Advantages of Developing Super Resolution Microscopy Techniques at Columbia University’s Microbeam II Endstation

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Abstract

This project aims to ultimately develop sub-diffraction level imaging techniques such as gated stimulation emission depletion (g-STED) in the microbeam II as a precursor to the application of fluorescence lifetime imaging (FLIM) as a future imaging technique. Laser maintenance and imaging software updates were performed prior to imaging any samples. Our system was tested using 10µm fluorescent beads and prepared slides to ensure that the microbeam II multiphoton imaging system was functioning with goals of being restored to full imaging quality. Further work to be completed hopes to ensure the integration of g-STED and FLIM as other imaging techniques capable of imaging biological samples at the Microbeam II end station. The TimeHarp 260 Nano computer card installed will allow for the performance of time correlated single photon counting with picosecond timing resolution down to 250ps. With the installation of the FLIM timing system, TimeHarp 260 software will be programmed with intentions of replacing the previous imaging system. This work was completed during a ten week REU program.

I. INTRODUCTION

Light microscopy methods used for imaging small objects are diffraction-limited in resolution. The diffraction barrier follows laws in physics discussing the difficulties faced while using an optical instrument to clearly image a sample with a tangential distance that is less than approximately half the wavelength of light which was used to image the sample. In recent years, different super-resolution microscopy techniques, forms of light microscopy often used to image sub-diffraction samples, have been found to successfully break the diffraction-imposed resolution limit originally proposed by Abbe[4] without causing minimal to no photobleaching or
photodamage to in vivo samples. The diffraction of light in far-field fluorescence microscopy limits the sharpness of a beam to be focused down to a spot of $\lambda/2NA$ where $\lambda$ is the wavelength and $NA$ is the aperture of the lens. When two fluorescent samples are spaced closer than the smallest size of the excitation beam, they are excited simultaneously. The images of the two objects end up larger than their spacing resulting in the impossibility to distinguish between the two object.

The importance of breaking the diffraction limit of 250nm is especially important in the advancement of biological studies through imaging. Most biomolecules are on the order of approximately 1-50nm in size.

Super-resolution techniques have been found to break this diffraction barrier. One of the more commonly utilized super-resolution microscopy techniques is known as STED, stimulation emission depletion. In this technique, the combination of a donut shaped CW STED laser for fluorescence depletion and a pulsed excitation laser aligned at the center of the donut are simultaneously used to produce high resolution images. The depletion laser works by using photons whose energy is not high enough to excite molecules but have a lower energy fitting the energy between the fluorescent state and the ground state. If the photon energy fits the specified energy gap, these photons are capable of sending molecules back down to the ground state instantly taking away the majority of the energy, sending the affected fluorophores into a dark state before fluorescing[3]. While almost all the excited molecules are forced to return to their ground state, the remaining excited dye molecules at the center of the excitation focus are able to be detected.

The smallest size the excitation beam can be reduced to cannot surpass the size of the diffraction limit $d = \frac{\lambda}{2n\sin \alpha \sqrt{1 + 1/I_s}}$ (1)

but is still able to achieve a smaller focal point when used in combination with a STED depletion beam. As the intensity of the depletion laser is increased, its area is also increased, therefore
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Reducing the size of the excitation beam and focal point.

\[ \frac{\lambda}{2 \cdot NA \sqrt{1 + \frac{I}{I_a}}} \]

\[ \frac{\lambda}{2 \cdot NA} \]

\[ I \]

**Figure 2:** a) The excitation beam cannot achieve a focal point smaller than its wavelength divided by 2 times the diffractive index times the sine of the largest angle that can be achieved. b) The area of the depletion beam increases with an increase in intensity resulting in a smaller focal point and increase in spatial resolution.

Improvements to the spatial resolution of this already super-resolution technique have been found to be possible. This improved super-resolution modality can be achieved by introducing a time-gating component to the data collection, making use of the fluorescence lifetime of fluorophores.

Often, the STED depletion laser is incapable of depleting all targeted fluorophores, causing some fluorophores to emit fluorescent light which in turn creates noise tainting image resolution. This time-gating detection method (g-STED), has been used in fluorescence microscopy for suppressing unwanted background, allowing photons to be detected shortly after the STED pulse has ended [8]. G-STED reduces the amount of time a fluorophore spends in the excited state, therefore increasing image resolution. By incorporating the time-gating system, we are able to control which part of the fluorophore emission decay will be used to construct the final image. G-STED allows for longer pulses with no loss in resolution and minimal photodamage.

In order to execute time-gated detection, the implementation of a time-correlated-single-photon-counting (TCSPC) card is necessary. The TCSPC card contributes to filtering in STED by detecting photon arrival times and collecting the data shortly after the initial activation of the excitation beam.

A multiphoton imaging system combines a multiphoton microscope with a single-cell single particle irradiator in order record high-resolution time-lapse images of particle induced focus formations within a single cell in real time [2]. The Microbeam II at RARAF has capabilities of imaging small 3-D tissue samples and organisms. In two-photon microscopy, two photons are excited and absorbed at the same time. The two photons combine to act as a single photon, resulting in a multiplication of the energy by a value of 2 and cutting the value of the wavelength in half. A super microbeam was installed to allow for higher energy photon excitation. By increasing the intensity of the STED beam, the depletion area will increase leading the point spread function (PSF) to be condensed into even smaller dimensions. PSF gives the volume from which the fluorescence signal is acquired. [3]

The PSF is approximated by

\[ \text{PSF}(r) \sim e^{-4r^2/\omega^2} \]  

(2)
where $\omega$ represents $1/e$ of the beam focus where the excitation beam radius is smallest. Flourescence Lifetime Imaging (FLIM), is a technique used to develop images based on the differences in the rate of decay of fluorescent molecules. The fluorescence lifetime is defined as the time a molecule remains in the excited state, prior to returning to its ground state as a result of the emission of a photon.

Every fluorescent molecule has its own fluorescence lifetime which can be used to characterize a sample in various environments and sample times. Two-dimensional images can be generated from the fluorescence lifetime measurements. Photodamage from photobleaching and phototoxicity limits the application of using fluorescence in imaging living samples, but by maximizing the ability to detect single photons during each excitation event the probability of photodamage is minimized.

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Figure 3: Improving Spatial Resolution with Time-Gating: a) Timing diagram for pulsed STED with time-gated detection. Excitation beam of negligible duration (blue) is immediately followed by the depletion pulse of duration $t_s$ (red), and the detection of spontaneous emission that occurs during the time window starting $t_g$ after the completion of the first excitation pulse and continues detecting up until the beginning of the next excitation pulses is used to create the final image (green). b) The probability of emitting via normal fluorescence as a function of position (black) when a uniform field of excited fluorophores (dashed black) are exposed to the pulsed STED beam. The probability of emitting via spontaneous emission after the STED pulse has completed (red, dashed), and this probability corrected for the decrease in image brightness (red, solid). c) The PSFs for confocal, STED, and time-gated STED detection. [6]

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scale. The start signal is determined by the electronics of the controlling the laser pulse, and a stop signal is determined by the arrival of a photon at a single-photon sensitive detector.

High resolution high efficiency lifetime FLIM techniques in multi-photon laser scanning microscopes can be achieved by combining TCSPC with a fast scanning technique. In order to get FLIM integrated into our setup, the TimeHarp 260 Nano was the time correlated single photon counter which was selected and installed in a computer to be used with the Microbeam II system. This computer chip is capable of picosecond resolution, down to 250ps.

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The custom design, seen in Figure 5b, has the laser beam following the path of the half-wave plate and beam splitter which in conjunction act as an attenuator to control beam power. After passing through a fast acting shutter, the mirrors direct the beam up through the microscope shaft and through a beam expander which increases the size of the beam to a size which will fill the entire back aperture of the objective lens. The scan lens focuses the scanned laser down to an image plane. The mirror then guides the beam through the tube lens and to a focal point within the specimen where the multiphoton absorption occurs. The emitted light is then deflected by the dichroic mirrors to an array of PMTs. The images are then constructed from the PMT signals.

Figure 4: Generalized setup of a Fluorescence Lifetime Imaging microscope. [7]

II. MATERIALS AND METHODS

Microbeam II
The Microbeam II end station located at Columbia University’s Radiological Research Accelerator Facility (RARAF) focuses on imaging nanoscale biological related samples through fluorescence microscopy. A custom developed multiphoton imaging system built into the end station of Microbeam II allows for the investigation of live cell samples at predetermined depths. [2]
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Figure 5: (a) Multiphoton microscope at RARAF used to observe irradiated cells and tissues. (b) Diagram of the optics path on the multiphoton microscope located at RARAF. [2]

**STED Microscopy**

Developed by Stefan Hell, stimulated emission depletion microscopy is a super-resolution fluorescence imaging technique typically performed through a confocal microscope. By exciting a small set of fluorophores with an excitation laser, quickly followed by a doughnut shaped depletion laser sending fluorophores back to their ground state. The remaining excitation volume can be reduced to a point sized area, creating a sub-diffraction excitation volume.

The STED laser is typically fired shortly after the initial excitation beam in order to allow excited fluorophores to undergo vibrational relaxation ensuring that the majority of fluorophores then targeted by the STED pulse will be pushed out of their excited state and back into the ground state.

**g-STED Microscopy**

Gated stimulated emission depletion microscopy reduces the amount of time that a fluorophore spends in the excited state. It utilizes the same techniques and procedures as described with STED imaging, except it introduces a time-gating component. The time-gating system allows the user to control which part of the light decay will be used in constructing the final image therefore improving image resolution.

**FLIM**

Fluorescence Lifetime Imaging is an imaging technique which produces images based on the differences in the rates of exponential decay from fluorescent samples. Fluorescence techniques are noninvasive and nondestructive making them popular for imaging live specimens. Data recording of FLIM utilizing single photon counting to detect the time between the laser pulse and the arrival time of the photons [1].
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TimeHarp 260

TimeHarp 260 is a time correlated single photon counting (TCSPC) device with picosecond resolution timing. TCSPC is a vital application in the area of applied physics, including that of fluorescence lifetime measurements. This device provides a high timing resolution with a very short dead time.

The imaging software used with the FLIM imaging system is the TimeHarp 260. It’s primary mode of operation is its interactive histogramming mode. The control panel allows for the user to set the resolution, time of data collection, and to select a specific block of memory to store measurements. Trace mapping allows for up to 8 curves to be selected and displayed at once. The user has the ability to select different colors to represent each memory block. Two different histogramming modes are available to be selected from, Oscilloscope and Integration mode. The Oscilloscope histogramming mode repeatedly collects histograms with a fixed measurement time and displays you on the screen. This allows for the user to analyze the fast changes taking place in the histogram data. Integration mode collects the data over a longer acquisition times and only updates the display at regular intervals, allowing for the accumulation process to be easily observed.

The Time-Tagged Time-Resolved (TTTR) mode records the individual count events directly to the hard disk with respect to the beginning of the entire measurement. This mode is useful in identifying photon bursts which are valuable in detecting single molecules. These features allow for an ultra-fast Fluorescence Lifetime Imaging. Within Time-Tagging modes, the user has the option to choose between T2 and T3 mode.

T2 mode will record events from all the channels independently, recording arrival times of the event with respect to the overall measurement start time, and indicating specifically which channel it came from. This is done by ensuring that all the timing inputs of the TimeHarp 260 and the SYNC input are both functionally identical. The highest resolution for timing on our device is 250 ps. Each event record consists of 32
bits, made up of 6 bits for the channel number and 25 bits for the time-tag. In the case of an overflow, an overflow record is included in the data stream, allowing for the data stream to be processed and recovered at full resolution, even in the theoretical case of an infinite time span. Dead times only exist within a singular channel and not across the channels meaning that cross correlations can be calculated down to zero lag time.

T3 mode is intended for allowing high sync rates which could not be handled in the T2 mode. This is done by again employing the sync divider as in histogramming mode, therefore reducing the sync rate eliminating the issue of channel dead time. The event will only be recorded if they arrive in the instance of a photon event.

### III. PRELIMINARY TESTING

Before testing the imaging capabilities on the multiphoton system, issues regarding the laser and mirror systems needed to be addressed. Overheating mirrors were responsible for a shutdown of the entire multiphoton system. This issue was temporarily resolved by adjusting and cleaning out the cooling system allowing mirrors to maintain a normal temperature. In addition to the mirrors, the laser was not providing the necessary amount of power in order to successfully run multiphoton imaging.

During the time of this experiment, the Ti:Sa Chameleon Ultra II laser would provide approximately 1W of power at 1010nm, and provide approximately 3.5W at its peak power wavelength of 800nm. At other times, for unknown causes, the laser would provide approximately 1.2 W at 800nm and approximately 0W at 1010nm.

### IV. TESTING AND RESULTS

To test out the functionality of the laser and the system, 10µm sized green fluorescent beads were set on the stage to image. With a maximum laser intensity of 3.5 W and a wavelength of 1010nm, the two photon system was able to work at 505nm for the excitation of the fluorophores.

![Image of 10µm sized green fluorescent bead imaged at laser intensity of 3.5 W with wavelength of 1010nm.](image)

This image successfully taken with the multiphoton imaging system at the Microbeam II endstation is the first image to be successfully captured since the installation of the new super beam Chameleon Ultra II laser.

In previous experiments with the prior laser, the Microbeam II endstation was capable of imaging samples down on the nano scale.
Figure 10: a) 10µm sized green fluorescent bead taken with multiphoton imaging system at RARAF in 2017 (post new laser installation). b) Image of 10µm standard cell taken with multiphoton imaging system at RARAF in 2016. c) Image of 253nm sub-diffraction sized beads taken with multiphoton imaging system at RARAF in 2016.
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Figure 10 compares the images of green fluorescent beads taken with the multiphoton imaging system at RARAF before the installation of the new laser, and after the installation of the new laser. Compared to the image resolution of the 253nm bead taken from the previous year, the current image resolution of the 10 µm bead does not match up in quality despite its much larger size. It will be important for future work to discuss where the laser is losing its power and or its fluorescence in order to image smaller sized samples.

V. CONCLUSIONS

While the capability of capturing images at the Microbeam II endstation at RARAF has successfully resumed, troubleshooting still needs to occur in order to successfully maximize image resolution. Once maximum power is able to be generated out of the laser, the implementation of other imaging techniques will be able to successfully run on the Microbeam II endstation. STED imaging will allow for the successful imaging of sub-diffraction sized in vivo samples with minimized photodamage and photobleaching. Once STED is implemented, the system will be upgraded to make use of the new TCSPC unit to allow for the integration of the time-gated STED imaging to further improve spacial resolution.

With the purchase of the new pre-amplifier PMT which is now compatible with single photon counting, fluorescence lifetime imaging is expected to be able to function properly once the laser is properly functioning.

These super resolution imaging techniques will be used to further investigate biological samples that require imaging beyond the diffraction limit.

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