Preventing Photobleaching by Intensity Modulation of Continuous Wave Laser

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Light microscopy is non-destructive and preferred over electron microscopy for imaging biological samples. Fluorescence light microscopy is particularly useful for tracking individual molecules or staining certain organelles, which may not be clearly visible during bright-field observation. Fluorescence signals are often limited by the effect of photobleaching, which reduces the signal intensity over time. Attempting to increase the signal by increasing the intensity of the excitation laser only adds to the photobleaching, and is not effective. Molecules undergo photobleaching from the triplet state, which has a theoretical lifetime of a few μs in fluorescence molecules such as Rhodamine 6G (R6G), used in this study. When molecules are continuously excited further from the triplet state, the risk of photobleaching increases. If the molecules can first relax to the triplet state, they can then be safely excited again to release more photons, which results in a gain in fluorescence signal without photobleaching. In this study, we modulate the intensity of a continuous wave (CW) laser with an acousto-optic modulator (AOM) at a frequency range of 0.1 – 10 MHz. Preliminary results suggest that amplitude modulation causes a decrease in fluorescence decay and increased fluorescence signal in R6G thin film samples compared to CW laser excitation. Decay rate and initial fluorescence signal were found to be correlated with excitation intensity. We find that manipulating the modulation parameters gives a frequency and wave shape that together reduce photobleaching in fluorescent molecules. Incorporating this modulation scheme into fluorescence microscopy should significantly improve fluorescence imaging for biological and other applications.

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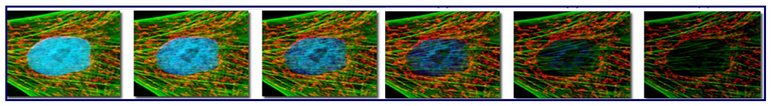
SUMMER NANOTECHNOLOGY STUDY PROGRAM IN JAPAN

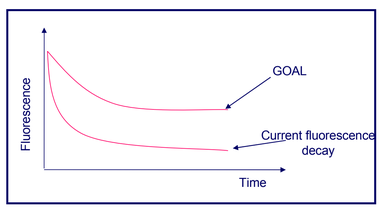
Abstract

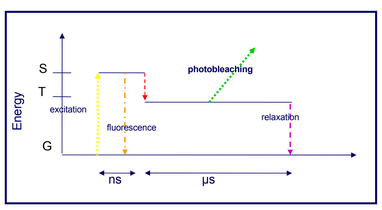
Light microscopy is non-destructive and preferred over electron microscopy for imaging biological samples. Fluorescence light microscopy is particularly useful for tracking individual molecules or staining certain organelles, which may not be clearly visible during bright-field observation. Fluorescence signals are often limited by the effect of photobleaching, which reduces the signal intensity over time. Attempting to increase the signal by increasing the intensity of the excitation laser only adds to the photobleaching, and is not effective. Molecules undergo photobleaching from the triplet state, which has a theoretical lifetime of a few µs in fluorescence molecules such as Rhodamine 6G (R6G), used in this study. When molecules are continuously excited further from the triplet state, the risk of photobleaching increases. If the molecules can first relax to the ground state, they can then be safely excited again to release more photons, which results in a gain in fluorescence signal without photobleaching. In this study, we modulate the intensity of a continuous wave (CW) laser with an acousto-optic modulator (AOM) at a frequency range of 0.1 – 10 MHz. Preliminary results suggest that amplitude modulation causes a decrease in fluorescence decay and increased fluorescence signal in R6G thin film samples compared to CW laser excitation. Decay rate and initial fluorescence signal were found to be correlated with excitation intensity. We find that manipulating the modulation parameters gives a frequency and wave shape that together reduce photobleaching in fluorescent molecules. Incorporating this modulation scheme into fluorescence microscopy should significantly improve fluorescence imaging for biological and other applications.

Purpose

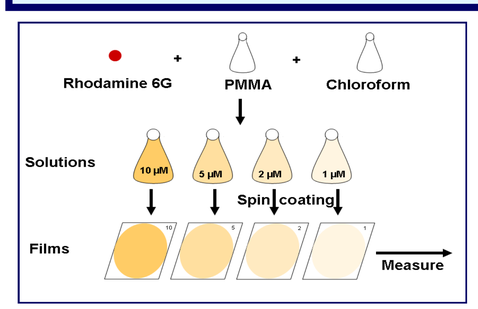


- Fluorescence microscopy
  - Useful for imaging biological samples
  - Tracking individual molecules or staining organelles or proteins with fluorescent dyes
  - Limited by decrease in signal due to photobleaching
- **Goal:** reduce photobleaching to reduce fluorescence decay and increase fluorescence yield
- Fluorescent molecules
  - Excited from ground state to singlet state by light
  - Molecules move from singlet to triplet state (T1 or Tn) and are more susceptible to photobleaching
  - Molecules relax for 3-5 microseconds (lifetime of triplet state) before being excited again





Sample Preparation



Samples prepared using:
- Fluorescent Molecule: Rhodamine 6G
- Polymer Matrix: PMMA
- Solvent: Chloroform
- Method: Spin coating
- Concentrations: 10 µM, 5 µM, 2 µM, 1 µM

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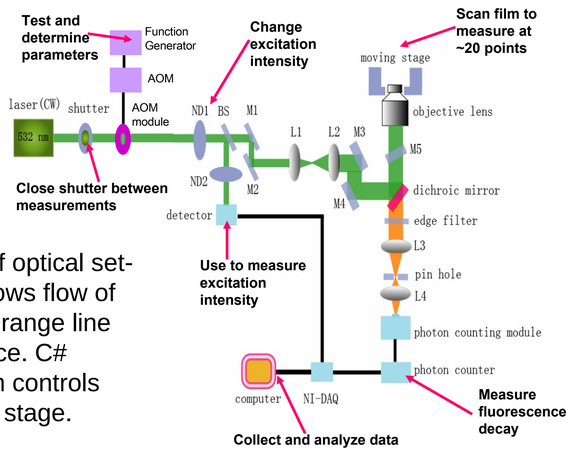
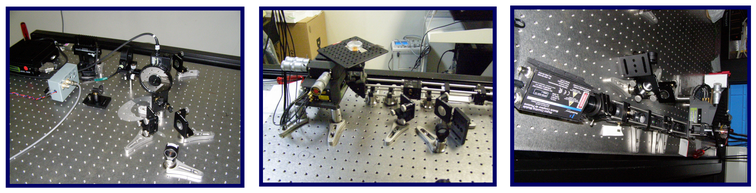
Optical Set-Up

(Below) Scanning Stage: Sequence of Measurements.

17	18	19	20
13	14	15	16
9	10	11→12	
5	6→7	8	
1→2	3	4	

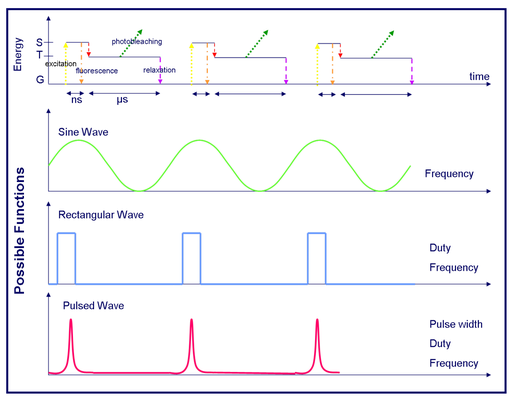


(Right) Diagram of optical set-up. Green line shows flow of excitation laser. Orange line shows fluorescence. C# computer program controls shutter, ND1, and stage.



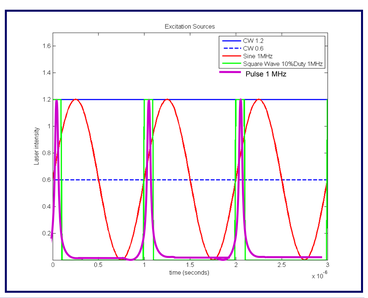
Experimental Optical Set-Up: (Left) excitation laser, shutter, ND filter, and detector to measure excitation intensity. (Middle) scanning piezo-electric stage and dichroic mirror. (Right) photon counting module collecting fluorescence from sample and objective lens.

Laser Modulation

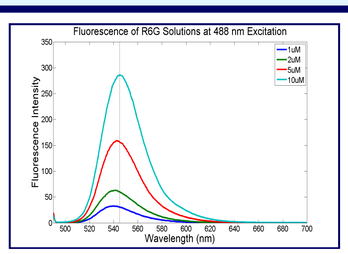


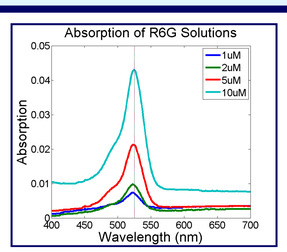
(Left) Modulation patterns line up with excitation and relaxation cycles to only excite molecules from the ground state. Possible modulation functions are shown along with parameters that can be varied.

(Right) Sine, square, and pulse waves with a frequency of 1 MHz are shown. These are compared to two levels of continuous wave (CW) with no modulation. CW 1.2 has the same peak intensity and CW 0.6 has the same average power as the 1 MHz sine wave.



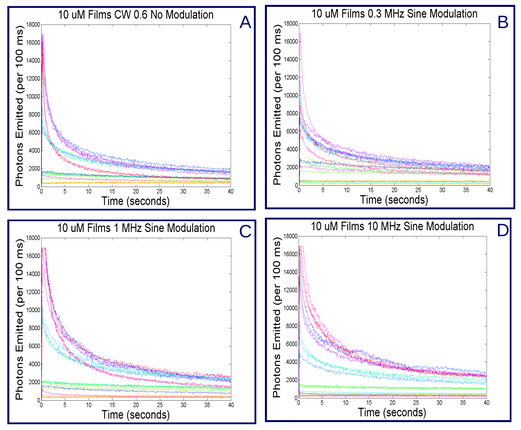
Optical Properties





(Left) Fluorescence and (Right) absorption spectra are shown to examine the optical properties of Rhodamine 6G. The molecules are excited at 488 nm to observe the fluorescence spectra. Both spectra show a red shift with an increase in concentration. Both fluorescence intensity and absorption increase with increasing concentrations.

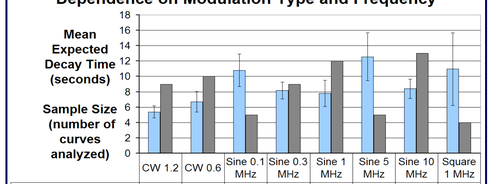
Results

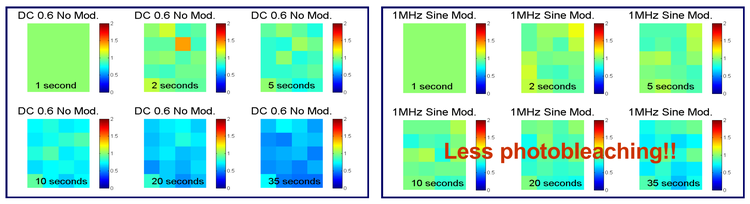


(Left) Fluorescence vs. time graphs for 4 modulation conditions. (A) CW no modulation. (B) 0.3 MHz sine modulation. (C) 1 MHz sine modulation. (D) 10 MHz sine modulation. Comparing (A) and (C) shows that sine modulation at 1 MHz gives a slower decay rate when compared to no modulation case. Comparing (B), (C), and (D) shows the effect of frequency. The optimum frequency still needs to be determined.

(Right) Analysis of mean expected decay time determined from exponential fitting of curves shown above. Blue bars show a definite increase in lifetime when modulation is used.

Analysis of Mean Expected Decay Times for R6G Films and Dependence on Modulation Type and Frequency





(Above) Normalized film fluorescence over time 'imaged' with photon counter. Fluorescence intensity is normalized by the 1 second time point to remove batch film effects. (Left) CW no modulation. (Right) 1 MHz sine modulation.

Future Research

1. Determine exactly which modulation parameters are most effective in preventing photobleaching.
2. Examine modulation effect in different film concentrations.
3. Repeat pulsed and square waves with a faster AOM and detector.
4. Incorporate modulation into imaging microscope to try with biological samples.

References

Donnert G, Eggeling C, Hell SW. Major signal increase in fluorescence microscopy through dark-state relaxation. *Nature Methods.* 2007;4(1): 81-86.

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Acknowledgments

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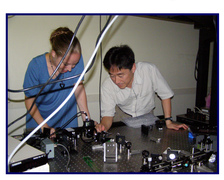

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