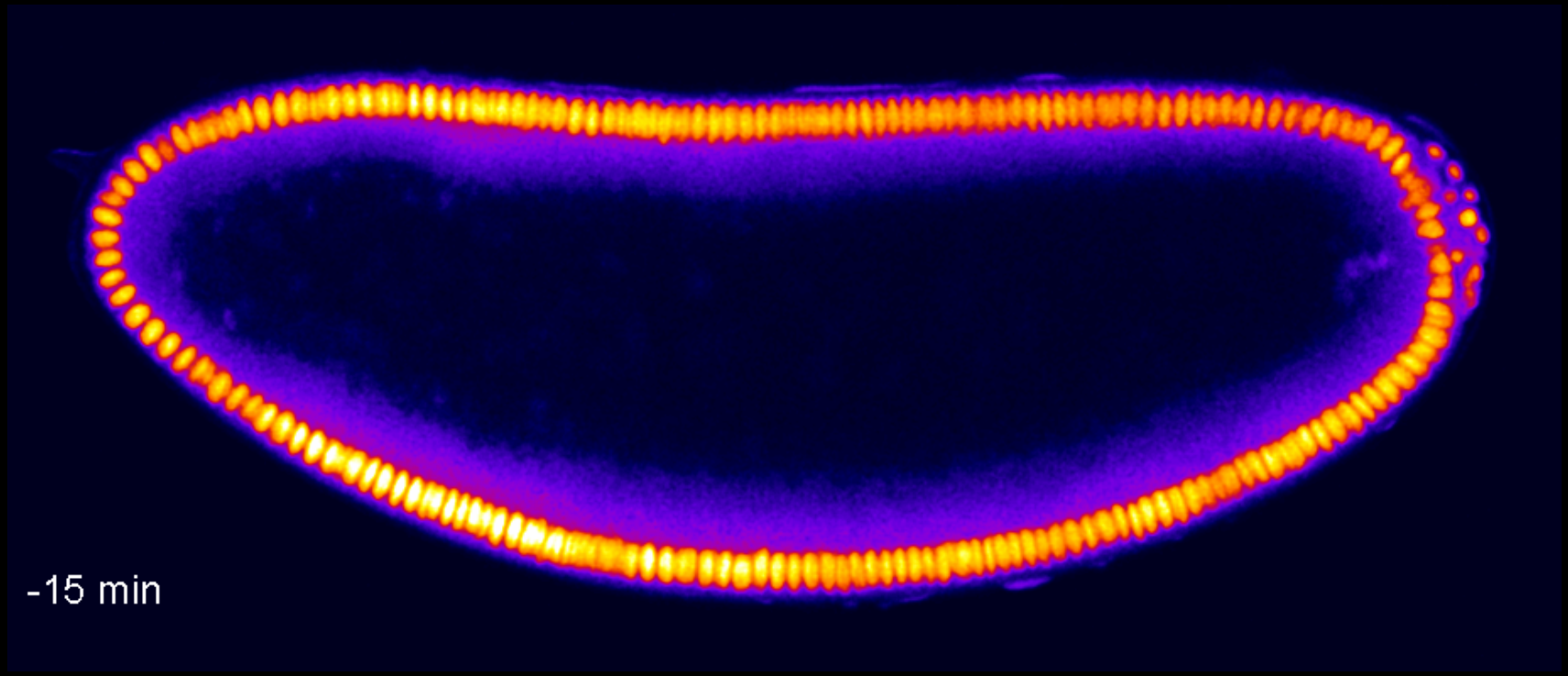
FRAP Overview

- Excite Green Fluorescent Protein (GFP) fluorophore with low energy 488nm Argon laser
  - Detect emission of a lower wavelength signal
- Maximum laser power is used to photobleach GFP for analysis
- Returning to low energy emission allows quantification of the remaining unbleached GFP-coupled proteins

Drosophila Embryos

- FRAP assay performed on two transgenic types:
  - Wild-type embryos ubiquitously expressing histone (H2A-GFP)
  - Wild-type embryos ubiquitously expressing nuclear localization signal (NLS-GFP)

Nuclear Labeling of NLS-GFP



Supatto, W et al. *PNAS* 2005

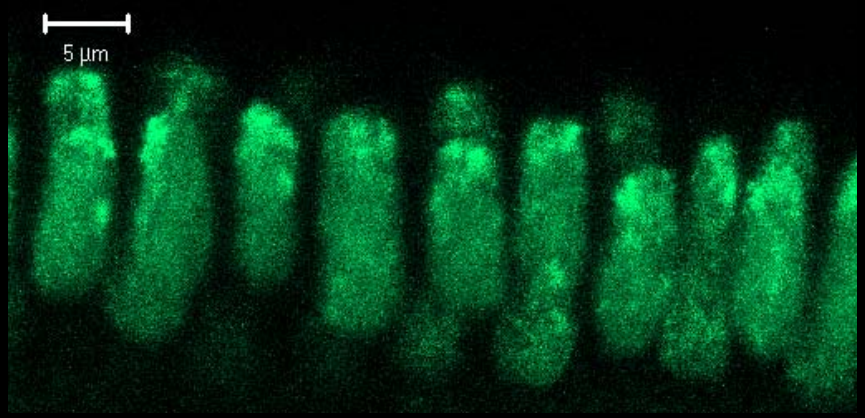
Protocol

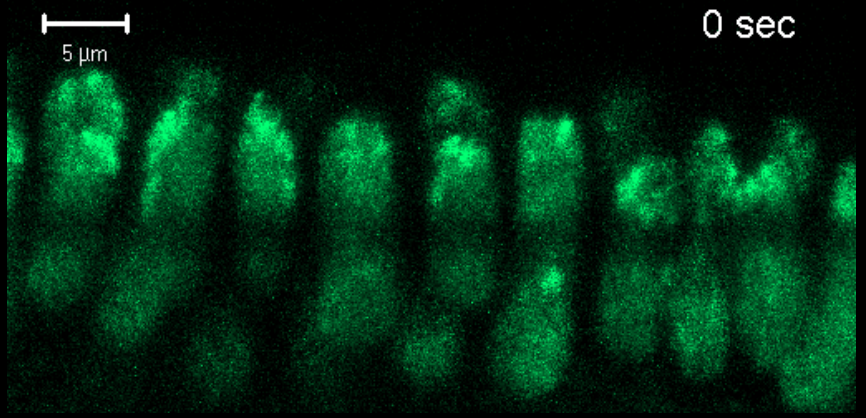
- Embryo Preparation
  - De-chorionated and placed on microscope slide after 14 developmental cycles (2.5 hours)
  - After isolation, the nuclei remain as a single epithelial layer at the edge of the yolk for approximately 30-40 minutes
    - Gastrulation occurs after this stationary period

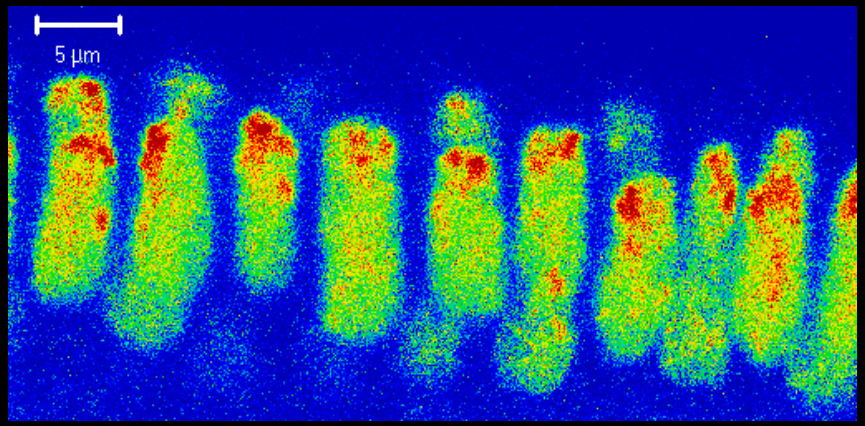
Protocol

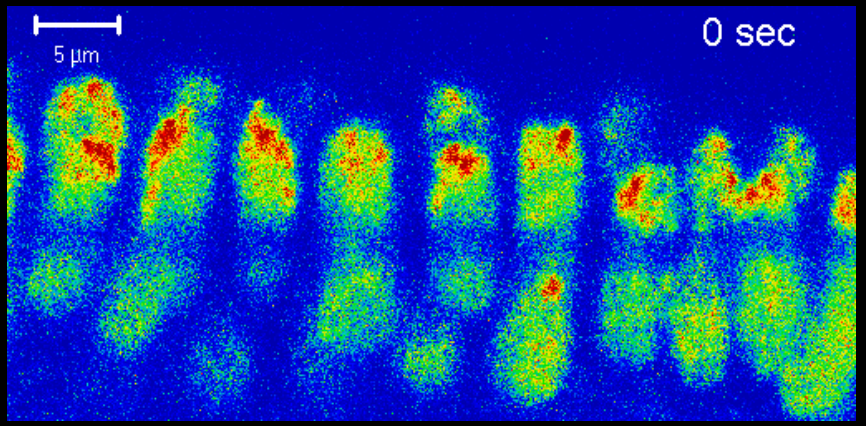
- Imaging
  - 63x, 0.9 NA water Acroplane Confocal Microscope objective
  - Seven 1.5 micron z-sections are taken between a nuclear layer to allow full planar photobleaching
- Bleaching and Time Lapse
  - Define a region of interest and bleach the middle section with 100% laser power
  - Take 7 z-sections every 10 seconds to quantify fluorescence recovery

H2A Experiment 1

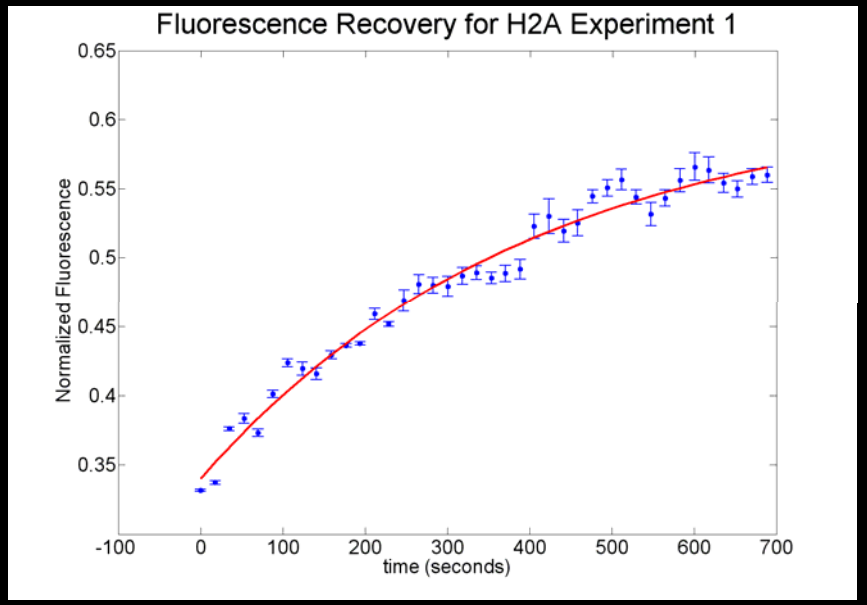


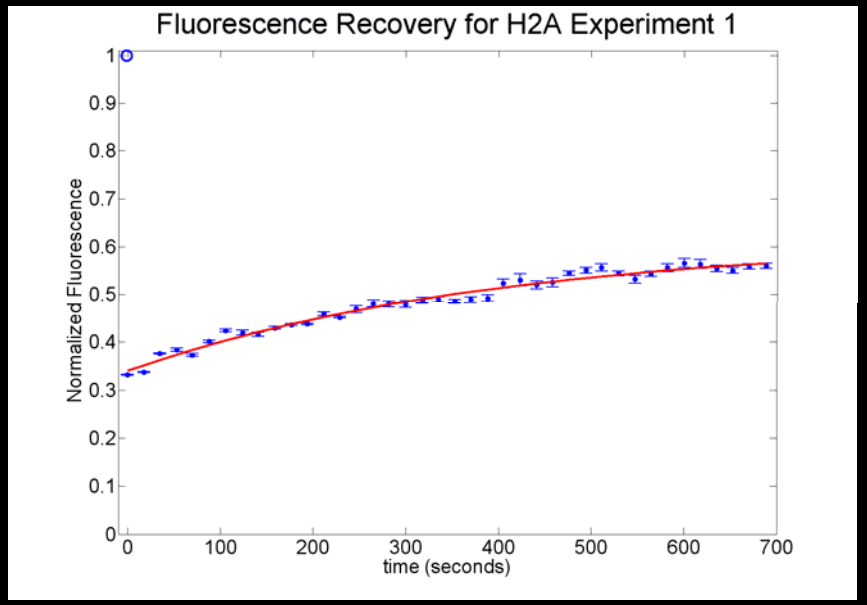






H2A Experiment 1



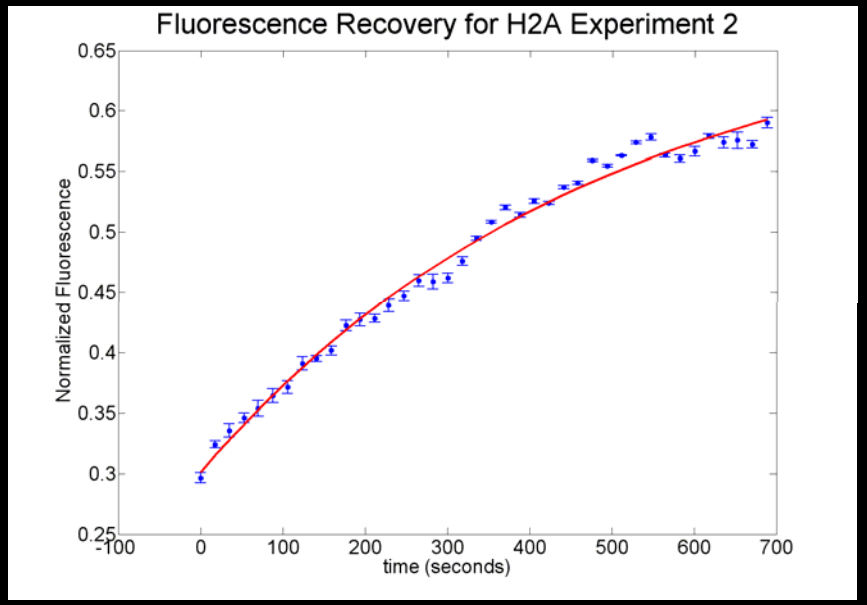


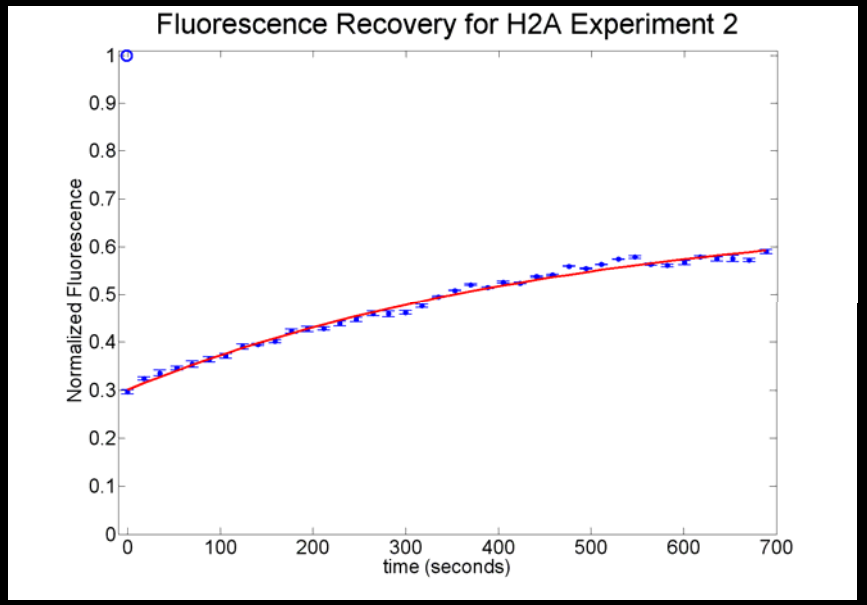
$$f(t) = (A - B)\,(1 - e^{-(t - t_0)/\tau}) + B$$

$$D_{eff} = \frac{\omega^2}{\tau}$$

Calculated D_{eff} was between 0.0094 and 0.0139 $\mu m^2/sec$

H2A Experiment 2



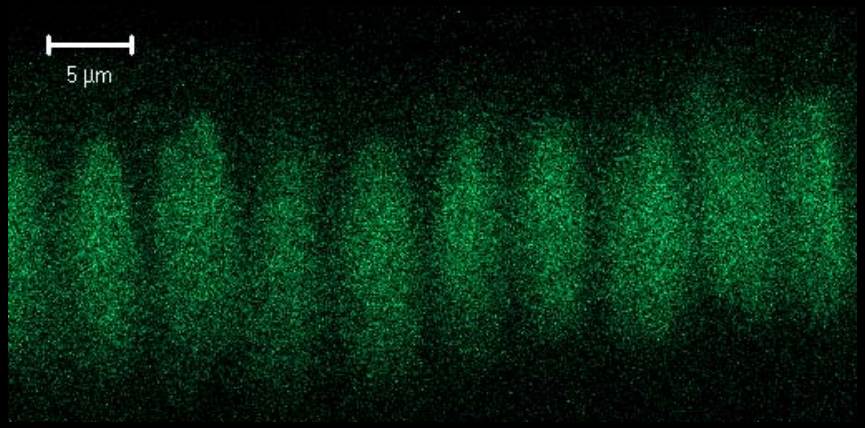


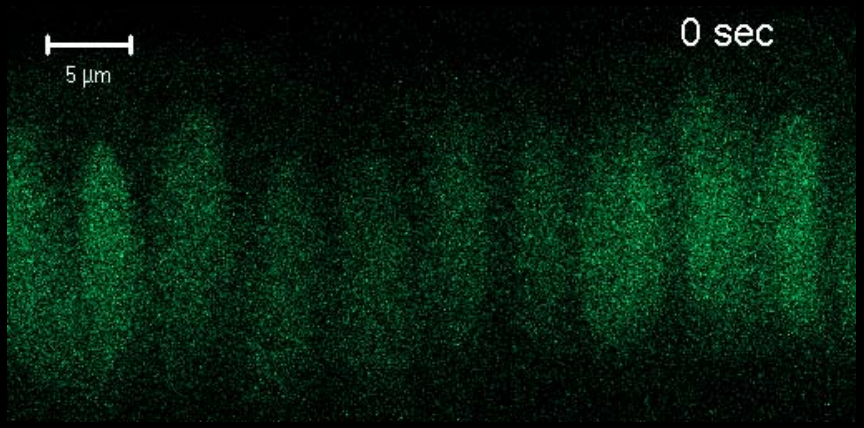
$$f(t) = (A - B)\,(1 - e^{-(t - t_0)/\tau}) + B$$

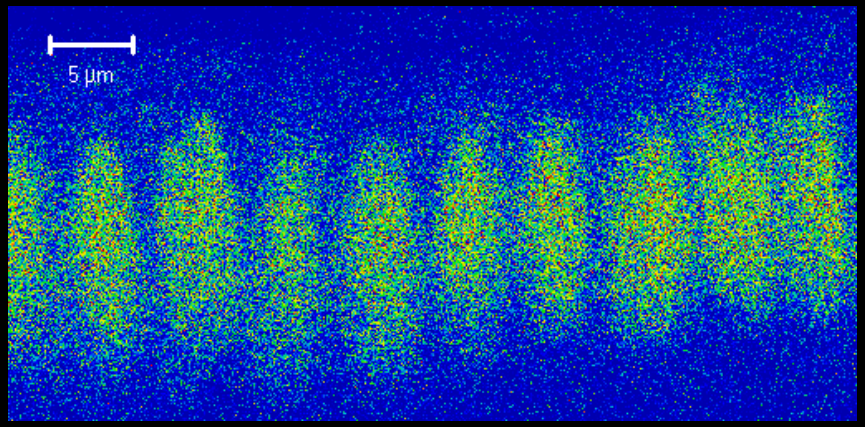
$$D_{eff} = \frac{\omega^2}{\tau}$$

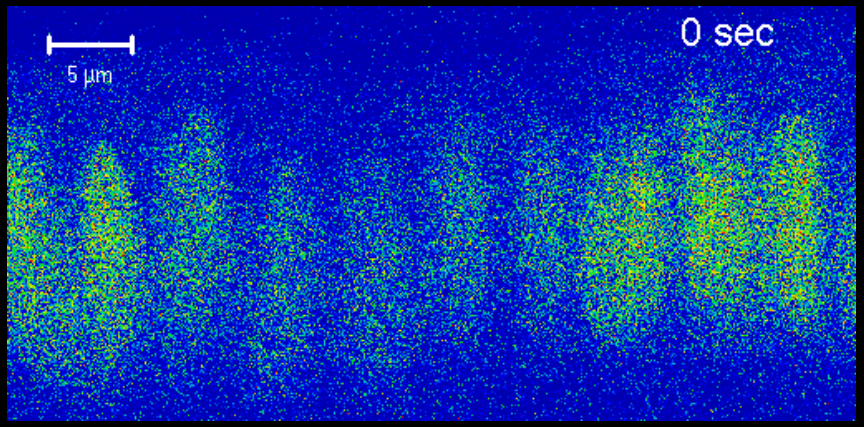
Calculated D_{eff} was between 0.0079 and 0.0117 $\mu m^2/sec$

NLS Experiment

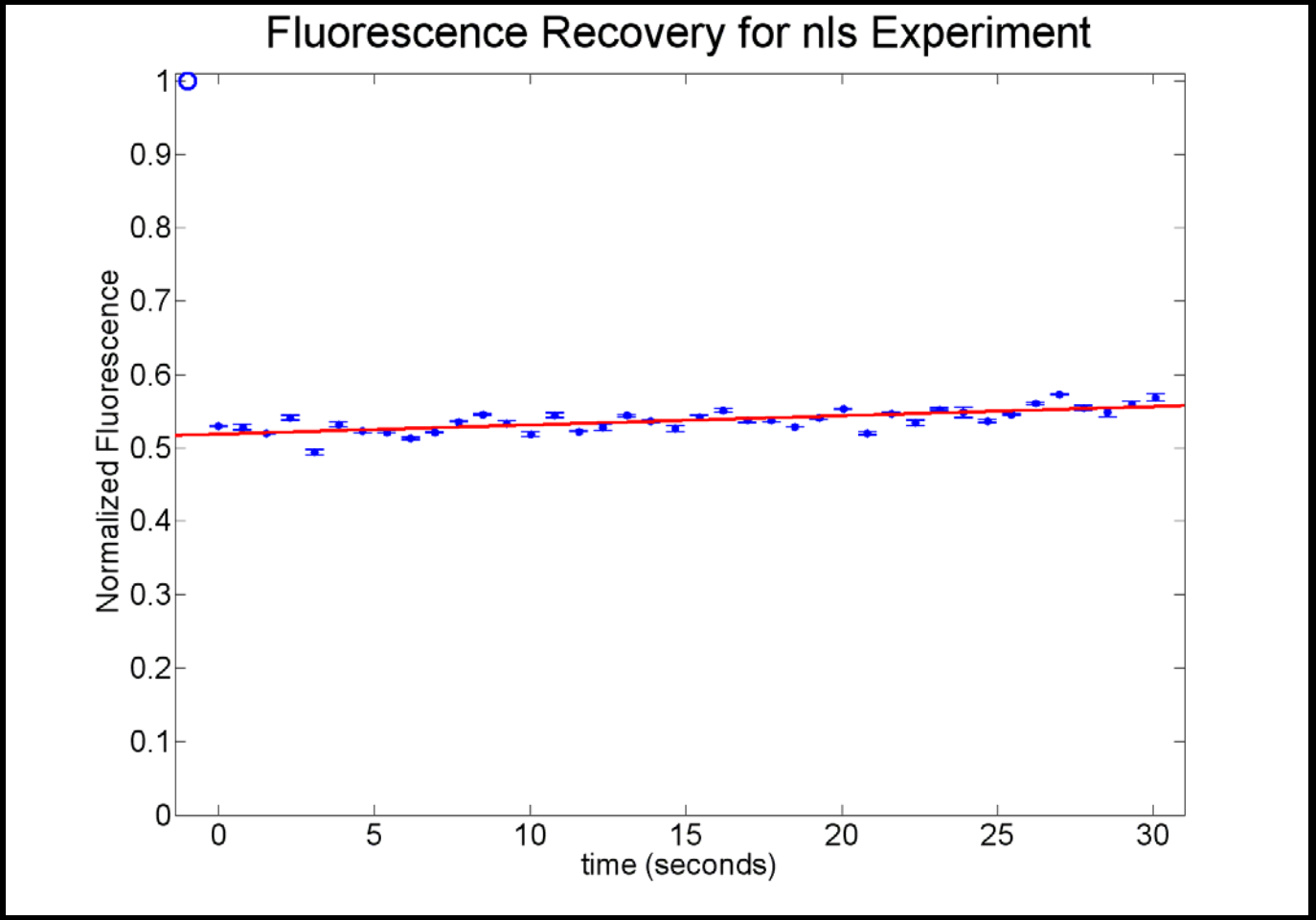






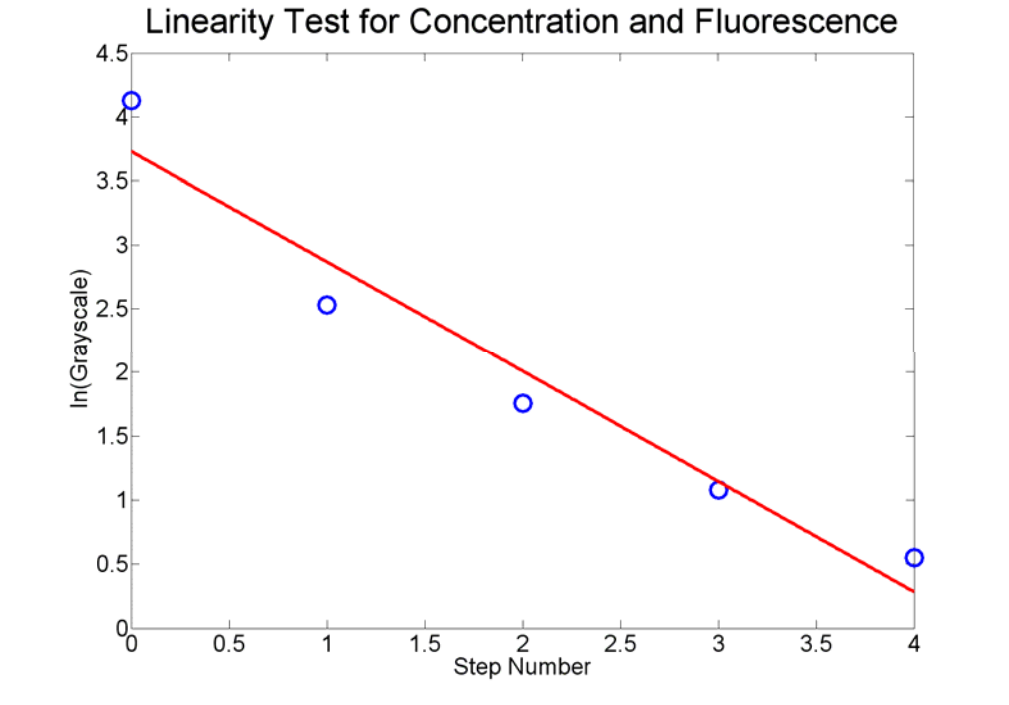


NLS Experiment



Slope was calculated to be approximately 0.0012 sec^{-1}

Control 1: Linearity



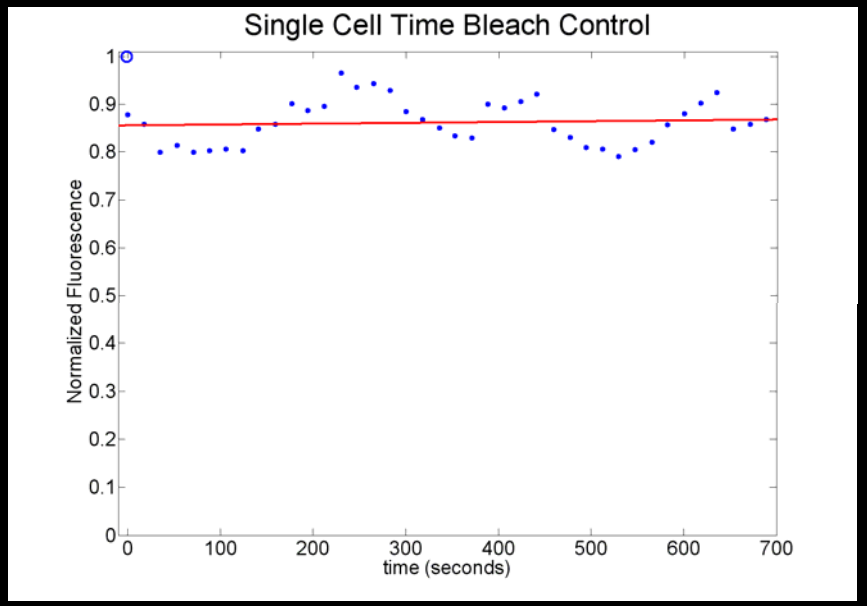
$$f_0 = \alpha C_0$$

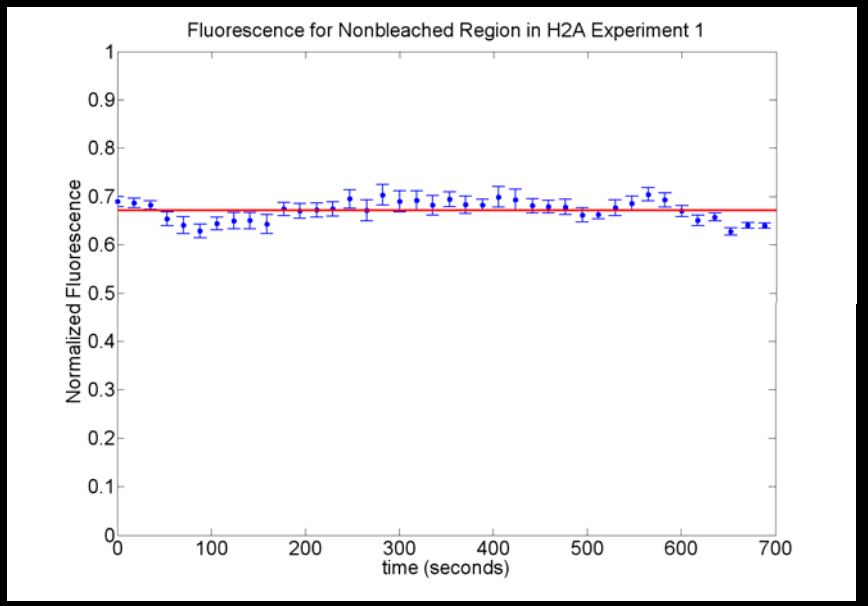
$$C_n = \gamma^n C_0$$

$$f_n = \alpha C_n$$

$$ln(f_n) = nln(\gamma) + ln(\alpha C_0)$$

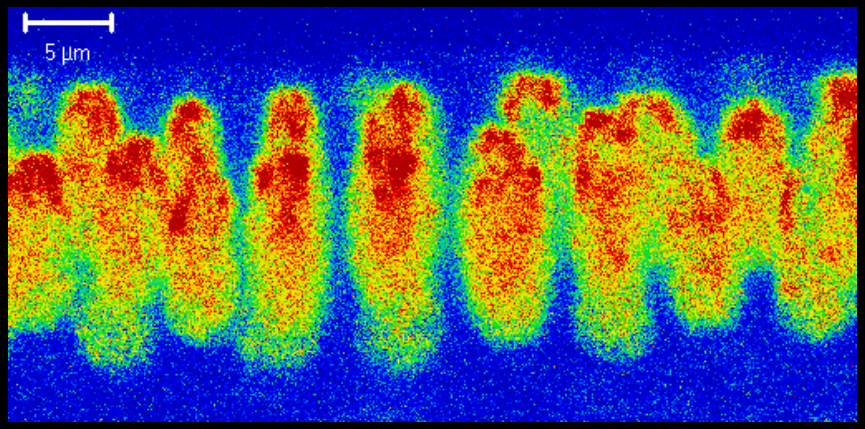
Control 2: Time Bleach

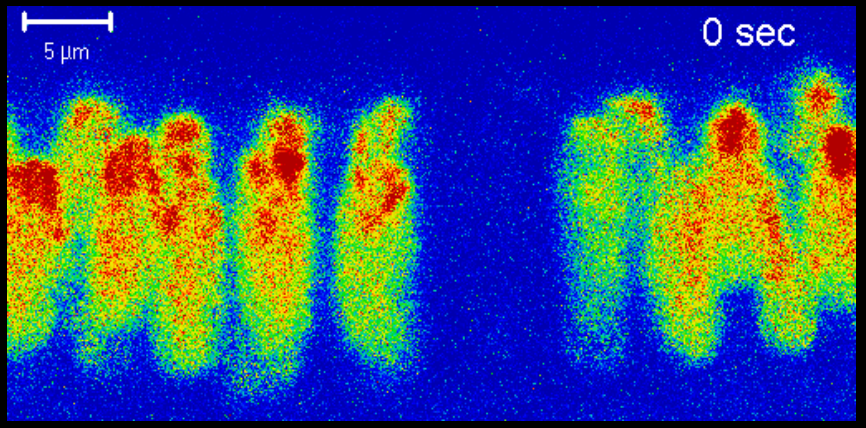


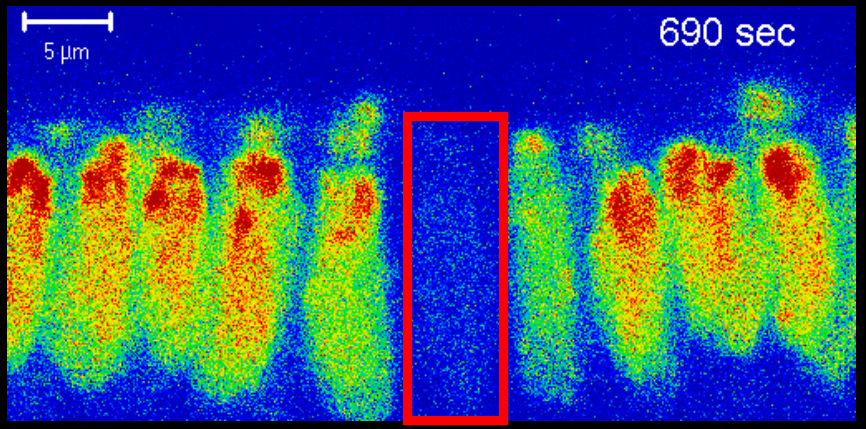


- No significant photobleaching at 2% laser power for 690 seconds

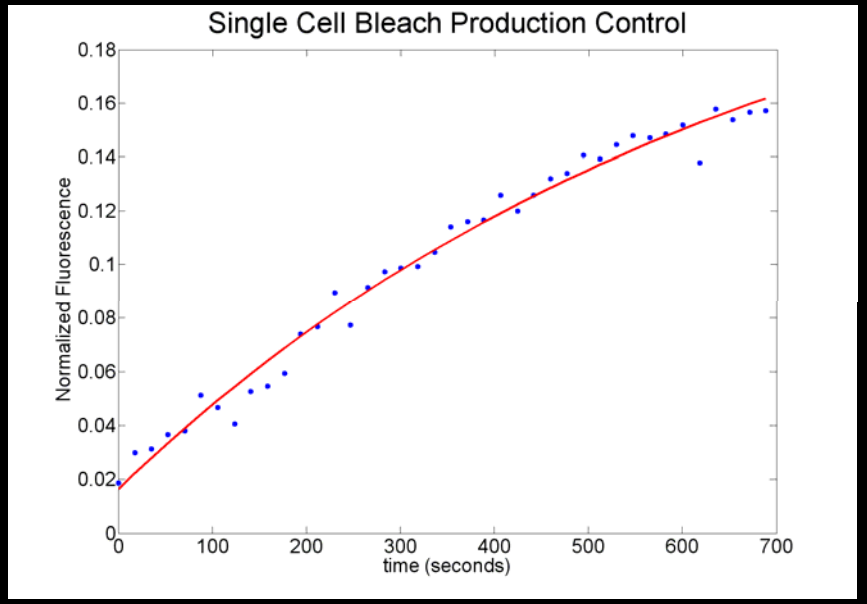
Control 3: Single Nucleus Bleach

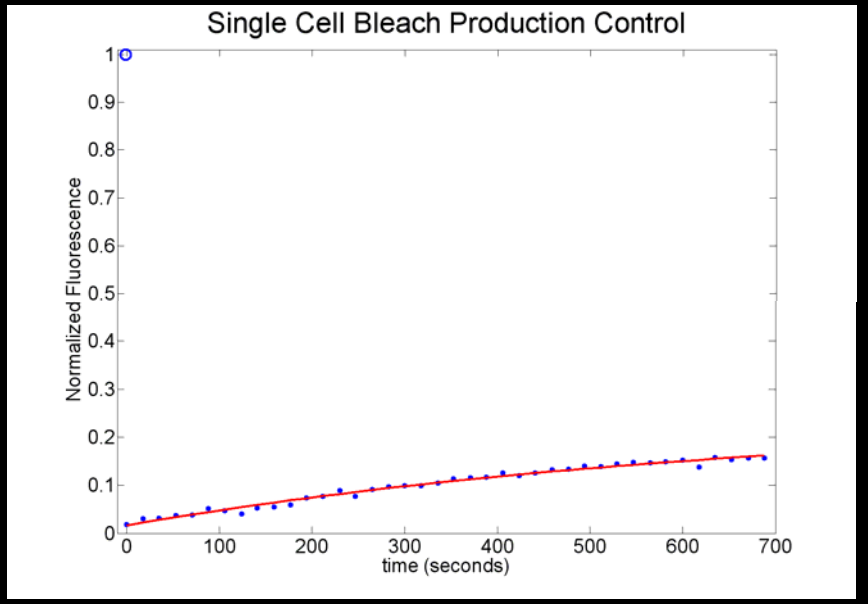






Control 3: Single Nucleus Bleach

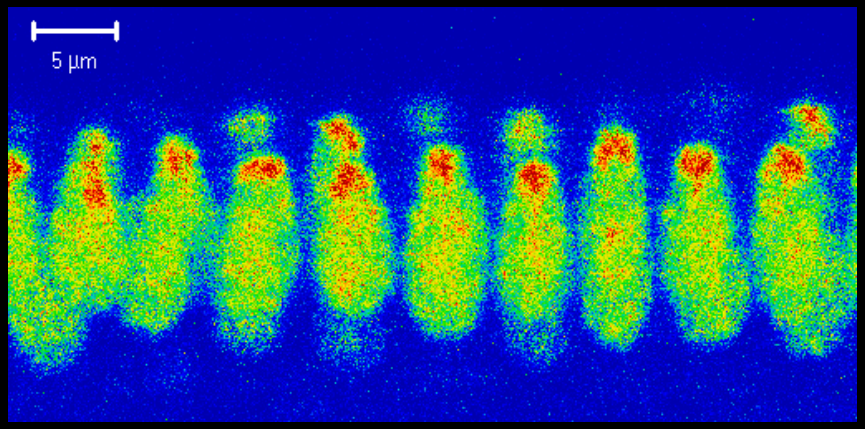


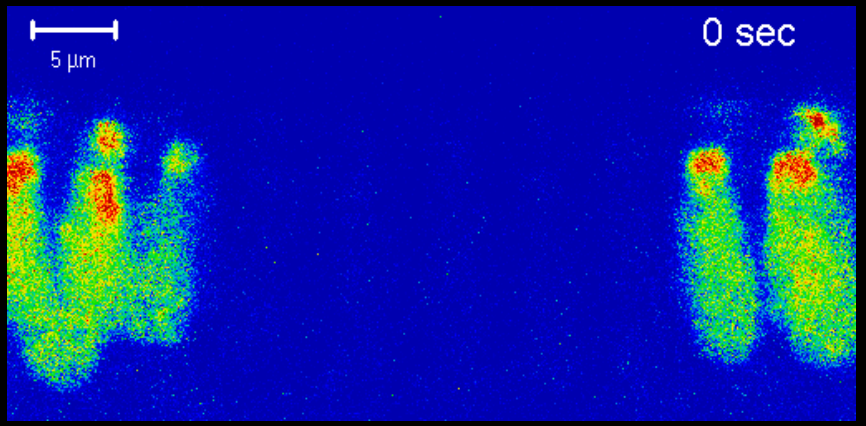


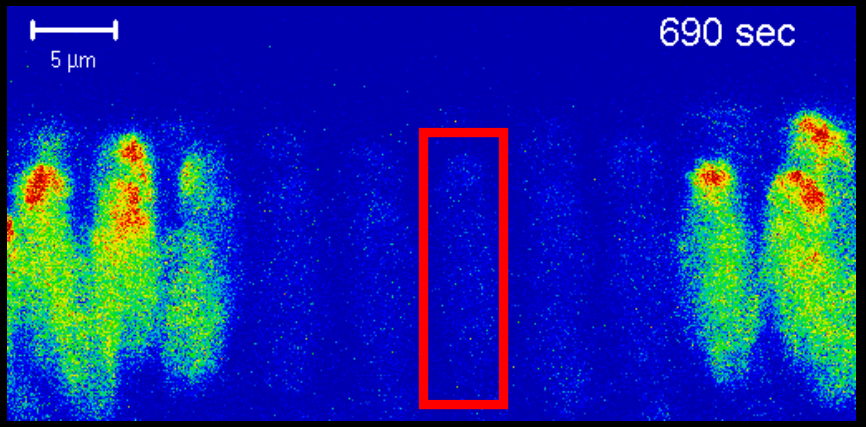
- Signal is restored above background levels after one entire nucleus is photobleached

Calculated D_{eff} was between 0.0056 and 0.0083 $\mu m^2/sec$

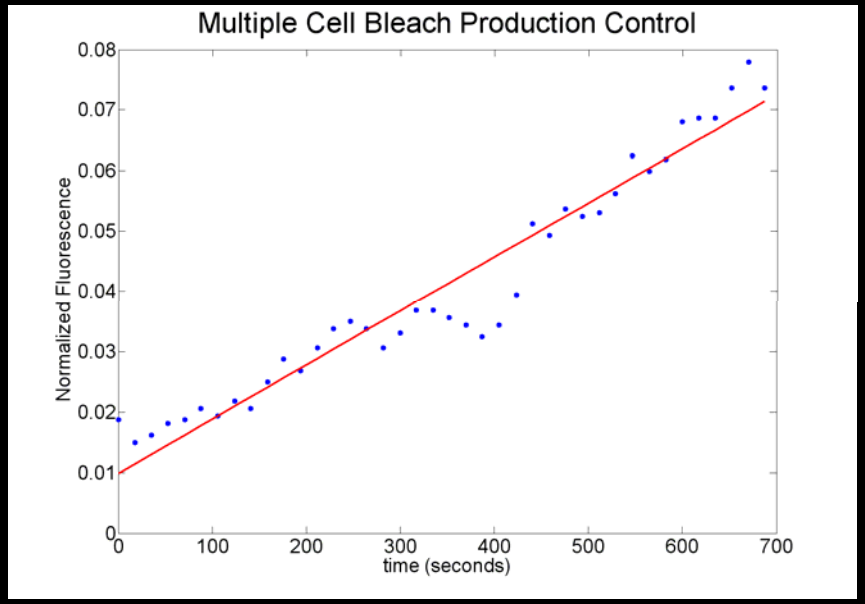
Control 4: Five Nucleus Bleach

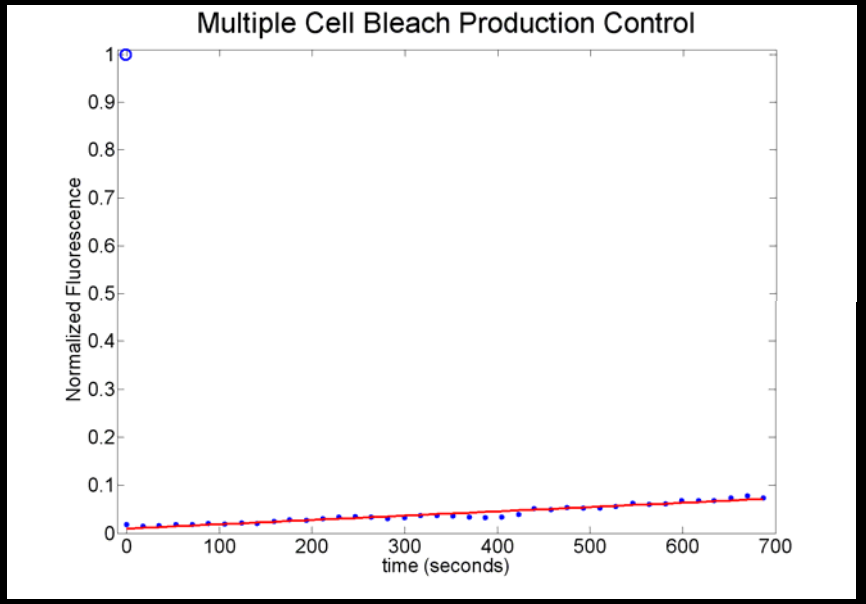






Control 4: Five Nucleus Bleach





- Central nucleus no longer regains signal in a diffusion-like manner

Conclusions

- Motion of H2A within Drosophila embryo nuclei can be modeled by simple diffusion
  - D_{eff} was calculated to be between 0.0079 and 0.0139 $\mu m^2/sec$
  - D_{eff} of free GFP is approximately 87 $\mu m^2/sec$
  - Kicheva et al (2007) reported Dpp-GFP to have a D_{eff} of approximately 0.10 $\mu m^2/sec$ during Drosophila wing development

Conclusions

- NLS diffusion is too rapid for manual FRAP experiments
  - NLS fluorescence returns to a stable level in less than 1 second
  - Relative size (kDa) between GFP and NLS may contribute (GFP is 27kDa)
  - Better time resolution may be needed to observe an exponential recovery

Conclusions

- Single cell control indicates that H2A can migrate between nuclei
  - Cell membranes are not fully formed between nuclei
  - Multiple cell control verifies that production is unlikely
  - Since H2A is expected to constantly bind DNA, why is it diffusing between nuclei in the embryo?

Future Propositions

- H2A experiments must be redone
  - Include diffusion between nuclei
  - Perform a complete embryo bleach to be sure of no generation
  - Perform for longer time intervals (20 minutes instead of around 10)
- NLS experiment must be performed with higher time resolution

Future Propositions

- Nuclear bleach experiments must be performed to better quantify diffusion between nuclei
  - Bleach 1, then 2, then 3, etc. to see whether the D_{eff} is approximately the same
  - Perform full nuclei bleaches at different stages of development
    - Nuclei change size during different stages of development (larger at early stages)

References

- “1kb DNA Ladder,” from New England Biolabs webpage, Last accessed on Sept 22, 2007; URL: http://www.neb.com/nebecomm/products/productN3232.asp
- Axelrod, D et al. “Mobility measurement by analysis of fluorescence photobleaching recovery kinetics,” *Biophys J*, Vol. 16, 1055-1069, 1976.
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- Kicheva, A et al. “Kinetics of morphogen gradient formation,” *Science*, Vol. 315, No. 5811, 521-525, 2007.
- Kim, I et al. “Cell-to-cell movement of GFP during embryogenesis and early seedling development in Arabidopsis,” *PNAS*, Vol. 102, No. 6, 2227-2231, 2005.
- Supatto, W et al. “*In vivo* modulation of morphogenetic movements in *Drosophila* embryos with femtosecond laser pulses,” *PNAS*, Vol 102, No. 4, 1047-1052, 2005.

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- Scott Fraser, Ph.D. – Caltech Professor
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