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Instrumentation for Single Molecule Fluorescence Lifetime Imaging in Living Systems

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14. ABSTRACT
Developed was a confocal attachment, that incorporated a fast piezo scanner, a high NA microscope objective, and a picosecond super continuum laser that was interfaced with an existing Guide Star microscope and TTTR-TCSPC instrumentation to create a fluorescence lifetime imaging (FLIM) capability with single molecule sensitivity along with its current state-of-the-art super-resolution capability. This instrument was calibrated to account for the absolute number of photons emitted from an individual probe molecule; giving rise to the real-time quantum calibration of probes in situ.

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Instrumentation for Single Molecule Lifetime Imaging in Living Systems

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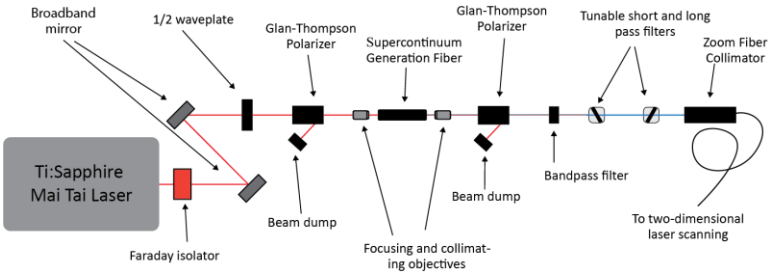
I. Objectives – The principle objective of this work was to build a fast 3D scanning Fluorescence Lifetime Imaging (FLIM) attachment for the Guide Star single molecule fluorescence microscope developed under AFOSR grant **FA9550-14-1-0272** and to interface the attachment with time-correlated single photon counting (TCSPC) and a time-tagged time-resolved (TTTR) data collection capability. Specifically, the confocal attachment was to incorporate a fast laser scanner, a 3D piezo sample scanner to keep mobile samples in the field of view, high NA microscope objectives (both water and oil immersion), and a picosecond super continuum laser allowing for full spectroscopic range throughout the visible and near infrared regions of the electromagnetic spectrum and TTTR-TCSPC instrumentation to create a 3D FLIM capability with single molecule sensitivity along with the current state-of-the-art super-resolution capability (previously developed). The second objective was to calibrate the instrument to account for the absolute number of photons emitted from an individual probe molecule. The second goal would provide the capability to carry out real-time quantum calibration of probes ***in situ***. The scanning capability of the proposed instrumentation has radically enhanced our capacity to **quantitatively** carrier out dynamic single molecule experiments ***in vivo***; which is of prime importance to future AFOSR technology.

II. Results of Effort – In building the instrument, we have met all goals set out in the original DURIP proposal that was summarized in section I. The individual accomplishments are discussed in section III below. Overall, a sophisticated confocal microscope attachment was designed, assembled, calibrated, and tested. It has capabilities in 3D FLIM, FCS, single-molecule tracking, absolute photon counting, and single-molecule fluorescence spectroscopy. Using a specially designed piezo sample stage and field programmable gate array, it also has the capability to track and keep a single molecule or protein that is freely diffusing in an aqueous solution, within the confocal volume of the microscope for 1 minute (or until it photobleaches).

III. Accomplishments / New Findings

(1) **Super Continuum Laser.** The original plan was to purchase a super continuum laser from a commercial supplier (PicoQuant) that could be interfaced with exiting TTTR-TCSPC equipment. Unfortunately, the supplier had temporarily discontinued sales in the US due to technical difficulties associated with the units. After consultation with other suppliers, it was decided that in order to fulfill the specification of the instrument design, it was best to build the super continuum laser using a femtosecond Ti:Sapphire laser from our laboratory. A schematic and photograph of the system is presented in figure 1.



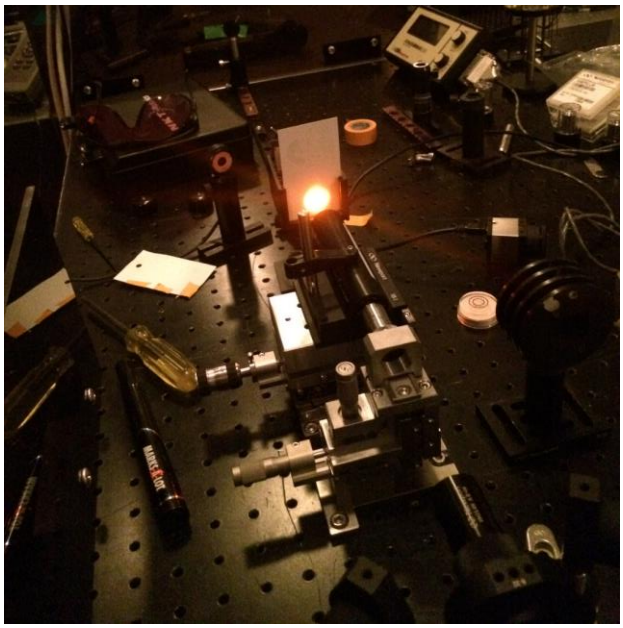


Figure 1. (Top) Schematic of the super continuum generation chamber, output power control scheme, tuning chamber, and fiber coupler. (Bottom) Photograph of the super continuum generation.

The laser consists of four parts. The first part generates the super continuum. It consists of a Femtosecond Ti:Sapphire laser (Spectra Physics Mai Tai). A Faraday isolator to prevent back reflections into the pump laser. Two folding mirrors to steer the pump beam. A Glan-Thompson polarizer and beam dump to regulate the power entering the super continuation generation fiber. Power regulation at this point is important because it determines the spectral peak in the power spectrum and pulse broadening in the fiber. After the Glan-Thompson polarizer the beam is launched into the super continuum generation fiber with a microscope objective. We have used a 40x objective which generates more power and a 20x objective which out performs the 40x in terms of stability. After the pump beam enters in the fiber it generates a super

continuum from 420nm – 1600nm. The super continuum beam is then collimated by a second 20x microscope objective.

The power generated by the super continuum laser is far greater than is needed in most linearly excited confocal fluorescence experiment. Therefore, a module was built to attenuate the final output power using a second a Glan-Thompson polarizer and beam dump. After the power selection module, a module for wavelength selection was added. This was accomplished with a pair of tunable band pass filters (long pass and short pass) mounted on precision rotation stages. In this way the wavelength selection and bandwidth could be continuously tuned. Finally, the laser is launched into a single mode polarization preserving fiber with a zoom collimator. The polarization preserving fiber is important because of the acousto-optic beam deflectors built into the microscope (see below). The fiber is then coupled directly into the microscope.

(2) **Confocal Attachment:**

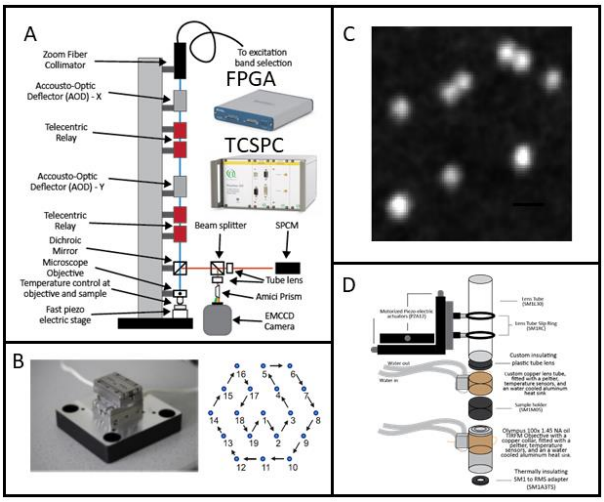


Figure 2. (A) Schematic of confocal attachment, (B) sample stage scanner, (C) confocal image of single Alexa 647 molecules on cover-glass, (D) prototype temperature control system.

Depicted in Figure 2A is the confocal attachment. The laser fiber from the super continuum laser is coupled to a zoom collimator that produced a 1mm collimated laser beam. The beam then passes through an acousto-optic beam deflector (AOD) which gives an optimal first-order deflection when coupled with polarized light. The AOD is arranged to sweep through an angle in the xz-plane. The deflected light was then passed through a telecentric relay constructed from a pair of 30mm Erfle eyepieces. The telecentric relay converted the angular displacement of the laser beam into vertical displacement in the x-direction. The x-deflected beam was then

coupled into a second AOD oriented in the yz-plane and telecentric relay to complete the fast (nanosecond/pixel) laser scanner.

The scan beam was then passed through a dichroic mirror and coupled into a high numerical aperture (NA) microscope objective and onto the sample. The sample was then mounted onto a fast xy-piezo stage customized for our application by Physik Instrumente Gmbh & Co and interfaced with a field programmable gate array (FPGA) from National Instruments. This combination allows from spatial positioning (from 0.05 – 12 µm) with >200 µs between steps. The combination of fast laser scanning (ns per pixel) and sample positioning is an enabling technology that allow one to continually make measurements on a single molecule or protein as it diffuses in an aqueous media or inside a cell.

Next, the fluorescence from the sample is collected by the high NA objective and reflected at 90^{o} into the detection arm of the microscope. The light from the sample then passes through a lens tube, a long pass filter and into the 30:70 beam splitter. The 30% arm is focused onto an EMCCD camera for super resolution imaging or spectroscopic imaging. In order to perform spectroscopic imaging an amici prism spectrometer is inserted into the attachment. The spectrometer consists of a short fiber (30 µm) pinhole directly incorporated into a fiber collimator which passes through an amici prism and imaged onto the EMCCD camera. The 70% arm is directly imaged onto the APD (30 µm active area and 30 ps TCSPC time resolution).

The signal from the APD and EMCCD camera are then synchronized through time-tagging and sharing of a common global clock. The time-tagging and TCSPC is accomplished by interfacing the output of the APD to a PicoHarp 300 (from PicoQuant). The entire system including laser scanning, sample scanner, synchronization between EMCCD and PicoHarp is implemented on the FPGA using a LabView SPI interface and custom software. In this way FLIM, FCS, spectroscopy or super-resolution microscopy and particle tracking is seamlessly integrated into a single instrument.

(3) **Quantum Calibration.**

The instrument was assembled, in part, to support AFOSR grant (**FA9550-18-1-0344**) “Universal Quantum Yield Standards for Stochastic Biophysics”. In order to carry out this work the DURIP instrument was calibrated in order to account for the attenuation through all optical paths of the microscope, the quantum efficiencies of the detector, and the collection efficiency of the instrument. This was accomplished using low-intensity FCS of known quantum standards and following the methods of Kempe et. al, J. Phys. Chem. B 2015, 119, 4668−4672, were they used LI-FCS to

determine the quantum yields of unknown fluorophores. The method was further adapted to take into account collection depth (z-direction). This was accomplished by repeating the calibration as a function of sample depth. This corrected for errors in the extracted collection efficiency of the microscope. The errors are caused by any index of refraction mismatch between the objective/index matching fluid and sample. Once the attenuation factor (g) and collection efficiency (CE) are known, the absolute number of photons emitted from a sample can be determined.

IV. Personnel Supported

While this was an instrument grant (DURIP) and there were no direct support for personnel, the construction of the confocal attachment was accomplished with a few key graduate students who received specialized training from Professor Brozik. Professor Brozik worked side-by-side with student researchers to ensure completion of the new instrument. Students included:

a. Mr. Evan Taylor (Graduate Student; Confocal design and TTTR-TCSPC)
b. Ms. Jessica Carder (Graduate Student; Construction of Super-continuum laser, laser scanning, and software interface)
c. Mr. Michael Martinez (Graduate Student; Construction of Amicini Prism Spectrometer)

V. Publications –

The instrument was completed in May 2019, we have used the new instrumental capability to make quick progress on the stochastic quantum standardization of fluorescent probe molecules, the measurement of intersystem crossing rates, and was used to solve an outstanding problem in the study of purinergic receptors. These will be published shortly with the acknowledgment of this DURIP grant (**FA9550-18-1-0395**) and AFOSR grant (**FA9550-18-1-0344**).

VI. Interactions / Transactions

a. Meetings and Conferences.

i. James A. Brozik, "(Bio)molecular machines, Markov walks, and single molecule tracking: Ion channels, enzymes, and synthetic polymers", Emerging Frontiers in Fluorescence Microscopy: From Single Molecules to Super Resolution; ACS Spring 2019 National Meeting in Orlando, FL, March 31 – April 4, 2019.
ii. Evan L. Taylor, Alex D. Dixon, Boris Lam, and James A. Brozik "Quantum standardization of single-molecule fluorescence probes using Low Intensity Fluorescence Correlation Spectroscopy and Time-Tagged Time Correlation Single Photon Counting" 74th

Northwest Regional Meeting of the American Chemical Society, Portland OR, June 16 – 19th 2019.

iii. J. A. Brozik, Adam O. Barden, and Adam N. Goler "Stochastic Quantum Standards and Instrumentation for Single Molecule Biophysics" AFORS Program Review, May 6-10 2019.

b. Consultative / Advisory Functions – These functions have been as a reviewer for grant submission from:
   i. National Institutes of Health – Panel Member ZRG1 SBIR T10
   ii. Air Force Office of Scientific Research – MURI Program "Using Electromagnetic Energy to Control Biological Systems"
   iii. Air Force Office of Scientific Research – Reviewer; Individually Assigned Single PI Proposals
   iv. Center for Integrated Nanotechnology – Sandia National Laboratories and Los Alamos National Laboratory

c. Technology Assists / Transitions / Transfers – The confocal attachment, fast laser and sample scanning and the incorporation of the super continuum laser are important technical advanced needed for fundamental scientific research. The instrumentation developed as a result of this grant research will be of universal interest to the biophysics community, especially the quantum calibration procedure. The instrumental designs, algorithms, spatial calibration, quantum calibration procedures will be submitted for publication along with the fundamental scientific work on stochastic quantum standardization for fluorescent probe molecules supported by AFOSR grant (**FA9550-18-1-0344**).

VII. New Discoveries, Inventions, or Patent Disclosures – No new inventions and no new patent disclosures.

VIII. Honors / Awards – Dr. Brozik was promoted to full professor at Washington State University.