Super Resolution Pump-Probe Microscopy With Point Spread Function Engineering

Farzaneh Mohajerani

University of Texas at El Paso, frz.mohajerani@yahoo.com

Follow this and additional works at: https://digitalcommons.utep.edu/open_etd

Part of the Physics Commons

Recommended Citation
https://digitalcommons.utep.edu/open_etd/1301

This is brought to you for free and open access by DigitalCommons@UTEP. It has been accepted for inclusion in Open Access Theses & Dissertations by an authorized administrator of DigitalCommons@UTEP. For more information, please contact lweber@utep.edu.
SUPER RESOLUTION PUMP-PROBE MICROSCOPY WITH
POINT SPREAD FUNCTION ENGINEERING

FARZANEH MOHAJERANI

Department of Physics

APPROVED:

________________________________________________________________________

Chunqiang Li, Ph.D., Chair

________________________________________________________________________

Felicia Manciu, Ph.D.

________________________________________________________________________

Chuan Xiao, Ph.D.

________________________________________________________________________

Bess Sirmon-Taylor, Ph.D.
Interim Dean of the Graduate School
SUPER RESOLUTION PUMP-PROBE MICROSCOPY WITH POINT SPREAD FUNCTION ENGINEERING

by

FARZANEH MOHAJERANI

THESIS

Presented to the Faculty of the Graduate School of The University of Texas at El Paso in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Physics
THE UNIVERSITY OF TEXAS AT EL PASO
August 2014
Acknowledgements

Foremost, I would like to express my sincere gratitude to my advisor Dr. Chunqiang Li for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. Besides my advisor, I would like to thank the rest of my thesis committee: Dr. Felicia Manciu and Dr. Chuan Xiao who offered guidance and support. I also thank Dr. Vivian Incera, Dr. Efrain Ferrer, and Dr. Eric Hagedorn, for their kind help and advices during my graduate studies at University of Texas at El Paso. Finally I would like to thank my husband and my parents for always offering help and support.
# Table of Contents

Acknowledgements ........................................................................................................ iv  
Table of Contents ........................................................................................................ v  
List of Figures ............................................................................................................... vii  

Chapter 1: Super Resolution Optical Microscopy ......................................................... 1  
1-1 Resolution Limit in Optical Microscopy ................................................................. 1  
  1-1-1 Airy Disk and Rayleigh Criterion .................................................................... 1  
  1-1-2 Abbe Diffraction Limit ................................................................................... 3  
  1-1-3 Point Spread Function .................................................................................... 4  
1-2 Improving the Resolution ..................................................................................... 6  
  1-2-1 Near-field Scanning Optical Microscopy ....................................................... 6  
  1-2-2 Confocal and Multi-photon Microscopy ........................................................ 7  
  1-2-3 4Pi Microscopy and i5M .............................................................................. 7  
  1-2-4 Structured Illumination Microscopy ............................................................. 8  
1-3 Point Spread Function Engineering to Break the Resolution Limit ...................... 8  
  1-3-1 Stimulated Emission Depletion Microscopy ............................................... 8  
1-4 RESOLFT Concept ......................................................................................... 11  
  1-4-1 Saturated Structured Illumination Microscopy ........................................... 12  
1-5 Single Molecule Localization ............................................................................. 12  

Chapter 2: Imaging Non-fluorescent Molecules ......................................................... 14  
  2-1 Nonlinear Optical Microscopy ..................................................................... 14  
  2-2 Second Harmonic Generation Microscopy ................................................... 16  
  2-3 Third Harmonic Generation Microscopy ....................................................... 18  
  2-4 Coherent Anti-Stokes Raman Scattering Microscopy .................................. 19  
  2-5 Pump-probe Microscopy ............................................................................... 20  

Chapter 3: Super Resolution Pump-Probe Microscopy ................................................. 24  
  3-1 Schematic of the Microscope .................................................................... 24  
  3-2 Super Resolution Capability of SRPPM ...................................................... 26  
  3-3 Methods .................................................................................................. 29  
    3-3-1 Excitation .............................................................................................. 29  
    3-3-2 Stimulated Emission .......................................................................... 30
3-3-3 Point Spread Functions of Beams .........................................................31
3-3-4 Signal Detection ..................................................................................33
3-3-5 Defining Resolution in SRPPM ..........................................................33
3-3-6 Finding Best Intensities ......................................................................35
3-4 Calculations and Results ......................................................................37
  3-4-1 Resolution .........................................................................................41
  3-4-2 Total Power on the Sample ..............................................................41
3-5 Conclusion ..............................................................................................42
References ....................................................................................................43
Vita .................................................................................................................46
### List of Figures

- **Figure 1-1** Airy pattern after a circular aperture ................................................................. 1
- **Figure 1-2** The Rayleigh criterion for resolution ........................................................................ 3
- **Figure 1-3** Cross section of a 3D point spread function ............................................................. 4
- **Figure 2-1** Resonant and nonresonant CARS ........................................................................... 19
- **Figure 2-2** Input and output pulse trains of PPM ......................................................................... 21
- **Figure 2-3** Energy level diagram of pump-probe microscopy ..................................................... 22
- **Figure 3-1** Schematic of SRPPM .............................................................................................. 24
- **Figure 3-2** Pulse sequence of SRPPM ...................................................................................... 25
- **Figure 3-3** modes of (a) pump 1 beam and (b) pump 2 beam and the probe beam .................. 26
- **Figure 3-4** A molecule at the center of PSF ............................................................................... 27
- **Figure 3-5** Molecules in the peripheral part of PSF ................................................................. 27
- **Figure 3-6** Energy diagram of a pump-probe experiment ......................................................... 29
- **Figure 3-7** PSF of two pump beams and the probe beam ......................................................... 32
- **Figure 3-8** $\Delta I_s$ vs. position of the molecule on the focal plane for SRPPM ...................... 34
- **Figure 3-9** $\Delta I_s$ vs. the position of the molecule on the focal plane for PPM ..................... 35
- **Figure 3-10** Probability of excitation for two pump beams ....................................................... 36
- **Figure 3-11** Probability of excitation for two pump beams at saturated regime .................... 37
- **Figure 3-12** SRPPM signal profile for different intensities of two pump beams .................... 39
- **Figure 3-13** FWHM of $\Delta I_s/I_s$ vs. intensity of the doughnut pump beam ......................... 41
Chapter 1: Super Resolution Optical Microscopy

1-1 Resolution Limit in Optical Microscopy

1-1-1 Airy Disk and Rayleigh Criterion

Focusing light by an objective lens will not make a point on the focal plane, instead because of diffraction it will make a pattern known as Airy disk as shown in figure 1-1.

\[
\text{Figure 1-1 Airy pattern after a circular aperture}
\]

In 1834 Airy calculated the central disk radius for a light with wavelength \( \lambda \) after an aperture with diameter \( d \) and showed that the angular position (from the center) for the first minimum of this pattern is given by

\[
\sin \alpha = \frac{1.22\lambda}{d}.
\]

where \( \alpha \) is the half-angle of the maximum cone of light that can enter or exit the lens.

This calculation can be done by considering the Fraunhofer diffraction pattern of a circular aperture. The intensity of the Airy pattern is given by the squared modulus of the Fourier transform of the circular aperture:
\[ I(\theta) = I_0 \left( \frac{2 J_1(k \text{asin}\theta)}{k \text{asin}\theta} \right)^2 \] 1-2

where \( I_0 \) is the maximum intensity of the pattern at the Airy disc center, \( J_1 \) is the Bessel function of the first kind of order one, \( k \) is the wavenumber, \( a \) is the radius of the aperture, and \( \theta \) is the angle between the axis of the circular aperture and the line between aperture center and observation point. Considering \( x = k \text{asin}\theta \), the zeros of \( J_1(x) \) are at

\[ x = k \text{asin}\theta \approx 0, 3.8317, 7.0156, 10.1735, \ldots \] 1-3

So, the first dark ring in the diffraction pattern occurs where

\[ k \text{asin}\theta \approx 3.8317 \] 1-4

or

\[ \sin \theta \approx \frac{3.83}{ka} = \frac{3.83 \lambda}{2\pi a} = 1.22 \frac{\lambda}{2a} = 1.22 \frac{\lambda}{d} \] 1-5

In optical microscopy, the emission from a spot in the specimen will produce an image with the same airy pattern. The effect will become noticeable with high magnifications.

A single isolated spot can be detected if it is given enough illumination. However for two points close to each other, there will be an overlap of two diffraction patterns. Making two points closer to each other, at some point this overlap will make it difficult to say whether there are two objects or just one. For two patterns to be distinguishable, Rayleigh suggested that the minimum distance between two points should be equal to half of the airy disk. In this case the maximum of one pattern will lie over the first minimum of the other and the intensity between two peaks will fall to 80% of maximum.
Figure 1-2 The Rayleigh criterion for resolution

Considering $\lambda_0$ as the free-space wavelength, $n$ as the refractive index of the immersion medium, and $\theta_0$ half the angle subtended by the lens at the object, Rayleigh criterion defines the resolution $y_R$ as

$$y_R = \frac{0.61 \lambda_0}{n \sin \theta_0}$$

1-6

The use of immersion oil obviously improves the resolution. The product $n \sin \theta_0$ is called numerical aperture (N.A.) which will determine the ability to image fine detail. To get the best resolution N.A. should be as large as possible but there are practical limits. With the best N.A. about 1.3 resolution limit will be about half of the wavelength i.e. for the green light the resolution limit will be about 250 nm.

1-1-2 Abbe Diffraction Limit

Although Rayleigh criterion defines resolution limit with a relatively simple analysis, it assumes that the point objects considered behave like self-luminous incoherent sources which is applicable to stars viewed through a telescope but not strictly correct in a microscope. Moreover it is not clear how the effect of diffraction on a complex object can be predicted and what details are affected.
In 1873 Ernst Abbe introduced the idea of using Fourier analysis to predict the form of the image produced by an objective of limited aperture. Abbe’s Fourier analysis approach not only shows an alternative expression to derive the resolution limit, but also is able to explain how the phase microscope works [1].

1-1-3 Point Spread Function

The impulse-response of an optical system is called point spread function (PSF) [2]. For an imaging system with a circular aperture with diameter D at distances $d_1$ and $d_2$ from the object and image planes, respectively, the impulse-response function is

$$h(x, y) = h(0,0) \frac{2J_1(\pi D \rho / \lambda d_2)}{\pi D \rho / \lambda d_2}, \quad \rho = (x^2 + y^2)^{1/2},$$

where $\lambda$ is the light wavelength, $|h(0,0)| = \pi D^2 / 4 \lambda^2 d_1 d_2$ and $J_1(.)$ is the Bessel function. This is circularly symmetric function and cross section is shown in Fig. 1-3. It drops to zero at a radius $\rho_s = 1.22 \lambda d_2 / D$ and oscillates slightly before it vanishes. The radius $\rho_s$ is therefore a measure of the size of the blur circle. If the system is focused at $\infty$, $d_1 = \infty, d_2 = f$, and $\rho_s = 1.22 \lambda f / D$.

![Figure 1-3 Cross section of a 3D point spread function](image)
In optical microscopy, the three-dimensional (3D) intensity distribution of the image is called point spread function. The full width at half-maximum (FWHM) of this PSF determines the resolution of the image, where two points closer than this length cannot be resolved. Improving the resolution is equivalent to narrowing the PSF. In conventional microscopes, the FWHM of the point spread function is about $\frac{\lambda}{2\, n \, \sin \, \alpha}$, where $\lambda$ is the wavelength, $n$ is the refractive index, and $\alpha$ is the semi-aperture angle of the lens. Without changing the principal method, the spot size can only be decreased by using shorter wavelengths and larger aperture angles. Because of the wavelength limit (350 nm) in biological application and technical limit of 70 degrees for lens half-aperture, in the best case 180nm resolution is achievable [3].

The optical transfer function (OTF) is defined as the Fourier transform of the impulse-response of the optical system, also called the point spread function. The optical transfer function is thus readily obtained by first acquiring the image of an incoherent point source, and applying the two-dimensional discrete Fourier transform to the sampled image. Such a point-source can, for example, be a bright light behind a screen with a pin hole, a fluorescent or metallic microsphere, or simply a dot painted on a screen. Calculation of the optical transfer function via the point spread function is versatile as it can fully characterize optics with spatial varying and chromatic aberrations by repeating the procedure for various positions and wavelength spectra of the point source.

The resolution limit is best understood in the frequency world. The image is described as being composed of spatial frequencies transmitted by the microscope. As it is mentioned before, the optical properties of the imaging device are described by the optical transfer function (OTF). Hence, the resolution limit is given by the highest frequency passed. Fourier mathematics
explains the intertwinement of point spread function and OTF: The sharper the point spread function, the broader the OTF [3].

1-2 IMPROVING THE RESOLUTION

1-2-1 Near-field Scanning Optical Microscopy

The first strategy to overcome the diffraction limit is to avoid focusing light. By using ultra-sharp tips or tiny aperture to localize the interaction of the light with the specimen, near-field microscope can image it to subdiffraction dimension; however this approach is limited to imaging the surface [4].

In practice, by placing the nanosized detector aperture adjacent to the specimen at a distance much shorter than the illumination wavelength, non-propagating light waves generated at the surface can be detected. Resolution is independent of the wavelength of illuminating light and limited only by the physical size of the aperture rather, where lateral and axial resolutions of 20 nanometers and 2 to 5 nanometers, respectively, can be achieved [30,31].

Because they do not require fluorescence, other contrast mechanisms that involve Raman scattering, spectroscopy, interference, polarized light, absorption, or some other type of optical signal can be used in near-field microscopy techniques allowing for imaging of non-emissive materials, such as semiconductor surfaces or thin films. Depending on tip quality and the defined field strength, resolutions in the range between 2 and 15 nanometers have been achieved [30,31]. However, the specimen must have a relatively smooth surface in order to avoid a collision with the probe and possible damage to both entities. Using near-field scanning optical microscopy, the first super resolution image of a biological sample, was obtained by Eric Betzig in 1992[5]. In recent years, near field scanning optical microscopy (NSOM) has been used to
investigate several membrane proteins in the nanoscale. However, this approach is not suitable for intracellular imaging.

1-2-2 Confocal and Multi-photon Microscopy

Among far-field microscopy techniques, confocal microscopy and multi-photon microscopy are among the most widely used to enhance the spatial resolution [6, 7]. Confocal microscopy, by adding a pinhole for detection is able to have a factor of $\sqrt{2}$ improvement in the spatial resolution. Although, nonlinear absorption processes in multi-photon microscopy reduce the effective size of excitation PSF, the increased excitation wavelength results in a gain in PSF. Hence, the main advantage of confocal and multi photon microscopy is the capability of optical sectioning in 3d by eliminating out-of-focus background and they cannot effectively improve the resolution beyond the diffraction limit.

1-2-3 4Pi Microscopy and I$^5$M

Microscope objectives collect light from only one side which results in an elongated PSF in axial direction. Improving the axial resolution has been possible by using two opposing objectives for excitation and/or detection. Two techniques, 4Pi microscopy and I$^5$M can improve the axial resolution to $\sim$100 nm [32].

In the ideal case, the use of two objectives would result in a point spread function that is symmetrical in the axial and lateral dimensions. Because the quality of the interference pattern is disrupted when traveling through thick tissues, these techniques are generally limited to use with thin specimens.
1-2-4 Structured Illumination Microscopy

Applying a patterned illumination field to the sample increases the spatial resolution in structured illumination microscopy (SIM). Mixing the spatial frequencies of the illumination pattern with those of the sample features, shifts the high-frequency features to lower frequencies that are detectable by the microscope. Creating this periodic illumination patterns is possible through the interference of multiple light sources in the axial direction and/or lateral directions. A high resolution image will be reconstructed from a series of images with illumination patterns of different phases and orientations, typically from a total of 9-15 images. No unusual properties are required from the sample or the fluorophore compared to conventional fluorescence microscopy. The illumination pattern itself is also limited by the diffraction of light, so SIM can only improve the resolution by a factor of 2 by combining two diffraction limited sources of information. A resolution of ~100 nm in the lateral direction and ~300 nm in the axial direction is achievable by SIM [8].

1-3 Point Spread Function Engineering to Break the Resolution Limit

Super-resolution microscopy is achievable by mean of sub-diffraction-limit features in the excitation pattern so that small-length-scale information can be read out. Super-resolution techniques including stimulated emission depletion microscopy (STED), reversible saturable optical fluorescence transitions (RESOLFT), and saturated structured-illumination microscopy (SSIM) share the idea of “patterned excitation” approach.

1-3-1 Stimulated Emission Depletion Microscopy

STED microscopy was introduced by Stephan Hell in 1994 and it was subsequently demonstrated experimentally [9,10]. To eliminate the resolution-limiting effect of diffraction
without eliminating diffraction itself, STED uses a second laser (STED laser) to quench the fluorescence emission from the fluorophores located off the center of the excitation. Stimulated emission of the excited-state fluorophore brings it back to the ground state and it happens when the wavelength of the STED laser matches the wavelength associated to the energy difference between the excited state and the ground state. This process effectively depletes the excited-state fluorophores capable of fluorescence emission.

Although the STED laser itself is diffraction limited, its spatial pattern with zero intensity at the center of the excitation laser focus and nonzero intensity at the periphery brings the super-resolution capability to this technique. Because of the stimulated emission, the STED beam depletes the molecular fluorescent state everywhere within the focal region, except at the zero intensity point and its proximity; thereby the spatial extent of effective molecular excitation is confined. Typically, a doughnut shaped distribution is the best choice to obtain the most uniform resolution increase in the focal plane. This spatial pattern is achieved by transmitting the STED laser through a phase mask with a phase retardation of $\pi$ over a central circular area before focusing it on the sample [11].

Point spread function engineering, which is manipulation of the shape and size of the effective fluorescent spot beyond the diffraction limit is the critical point in STED microscopy. The depletion of the excited state is driven by de-excitation through stimulated emission and the saturation of this transition provides the non-linear response that allows for decreasing the size of the effective fluorescent spot beyond the diffraction limit. Hence, the resolution of the STED microscope is a function of the spatial distribution and magnitude of the intensity of the STED beam. There is no theoretical limit to the ultimate achievable value. The dependence of the
resolution on the applied intensity of the STED beam is shown to increase with the inverse square root of the saturation level [11].

This relation between resolution of STED microscopy and saturation factor can be roughly estimated as follows: The time evolution of the normalized populations of the two states $n_A$ and $n_B$ is given by

$$\frac{dn_A}{dt} = -k_{AB}n_A + k_{BA}n_B = -\frac{dn_B}{dt}$$  \hspace{1cm} 1-8

where $k_{AB}$ and $k_{BA}$ show the rates of $A \rightarrow B$ and $B \rightarrow A$, respectively. Independent from the initial state, after an illumination time

$$t \geq (k_{AB} + k_{BA})^{-1}$$  \hspace{1cm} 1-9

when equilibrium is approximately reached, the population of state A is given by

$$N_A = \frac{k_{BA}}{(k_{AB} + k_{BA})}$$  \hspace{1cm} 1-10

The rate of depletion of state A is given by $k_{AB} = \sigma I$, where $\sigma$ denotes the molecular cross-section, and the intensity is written as photon flux per unit area. Hence the equilibrium population can be written as

$$N_A(r) = \frac{k_{BA}}{(\sigma I(r) + k_{BA})}$$  \hspace{1cm} 1-11

And for the saturation intensity $I_s = \frac{k_{AB}}{\sigma}$,

$$N_A = 1/2$$  \hspace{1cm} 1-12

We can see when $I(r) \gg I_s$, all the molecules end up in B. If we choose $I(r) = I_{\text{max}} f(r)$ with $I_{\text{max}} \gg I_s$, molecules in state A are only found in the nodes of the diffraction limited distribution function $f(r)$. As an example if a standing wave $f(x) = \sin^2(2\pi x/\lambda)$ is chosen for illumination, a simple calculation shows that FWHM of the peaks of $N_A$ and hence the resolution of the microscope is then given by

$$\Delta x = \lambda \pi^{-1} \arcsin(\sqrt{k_{BA}/\sigma I_{\text{max}}}) \approx \lambda / \pi \sqrt{\zeta} \quad , \quad \zeta = I_{\text{max}} / I_s$$  \hspace{1cm} 1-13
which shows a resolution \( \Delta x = \lambda/100 \) is achievable by a saturation factor \( \zeta \approx 1000 \). In principle by increasing \( \zeta \), the spot of can be squeezed continuously.

**1-4 RESOLFT CONCEPT**

Reversible saturable optical fluorescence transitions (RESOLFT) was first introduced by Stefan Hell. RESOLFT is the theoretical foundation necessary for achieving resolution beneath the diffraction limit. The capability of being reversibly photoswitched between a fluorescent "on" state and a dark "off" state of a fluorophore is the key point of the group of RESOLFT techniques which are able to image beyond the diffraction limit. Stimulated emission depletion (STED), ground state depletion (GSD) and ground state depletion-individual molecule return (GSDIM) microscopy are different microscopy techniques which share the RESOLFT concept. Depending on the nature of the states in the fluorophores, they are suitable for different microscopy techniques.

GSD microscopy or Ground State Depletion microscopy uses the triplet state of a fluorophore as the off-state and the singlet state as the on-state, whereby an excitation laser is used to drive the fluorophores at the periphery of the singlet state molecule to the triplet state. This is much like STED, where the off-state is the ground state of fluorophores, which is why equation 1-13 also applies in this case. The \( I_s \) value is smaller than in STED making super-resolution imaging possible at much smaller laser intensity. Compared to STED though, the fluorophores used in GSD are generally less photostable and the saturation of the triplet state may be harder to realized [24].
1-4-1 Saturated Structured Illumination Microscopy

In SSIM (Saturated Structured-Illumination microscopy), applying a sinusoidal illumination pattern with a peak intensity close to that needed in order to saturate the fluorophores in their fluorescent state retrieves moiré fringes. High order spatial information of the fringes may be extracted by computational techniques. Once the information is extracted a super-resolution image is constructed.

SSIM requires shifting the illumination pattern multiple times, which effectively limits the temporal resolution of the technique. Because of saturating conditions, very photostable fluorophores are also required. These conditions also induce radiation damage to the sample which restricts the possible applications of SSIM. A resolution of 50-nm has been achieved by SSIM [25].

1-5 SINGLE MOLECULE LOCALIZATION

The image of a single fluorophore is a PSF which is a finite-sized spot because of diffraction. However, when the image is resulted from multiple photons emitted from the fluorophore, the precision of determining the fluorophore position can be much higher than diffraction limit. The image consisting of N photons can be considered as N measurements of the fluorophore position where each has the uncertainty determined by the PSF [26]. Hence the localization precision can be approximated by

$$\Delta_{loc} \approx \frac{\Delta}{\sqrt{n}}$$

where $\Delta_{loc}$ is the localization precision and $\Delta$ is the size of the PSF. This relation between localization precision and the photon numbers shows the capability of super-resolution microscopy beyond the diffraction limit.
It is not easy to resolve a fluorescently labeled biological sample by the single-molecule localization approach, where it contains thousands or even millions of fluorophores at a high density. A recent innovation has overcome this problem by using fluorophores that can be switched between a fluorescent and a dark state where fluorescent overlapping images are separated in time domain. In this approach, molecules within a diffraction-limited region can be individually imaged and localized since they are activated at different time points. The final super resolution image is constructed by a sufficiently large number of frames (1000-100,000) where each frame represents randomly blinking individual fluorophores. Several super-resolution microscopy techniques including STORM [27] and PALM [28] have implemented this approach and a lateral image resolution of ~20 nm is achieved [27].
Chapter 2: Imaging Non-fluorescent Molecules

2-1 NONLINEAR OPTICAL MICROSCOPY

Nonlinear optical phenomena have been discovered since the invention of the laser [13]. and have been extensively used over the years for spectroscopy [14].

Scattering can be treated as a process of radiation from field-induced primary sources and the simple linear relationship between induced source strength $S(\vec{r})$ and a driving field $E(\vec{r})$, is given by

$$S(\vec{r}) = 8\pi^2k^2\frac{\delta n(\vec{r})}{n}E(\vec{r})$$

where $k = n\frac{v}{c}$, $n$ is considered to be homogenous, and the index-of-refraction inhomogeneities have been incorporated in $\delta n(\vec{r})$.

To proceeding along the same line for nonlinear scattering, the only difference to be considered is that the induced sources will now be driven by nonlinear optical interactions. Hence, the generalized nonlinear tensorial relation can be formally written as

$$\vec{S}_{\nu s} = \vec{X}^{(1)}(-\nu_s; \nu_1):\vec{E}_{\nu_1} + \vec{X}^{(2)}(-\nu_s; \nu_1, \nu_2):\vec{E}_{\nu_1}\vec{E}_{\nu_2}$$

$$+ \vec{X}^{(3)}(-\nu_s; \nu_1, \nu_2, \nu_3):\vec{E}_{\nu_1}\vec{E}_{\nu_2}\vec{E}_{\nu_3} + \cdots$$

2-1

The function $\vec{X}^{(M)}$ are susceptibilities of order $M$ which can be considered as transfer functions; their inputs are a set of driving fields $\vec{E}_{\nu_1} ... \vec{E}_{\nu_m}$ of respective oscillation frequencies $\nu_1 ... \nu_m$, and their output is an induced source strength $\vec{S}_{\nu s}$ of oscillation frequencies $\nu_s = \sum_{m=1}^{M} \nu_m$. Susceptibilities are considered to be tensorial, and "$\cdot\cdot\cdot"$ indicates a tensor product [12].

Obviously, there is more than one interaction with the laser field(s) in nonlinear optical processes. Therefore, the laser should be particularly intense which can be fulfilled by utilizing a focused laser beam. It is further facilitated by using a pulsed laser, since the intensity at the
peak of a short pulse is many orders of magnitudes larger than the average intensity. For example a pulsed laser with an average power of 1 mW, generates 100 femtosecond pulses at a frequency of 80 MHz and peak intensity of each pulse is as high as 200 GW/cm² at the focal spot of a high-numerical aperture microscope objective. A 1 mW continuous-wave laser however, has only an intensity of 0.5 MW/cm² at the microscope focus.

Due to the second or higher-order dependence of nonlinear optical processes on laser intensity; they tend to occur mainly at the focus of the laser beam. This is the first advantage of nonlinear optical microscopy (NLOM) over standard optical microscopy that will bring very high axial resolution in images, leading to straightforward optical sectioning. In linear fluorescence microscopy the axial resolution is usually degraded due to the out-of-focus fluorescent background, unless special care is taken to reject such contributions, for example, by using a pinhole in front of the detector. A second advantage is limited photobleaching and photodamage, which increases the lifetime of a sample.

Two-photon excitation fluorescence (TPEF) microscopy has ushered the era of nonlinear optical microscopy, since the first introduction by Denk, Strickland and Webb in 1999 [15]. Similar to standard fluorescence microscopy, TPEF microscopy involves electronic excitation of fluorescent molecules in the sample and subsequent emission of photons at a shifted wavelength. However, two photons are absorbed in TPEF microscopy instead of one photon is absorbed by each molecule in standard fluorescence microscopy. Therefore, TPEF belong to a series of nonlinear optical phenomena.

Unfortunately, for many biological chromophores, non-radiative de-excitation channels efficiently compete with emission, rendering them effectively non-fluorescent [16]. This group includes melanin in the skin, hemoglobin in red blood cells, myoglobin in muscles, chlorophyll
in plants and others. Since fluorescence detection cannot be used for imaging these molecules, there is a high demand for imaging methods that do not rely on fluorescence, but are still sensitive enough to enable detectable signal from a small number of molecules within a biological sample.

Several modes of NLOM that do not depend on electronic transitions, and can therefore image unlabeled samples, have been proposed in recent years. Second-harmonic generation (SHG), third-harmonic generation (THG) and methods based on Raman scattering, including coherent anti-Stokes Raman microscopy and stimulated Raman microscopy, are explained below. Pump-probe microscopy and its application are also explained at last.

2-2 Second Harmonic Generation Microscopy

For SHG two input fields that vary harmonically with the same frequency \( \nu_1 = \nu_2 = \nu \) are combined by a second–order susceptibility \( \tilde{\chi}^{(2)} \) to yield an induced source at the sum frequency \( 2\nu \). Hence, the equation 2-1 can be written as

\[
\tilde{S}_{2\nu} = \tilde{\chi}^{(2)} (-2\nu; \nu, \nu) : \tilde{E}_\nu \tilde{E}_\nu
\]

Source \( \tilde{S}_{2\nu} \), in turn, will make a nonlinearily scattered field \( \tilde{E}_{2\nu} \) which represents the signal of interest in SHG microscopy.

Similar to a TPEF microscope, in an SHG microscope, the two input fields in equation 2-2 can be derived from the same laser beam since they are of the same frequency. As a result of focusing the laser beam, there will also be the 3D resolution capability for SHG similar to TPEF.

However, the main difference between SHG and TPEF is because of the fact that SHG is based on scattering. This means that SHG is a coherent phenomenon, whereas TPEF is based on fluorescence which is in-coherent. Hence, SHG and TPEF are expected to probe very different properties of the sample. Since coherent scattering is sensitive not only to the amplitude of the
illumination field but also to its phase [12] SHG depends much more intricately on both the excitation beam focus and the sample susceptibility distribution.

Susceptibilities $\mathbf{X}^{(M)}$ are tensors whose components characterize physical properties of a medium. If the medium exhibits a spatial symmetry, this symmetry must be present in $\mathbf{X}^{(M)}$. For such media that exhibits centrosymmetry, electric dipole approximation shows that all even ordered susceptibility tensors must vanish. This implies that only media that are noncentrosymmetric can produce SHG. This is another difference between SHG and TPEF where SHG can provide specific information about molecular orientation that cannot be achieved by TPEF.

Since SHG and TPEF can be obtained from the same instrument, complementary information can be found on noncentrosymmetric structure (SHG) and fluorescence (TPEF). Noncentrosymmetric structures are fairly common in biological tissue. Collagen, a structural protein, can exhibit striking SHG contrast due to its fibrilous geometry that can provide extraordinary large values of $\mathbf{X}^{(2)}$. Protein structures in muscle tissue, and microtubules present in cytoskeleton or in mitotic spindles also can exhibit SHG contrast without requiring tissue labeling. SHG imaging can also be used to image cell membranes. Here exogenous labelling is required because membranes themselves do not provide sufficient $\mathbf{X}^{(2)}$. Large electric fields on the order of $10^7 \, V/m$ across living cell’s membrane can lead to change in the centrosymmetric molecules’ effective second order susceptibilities, leading to change in their capacity to produce SHG signal.
2-3 Third Harmonic Generation Microscopy

Third harmonic generation (THG) microscopy can be considered as a natural extension of SHG microscopy to the third order susceptibility. In THG microscopy, the induced source at the sum frequency $3\nu$ is the result of three driving fields of frequency $\nu$, which can be shown as

$$\vec{S}_{3\nu} = \vec{X}^{(3)} (-3\nu; \nu, \nu, \nu) \vec{E}_{\nu} \vec{E}_{\nu} \vec{E}_{\nu}. \quad 2-3$$

The scattered field $\vec{E}_{3\nu}$ will rise because of the $\vec{S}_{3\nu}$ source. Similar to SHG, these fields are generally derived from a single laser beam. To obtain adequate power density pulsed lasers are considered for THG imaging.

Although THG and SHG look similar, they are different in many aspects. Obviously $S_{3\nu}$ in THG is proportional to $E_{\nu}^3$ instead of $E_{\nu}^2$ in THG. This implies that $I_{3\nu}$ is proportional to $I_{\nu}^3$ and as a result for same driving field frequency, THG is more tightly confined about the focal center than SHG. Hence THG will have a better 3D resolution.

Since THG relies on an odd-ordered susceptibility, it is not subject to a requirement of medium noncentrosymmetry. This is the second fundamental difference between THG and SHG which means scattering centers need not exhibit a preferred orientation to produce THG. Being free from noncentrosymmetry requirement, considerably broaden the variety of molecular species that can potentially produce THG. One might concern about the environment surrounding the sample to have a capability to produce background THG that might overwhelm a THG signal of interest. But since the THG cannot be generated from a focused beam in a large volume, this is not the case. Hence THG microscopy is typically background free. SHG microscopy on the other hand is background free for a different reason. Although SHG from a focused beam in a large volume is possible, it only can be detected if the volume exhibits noncentrocymetry; which is generally not the case in practice.
THG microscopy is mostly applicable for imaging of lipid vesicle trafficking in cells. Being densely packed, these lipid vesicles lead to strong third-order susceptibilities. They are also much smaller than a wavelength and because of being highly structured, they will have efficient THG [17].

2-4 Coherent Anti-Stokes Raman Scattering Microscopy

Coherent anti-Stokes Raman scattering (CARS) was reported by Marker and Terhune [18] for the first time and was subsequently established as a nonlinear optical microscopy technique by Sunney Xie [19]. Similar to THG microscopy, CARS microscopy is based on third-order susceptibilities, but with driving field of different frequencies.

In the CARS process, two beams interact with a sample via a wave-mixing process: a pump beam at frequency $v_p$ and a Stokes beam at frequency $v_s$. When the beat frequency $v_p - v_s$ matches the frequency of a Raman active molecular vibration, the resonance mode of the oscillators happens, hence a strong anti-Stokes signal is generated at $v_{as} = 2v_p - v_s$. The nonlinearly induced source $\vec{S}_{as}$ which gives rise to a scattered field $\vec{E}_{as}$ is given by

$$\vec{S}_{as} = \hat{X}^{(3)}(-v_{as}; v_p, v_p, -v_s): \vec{E}_p \vec{E}_p \vec{E}_s^*.$$  \hspace{1cm} 2-4

![Figure 2-1 Resonant and nonresonant CARS](image)
Among three realizations of equation 2-4 shown in Fig. 2-1 only the third one has a possibility of resonance enhancement which is useful for imaging. Here the difference frequency is adjusted to match the vibrational level of the specific molecule of interest and this molecule becomes preferentially revealed by CARS microscopy. The difference frequency can also be scanned, allowing the identification of many molecular species, which is called multiplex CARS. However, CARS microscopy is not background free and the aqueous environment itself can generate significant CARS background. Although the background might be nonresonant, it can affect detecting signal of interest.

There have been efforts to develop a background free CARS microscopy. Recording the CARS signal in the backward or epi direction which is referred as E-CARS is the most straightforward strategy. E-CARS is background free; however, it can only image sample structures that have very high spatial frequencies, such as interfaces or objects smaller than a wavelength. Polarization-sensitive detection is another strategy for background free CARS microscopy. It can also be combined with a clever use of pulse shaping of the pump and Stokes fields to introduce an additional phase mismatch that helps suppress non-resonant volume background [20].

CARS microscopy has been used to image vibrational modes of a variety of intercellular proteins, peptides and lipid droplets [21].

2-5 PUMP-PROBE MICROSCOPY

Various forms of pump-probe microscopy have been developed to image non-fluorescent chromophores. In these techniques including excited state absorption, stimulated emission, ground state depletion, and photothermal effect, generally a pump pulse is used to excite the
molecule of interest and subsequently the probe pulse interrogates the created transient state on a laser scanning microscope.

To achieve a high time resolution and catch the transient intermediates for non-fluorescent chromophores, pump and probe beams are in the form of ultrafast lasers. To manage a certain time delay between the pump pulse and probe pulse, they are temporally synchronized and spatially combined before being focused on the sample. This time delay is necessary to allow for dynamic evolution of the transient state. An acoustic or optical modulator is used to modulate the intensity of the pump beam at a high frequency, while the probe beam entering the objective is unmodulated.

![Figure 2-2 Input and output pulse trains of PPM](image)

The intensity of the probe beam is collected and detected by a photodiode after the interaction with the sample on the common focal volume. A lock-in amplifier will demodulate the readout of photodiode. The combined pump/probe beam is scanned across the sample point-by-point with a laser scanning microscope. A 3D image is then constructed with the amount of the modulation of the pump beam that is registered for each pixel. Input and output pulse trains of pump and probe beams, before and after interacting with the sample are illustrated in Fig. 2-2. Depending on the type of light-molecule interaction, the intensity of the probe beam could
increase or decrease. This gain or loss in the intensity will correspond to an in-phase or an anti-phase signal.

Signal strength $S$, is defined as the amount of intensity modulation generated to the originally unmodulated probe beam and is proportional to the pump beam intensity $I_{pump}$ and the probe beam intensity $I_{probe}$. Because of this quadratic intensity dependence, signal is only generated at the laser focus point, providing a 3d imaging capability for pump-probe microscopy similar to TPEF microscopy. This is also the reason that pump-probe technique is considered as a nonlinear optical microscopy.

Energy level diagrams of pump probe microscopy based on contrast mechanisms of excited state absorption, stimulated emission, and ground state depletion is shown in Fig. 2-3.
possible for the probe beam to interact with the chromophore on the excited state. Hence the modulated pump beam at frequency $f$ will result in a modulated lower intensity output probe beam at the same frequency $f$.

The light amplification aspect of stimulated emission has been used in stimulated emission microscopy for highly sensitive imaging of non-fluorescent chromophores. Such chromophores have very short-lived excited state and the excited state will de-excite through non-radiative decay instead of spontaneous emission. In the presence of a probe beam with the appropriate wavelength and after a certain time delay the excited chromophore is much more likely to be brought down to the ground state through radiative decay. Thus, there will be an increase in the probe beam intensity which can be extracted by frequency demodulation. Stimulated emission microscopy has been able to visualize chromoproteins, non-fluorescent variants of the green fluorescence protein, monitor lacZ gene expression with chromogenic reporter, and map transdermal drug distributions with histological sectioning [22].

In the ground state depletion microscopy, pump and probe pulses are both resonant with the absorption band of the chromophore of the ground state. Without the pump pulse, the absorbed probe pulse will be attenuated by the chromophore. When the chromophore is excited to the higher level by a pump beam, the absorption of the probe beam by the chromophore will be less, due to the depletion of the ground state population. Therefore, the intensity of the output probe beam will be higher in the presence of a pump beam. Ground state depletion microscopy has been able to detect an absorption signal from single gold nanoparticles and single organic dye molecules at room temperature [23].
Chapter 3: Super Resolution Pump-Probe Microscopy

3-1 Schematic of the Microscope

The setup of the super resolution pump-probe microscope (SRPPM) is shown in the Fig. 3-1. There are three beams shined on the molecules; two modulated pump beams at 500 nm and a probe beam 700 nm.

![Figure 3-1 Schematic of SRPPM](image)

A femtosecond (fs) Ti-Sapphire laser (wavelength 700-1100) nm is the laser source. To achieve a broader laser spectrum down to 500 nm, a nonlinear photonic crystal fiber (PCF) is pumped by the Ti/Sapphire laser.

After the wavelength extender (PCF), two 500 nm pump beams are divided by a splitter. The pump 1 beam is modulated by an acoustic-optic modulator at a few MHz, so that only a certain fraction of the pump pulses pass through as shown in the first row of Fig 3-2. The pump 2
beam is also modulated at same frequency of pump 1, and the pulse sequence is complimentary to pump 1 sequence as shown in the second row of Fig. 3-2.

![Pulse Sequences](image)

**Figure 3-2 Pulse sequence of SRPPM**

The pump 1 beam then passes through a $0-2\pi$ helical phase plate to obtain the doughnut shaped pattern at the focus shown in the left panel of Fig. 3-3. The pump 2 beam has a Gaussian mode in Fig. 3-3. The probe beam has a Gaussian mode as shown in the right panel of Fig. 3-3
These three beams will combine at the dichroic beam splitter and propagate through a polarizing beam splitter, then a quarter waveplate, and last the objective lens.

The probe beam pulse sequence will be synchronized to the pump sequence as shown in the third row of Fig. 3-2. The wavelength of both pump beams is the same and falls within the same transverse absorption band (around 500nm) of the gold nanorods. While the wavelength of the probe beam molecules locates in the longitudinal band of the gold nanorods (around 700nm).

Each laser pulse has a width about 100 fs. The delay between each pump-probe pulse pair will be a few hundred fs, which is shorter than the typical excited-state lifetime (ps to ns) of the chromophores [22]. This time delay can also suppress contributions from other instantaneous optical processes, such as two-beam two-photon absorption, cross-phase modulation and stimulated Raman emission.

**3-2 Super Resolution Capability of SRPPM**

The capability of super resolution comes from the fact that the pump 1 beam and the probe beam overlap only in the peripheral part of the focal point (Fig. 3-4(a)), while the pump 2 beam and the probe beam overlap in both peripheral and central parts of the focal point (Fig. 3-
If a molecule (red stars in Fig. 3-4(a) & (b)) that is much smaller than the focal point sits in the center of the focal area, it does not contribute to the stimulated emission process described in case 1 but it does contribute to the stimulated emission in case 2. If there is no molecule in the central area, the output probe beam intensity is the same in both case 1 and case 2. The probe beam intensity difference in case 1 and case 2 represents that the molecule’s contribution comes from the central area only, which is smaller than the diffraction limited focal point.

Therefore, by moving the sample using an automated translational stage within nanometer precision, we can achieve images beyond the diffraction limit. The real resolution will
be determined by the doughnut shape of the pump 1 beam. When two molecules are close to each other and fall within the normal Gaussian mode, they can’t be resolved by conventional microscope. With pump-probe microscope, in Case 1 both molecules are located in the peripheral area (Fig. 3-5(a)) that contribute to the stimulated emission process, and in Case 2 they are also contributing to the stimulated emission signal (Fig.3-5 (b)). Therefore, the detected modulation signal will be minimal which indicates there is no molecule in the middle area in Fig. 3-5 (a) & (b).
3-3 METHODS

Two stimulated emission are considered. In each of them the pump beam which is followed by a probe beam, will excite the molecules from the ground state to an excited state. The probe beam will then result in the stimulated emission of the excited molecules. Probe beam intensity will increase due to this stimulated emission, and this change in the probe beam intensity will be considered as the image of the sample.

![Energy diagram of a pump-probe experiment](image)

**Figure 3-6 Energy diagram of a pump-probe experiment**

3-3-1 Excitation

Number of the molecules on excited state $N_1$, are found by solving the corresponding differential equations with considering the initial conditions and physical parameters. The duration of excitation laser pulse $\Delta t$ and the absorption cross section of the molecules $\sigma_{abs}$, are physical parameters. In the following equations, the initial number of molecules on the ground state is shown as $N_{00}$, number of molecules on the ground state is $N_0$, and number of the molecules on excited state is $N_1$. By solving

$$\frac{dN_0}{dt} = -N_0 \frac{I_{pump}}{hv_{pump}} \sigma_{abs}$$

3-1

where
\[N_{00} = N_1 + N_0, \quad 3-2\]

we find

\[N_0 = N_{00} e^{-\frac{l_{pump}}{\hbar \nu_{pump}} \sigma_{abs} \Delta t}, \quad 3-3\]

and

\[N_1 = N_{00} (1 - e^{-\frac{l_{pump}}{\hbar \nu_{pump}} \sigma_{abs} \Delta t}). \quad 3-4\]

Here \(N_0\) is the number of molecules on Ground state after the laser pulse and \(N_1\) is the number of molecules on Excited state after the laser pulse. The absorption cross section \(\sigma_{abs}\), is considered to be \(10^{-16} \text{ cm}^2\) and the pulse duration \(\Delta t\), is \(200 \times 10^{-15} \text{s} \quad [22]\).

3-3-2 Stimulated Emission

There will be a time delay between pump and probe beam which will let the excited molecules to go through a vibrational relaxation to level 2 shown in Fig. 3-4. After an appropriate time delay, all the molecules are considered to be found on level 2 where the probe beam will bring them to level 3, resulting in the increase in stimulated emission. So the number of the molecules on level 2 is considered to be the same as excited molecules.

\[N_2 \approx N_1, \quad 3-5\]

Now by solving the differential equation for the stimulated emission, number of the molecules which will go through the stimulated emission \(N_3\), are found as

\[N_3 = N_2 \frac{l_{probe}}{\hbar \nu} \sigma_{stim} \Delta t. \quad 3-6\]

Again the pulse duration \(\Delta t\), is \(200 \times 10^{-15} \text{s}\) and the stimulated emission cross section \(\sigma_{stim}\), is considered to be \(10^{-16} \text{ cm}^2 \quad [22]\). By substituting \(N_2\) we will have:

\[N_3 = N_{00} (1 - e^{-\frac{l_{pump}}{\hbar \nu_{pump}} \sigma_{abs} \Delta t}) \frac{l_{probe}}{\hbar \nu} \sigma_{stim} \Delta t \quad 3-7\]
By integrating over the time for the pulse duration $\Delta t$, change in the probe beam intensity for each pulse will be found

$$\Delta I_s = N_{00}(1 - e^{-\frac{I_{pump}}{h\nu_{pump}} \sigma_{abs} \Delta t})I_{probe}\sigma_{stim}. \quad 3-8$$

Here $N_{00}$ corresponds to the distribution of the molecules on the focal plane and $I_{pump}$ and $I_{probe}$ are point spread functions of pump and probe beams respectively. Hence this $\Delta I_s$ will be a function of position.

### 3-3-3 Point Spread Functions of Beams

The pump 1 and pump 2 beams have different point spread functions. The pump 1 intensity on the focal plane is considered to be Gaussian with the 2-D point spread function:

$$I_{pumpG} = I_{pumpG_{max}} e^{-2\left(\frac{r}{\omega_0}\right)^2}, \quad \omega_0 = \frac{0.6\lambda}{NA} = \frac{\lambda}{2} \quad 3-9$$

2-D Point spread function on the focal plane for the doughnut shape beam Pump 2, can be written as the superposition of two Hermit–Gaussian beams of orders (1,0) and (0,1) of equal intensities [2], which will result in

$$I_{pumpD} = I_{pumpD_{max}} 8\left(\frac{r}{\omega_0}\right)^2 e^{-2\left(\frac{r}{\omega_0}\right)^2}, \quad \omega_0 = \frac{0.6\lambda}{NA} = \frac{\lambda}{2} \quad 3-10$$

Probe beam also has a Gaussian point spread function.

$$I_{probe} = I_{probemax} e^{-2\left(\frac{r}{\omega_0}\right)^2}, \quad \omega_0 = \frac{0.6\lambda}{NA} = \frac{\lambda}{2} \quad 3-11$$

Three beam profiles are plotted in Figure 3-7.
Figure 3-7 PSF of two pump beams and the probe beam
3-3-4 Signal Detection

In a pump-probe microscope the small change in the intensity of the probe beam is detected as the image, but here the more important is the difference of two changes in the intensities of two consecutive pump probe events, one with Gaussian pump beam and the other with doughnut shaped pump beam. If there is any difference between the outputs of two pump-probe events, it means that there is an image and it is a super resolution image. As mentioned in 3-2 the position of the molecules with respect to point spread functions on the focal plane is really important.

3-3-5 Defining Resolution in SRPPM

We introduce a method to predict whether there will be a super resolution image signal or not. In the simulations, we move a single molecule on the focal plane and calculate the change in the intensity of the probe beam in two pump-probe events

\[ \Delta I_{s1} = N_{00}(1 - e^{-\frac{I_{pump}G_{abs}^2}{\sigma_{abs}^2}})I_{probe}\sigma_{stim} \quad 3-12 \]

\[ \Delta I_{s2} = N_{00}(1 - e^{-\frac{I_{pump}D_{abs}^2}{\sigma_{abs}^2}})I_{probe}\sigma_{stim} \quad 3-13 \]

For the single molecule on the focal plane, \( N_{00} \) will be a Dirac Delta function and we move it 1.2 microns in one dimension. We calculate the difference of two gains for different positions of the molecule.

\[ \Delta I_{s} = \Delta I_{s1} - \Delta I_{s2} \quad 3-14 \]

So \( \Delta I_{s1}, \Delta I_{s2} \) and eventually \( \Delta I_{s} \) will be functions of position. Whenever two gains (\( \Delta I_{s1} \) and \( \Delta I_{s2} \)) are different enough, there will be a descent \( \Delta I_{s} \), the image will be detected and it will be a super resolution image.
To quantify the resolution in this setup, this $\Delta I_s$ is plotted versus the position of the molecule in Fig. 3-8. FWHM of this plot is introduced as the resolution in this microscopy where the signal will be detectable for the molecules on the focal plane lying in that range.

**Figure 3-8** $\Delta I_s$ vs. position of the molecule on the focal plane for SRPPM

Calculations show that the FWHM of the graph is less than 50 nm for the following maximum beam intensities on the focal plane:

- Gaussian Pump beam: 500 $KW/cm^2$
- Doughnut Pump beam: 10 $MW/cm^2$
- Probe beam: 10 $KW/cm^2$

Figure 3-9 shows the same graph sketched for the pump probe experiment with the same intensities for one Gaussian pump and the Gaussian probe beam. Comparing FWHM of two graphs shows the Super resolution capability of SRPPM.
3-3-6 Finding Best Intensities

Figure 3-8 shows that the intensity difference of two stimulated beams will be detectable in the central region and it will be beyond the diffraction limit, but it also shows negative values of $\Delta I_s$ for peripheral parts. The question is: will this negative amount of $\Delta I_s$ disturb the result?

The answer is yes. Suppose we have a uniform distribution of molecules on the focal plane instead of a single molecule. Now $\Delta I_{s1}$ and $\Delta I_{s2}$ will be integral of all the stimulated emission signals over the focal plane. Since there are molecules within the radius of 30 nm of the center, there will be a good $\Delta I_s = \Delta I_{s1} - \Delta I_{s2}$ of that region. But there are also molecules in the peripheral part which will result in negative amounts of $\Delta I_s = \Delta I_{s1} - \Delta I_{s2}$. This negative $\Delta I_s$ eventually will decrease the overall $\Delta I_s$ output and we won’t be able to detect molecules in the central part effectively. When the density of molecules is more in the peripheral part we could even lose the signal detection completely. Here $\Delta I_s$ is the super resolution image and it is

Figure 3-9 $\Delta I_s$ vs. the position of the molecule on the focal plane for PPM
important to preserve it as high as possible so it can be detectable by lock in amplifier. Now we have to think where does this negative $\Delta I_s$ come from, and how to solve this problem.

Figure 3-10 shows the probability of excitation for molecules on the focal plane for both Gaussian pump and Doughnut pump beams for the same Maximum intensity of 0.1 MW/cm$^2$ of those beams. It can be seen that in the central part the probability of excitation for molecules is significantly higher after the Gaussian beam than after the doughnut beam. However, in the peripheral part it is more likely for the Doughnut beam to excite molecules. The more probability of the excitation for molecules, the stronger signal we will get.

![Figure 3-10 Probability of excitation for two pump beams](image)

Now the origin of negative $\Delta I_s$ is obvious and to solve the problem we have to match two point spread functions. It will not be easy to change point spread function as we desire. However the problem could be solved by increasing the intensities and going to the saturated regime. Fig. 3-11 shows the probability of excitation for molecules on the focal plane for both Gaussian pump and Doughnut pump beams. In this graph, Maximum intensity for Gaussian beam and Doughnut beams are 15 MW/cm$^2$ and 1 MW/cm$^2$ respectively.
3-4 Calculations and Results

To find the best intensities for two Pump beams, $\Delta I_s$ for the single molecule at different positions on the focal plane is calculated. Figure 3-12 shows $\Delta I_s / I_s$ for the single molecule plotted versus the position of the molecule for different intensities of Gaussian pump and Doughnut pump beams.

It can be seen that by increasing the Doughnut pump intensity in each graph, the resolution of the image improves. The Intensity of the Gaussian Beam is increased 10 times in each step from $10 \, kW/cm^2$ in Fig. 3-12 (a) to $10 \, MW/cm^2$ in Fig. 3-12 (d). Increasing the Gaussian Beam intensity results in eliminating the negative part in $\Delta I_s$ which will make the highest possible detectable signal.
(a) Intensity of the Gaussian beam 0.01 $MW/cm^2$

(b) Intensity of the Gaussian beam 0.1 $MW/cm^2$
Figure 3-12 SRPPM signal profile for different intensities of two pump beams.

(c) Intensity of the Gaussian beam 1 $MW/cm^2$

(d) Intensity of the Gaussian beam 10 $MW/cm^2$
From the calculated data, the $10\ MW/cm^2$ intensity for Gaussian beam and $10\ MW/cm^2$ for the Doughnut beam will make the highest possible signal and best resolution. It can also be seen from the graphs that increasing intensities to higher amounts will not change the resolution significantly.
3-4-1 Resolution

For the appropriate intensity of 1 MW/cm$^2$ for the Gaussian pump beam, and 10 KW/cm$^2$ for the probe beam, FWHM of $\Delta l_s$ graph is plotted versus the intensity of the Doughnut pump beam in Fig. 3-13. This FWHM can be considered as resolution in SRPPM.

![Graph showing FWHM of $\Delta l_s$ vs. intensity of the doughnut pump beam](image)

Figure 3-13 FWHM of $\Delta l_s/l_s$ vs. intensity of the doughnut pump beam

The graph shows that the resolution of 50 nm is reachable for 5 MW/cm$^2$ (5 x $10^{11}$ W/cm$^2$ peak intensity) intensity of the Doughnut Beam.

3-4-2 Total Power on the Sample

By integrating the point spread function of each beam on the focal plane, total power on the sample is calculated. For the Gaussian pump with the average intensity of 10 MW/cm$^2$, Doughnut beam with 5 MW/cm$^2$ and Probe beam with 10 KW/cm$^2$ total power of each beam on the sample will be:
Gaussian beam: 8.3 mW
Doughnut beam: 6.5 mW
Probe beam: 0.02 mW

3-5 CONCLUSION

We have introduced a super resolution pump-probe microscope (SRPPM) which will break the diffraction limit for imaging non-fluorescent molecules by point spread function engineering. Although in the STED microscopy the excited molecules should go through a stimulated emission by huge intensities of the Doughnut beam, our calculations for SRPPM shows that the intensities of pump and probe beams will not exceed $10 \, MW/cm^2$ which is compatible with bio imaging goals for this microscope.
References


Vita

Farzaneh Mohajerani was born in Tehran, Iran. She graduated from NODET (National Organization for Development of Exceptional Talents) high school, Tehran, and got her Bachelor of Science in Physics from Sharif University of Technology, Tehran. In the fall of 2012, she joined the University of Texas at El Paso to pursue a Master of Science degree in physics. During her study at UTEP, she conducted research in biophotonics. She has been awarded C. Sharp Cook Graduate Fellowship (2013), and Graduate Academic Excellence Award (2014) from the Department of Physics at University of Texas at El Paso.