Super Resolution Microscopy - Breaking the Diffraction Limit

Radiological Research Accelerator Facility

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Outline

Motivation

Fluorescence Microscopy
- Multiphoton Imaging
- Microbeam II

Super resolution Microscopy
- Abbe Diffraction Limit
- STED Imaging
- gSTED Imaging

Conclusions & Future Work
Motivation

- Biomolecules that require imaging are typically 1-50nm in size
- Most super resolution imaging techniques cause damage to biological cells
Multiphoton Imaging

- Two photons at the same time and at the same place with doubled wavelength
- Two lower energy photons can combine to have the same effect as one higher energy photon.

Multiphoton Microscopy

**Advantages**
- Improved axial resolution
- Higher depth of light penetration
- Reduced out of focus bleaching
- Broader excitation spectra

**Limitations**
- Higher bleaching in the focus
- More costly
- Broader excitation spectra
Multiphoton Imaging: Spatially Localized

- Single Photon Imaging (488 nm)
- Multiphoton Imaging (900 nm)
Microbeam II at RARAF

![Image of Microbeam II equipment]

**Diagram of Microbeam II Setup:**
- **Switch Mirror**
- **Scan Lens**
- **Scan Head**
- **Beam Expander**
- **Shutter**
- **Polarizing Cube**
- **Half-Wave Plate**
- **Beams Dump**
- **PMTs with Emission Filters**
- **Dichroic Mirrors**
- **Epi-Illumination Lamp**
- **Objective**
- **Specimen**
- **Coherent, Chameleon (140 fs, 705-950 nm)**

**Labeling:**
- **Laser**
- **Attenuator**

**Diagram Components:**
- **Intensity CCD Camera**
- **Switch Mirror**
- **Tube Lens**

The image illustrates the components and layout of the Microbeam II setup at RARAF, highlighting key elements such as the laser source, beam expander, shutter, and the path through the scan head and various optical filters and detectors intended for precise beam manipulation and specimen analysis.
Multiphoton Imaging

Standard Cell Image

Diffraction limited image of sub-diffraction beads

350 nm illumination => 253 nm
We are at the diffraction limit!
Multiphoton Imaging

Reset at Microbeam II
Multiphoton Imaging Reset

- 10µm green fluorescent bead
- 1010nm wavelength
Multiphoton Imaging Reset

- 10µm green fluorescent bead
- 1010nm wavelength

Image 1: 10µm scale
Image 2: 250nm scale
Motivation
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  - Multiphoton Imaging
  - Microbeam II
Super resolution Microscopy
  - Abbe Diffraction Limit
  - STED Imaging
  - gSTED Imaging
Conclusions & Future Work
Resolution is diffraction limited

“The microscope image is the interference effect of a diffraction phenomenon.”

Diffraction from different samples and interference between the diffractive beams gives rise to the image of the sample.

\[ d = \frac{\lambda}{2n\sin \alpha \sqrt{1 + I/I_s}} \]
The smallest resolvable distance \(d\) between two points may never be smaller than half the wavelength of the imaging light.

\[
d = \frac{\lambda}{2nsina\sqrt{1 + I/I_s}}
\]
Stimulated Emission Depletion (STED)

Super resolution technique

- Pulsed laser → excite fluorophores
- Depletion beam → force surroundings to ground state
- Resulting in → smaller point spread function → Greater Resolution!

Image: PubMed.gov
Stimulated Emission Depletion (STED)

- Not limited by the wavelength of light used
- Inhibiting the fluorescence at its outer part allows sharper image of focal point.

Image: Max Planck Institute for Biophysical Chemistry
Time Gated Stimulated Emission Depletion (G-STED)

Image: Optics Express
Fluorescence Lifetime Imaging (FLIM)

Building images through analysis of fluorescence lifetimes

- Separate fluorophores with similar spectra
- Minimize effect of photon scattering
Fluorescence Lifetime Imaging (FLIM)

Ti:Sa Chameleon Ultra II Laser

- Average Power 3 W
- Chameleon Tuning Range 680nm-1080nm
Fluorescence Lifetime Imaging (FLIM)

**PMT Amplifier**

- Rise Time: 0.23 ns
Fluorescence Lifetime Imaging (FLIM)

TimeHarp 260 Nano

- TCSPC Unit
- 250ps resolution
- Histogramming software
Fluorescence Lifetime Imaging (FLIM)

1) Data acquisition
2) Exponential fit of decay curves in each pixel
3) Lifetimes → color code
Conclusion

Breaking the 250nm diffraction limit

- Multiphoton Imaging ~250nm
- STED ~ < 20nm living cells
- g-STED
- FLIM

\[ d = \frac{\lambda}{2n \sin \alpha \sqrt{1 + \frac{I}{I_s}}} \]
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