Introduction to Two-photon Fluorescence Microscope

En-Kuang Tien
Introduction

• Two Photon Fluorescence Microscopy (TPM) greatly reduces photodamage and allows imaging of living specimens.
• TPM can image turbid specimens with submicrometer resolution down to a depth of a few hundred micrometers.
• TPM allows high-sensitivity imaging by eliminating the contamination of the fluorescence signal by the excitation light.
• TPM can initiate photochemical reaction within a subfemtoliter volume inside cells and tissues.
• TPM is perfect for fluorescence microscopy on relatively thick specimens.
Introduction

• Two-photon excitation Principle - first proposed by Maria Göppert Mayer(1931).
• Denk et al. investigated the potential of imaging two-photon excited fluorescence in a scanning microscope with ultrafast pulsed lasers(1990).
• The phenomenon of two-photon excitation arises from the simultaneous ($10^{-18}$ seconds) absorption of two photons in a single quantitized event.
• Fluorescence emission varies with the square of the excitation intensity.
• The photon density must be approximately one million times required to generate the same number of one-photon absorptions (mode-locked pulsed lasers).
Physical Basis for Two-Photon Excitation

The transition probability between the molecular initial state \(|i\rangle\) and the final state \(<f| :\)

\[
P \sim \left| \sum_m \frac{\langle f | \vec{E}_\gamma \cdot \vec{r} | m \rangle \langle m | \vec{E}_\gamma \cdot \vec{r} | i \rangle}{\varepsilon_\gamma - \varepsilon_m} \right|^2
\]

\(\vec{E}_\gamma\): Electric field vector of the photons.
\(\vec{r}\): Position operator.
\(\varepsilon_\gamma\): Photonic energy.
\(\varepsilon_m\): Energy difference between the state \(m\) and the ground state.

The dipole operator has odd parity (i.e. absorbing one photon changes the parity of the state), and the one-photon transition moment \(\langle f | \vec{E}_\gamma \cdot \vec{r} | i \rangle\) dictates that the initial and final states have opposite parity. The two-photon moment \(\langle f | \vec{E}_\gamma \cdot \vec{r} | m \rangle \langle m | \vec{E}_\gamma \cdot \vec{r} | i \rangle\) allows transition in which the two states have the same parity.
Optical Properties

$$NA = \sin(\alpha)$$

$$I(u, v) = \left| 2 \int_0^1 J_0(v \rho) e^{\frac{iu \rho^2}{2}} \rho d \rho \right|^2$$

where \( u = 4k \sin^2(\alpha/2)z \)

\( v = k \sin(\alpha)r \)

\( k = 2\pi / \lambda \)

One-photon point spread function (PSF) : \( I(u, v) \)

Because TPE depends on the square of incident photon flux

$$\Rightarrow$$ Two-photon point spread function : \( I^2 \left( \frac{u}{2}, \frac{v}{2} \right) \)
A comparison of one- and two-photon excitation.
Optical Properties

- **Three-Dimensional Localization of the Excitation Volume**
  - Femtoliter size focal volume
  - Excitation probability $\propto I^2$, the absorption happens near focal plane
  - Discriminate the fluorescence outside the focal plane
  - Greatly improved axial depth discrimination and improvement in image contrast.
  - Reducing the region of photointeraction significantly and decreases total specimen photobleaching

- **Reduced Attenuation in Biological Specimens**
  - Rayleigh scattering: the scattering cross-section $\propto 1/\lambda^4$
  - The tissue “optical window” at 700–1000 nm.

- **High Signal-to-Background Ratio Fluorescence Detection**
  - the excitation wavelength is much farther and highly efficient filters can be applied to eliminate the excitation.
Two-Photon Microscope Instrumentations

- **Laser Light Source**
  - Femtosecond pulse laser
    - Mode-Locked Ti-Sapphire
      - 700~1000nm tunable wavelength
      - 100 fs pulse at 80 MHz repetitions rate
    - Cr-LiSAF Laser
  - Pulse-compressed Nd-YLF lasers

- **Picosecond pulse laser (lower excitation efficiency)**
  - Mode-locked Nd-YAG(100 ps)
  - Mode-Locked Ti-Sapphire
  - Pulsed-dye lasers (1 ps).

- **Continuous-wave (cw) laser**
  - ArKr laser
  - Nd-YAG laser
Two-Photon and Laser Sources

The number of photons absorbed per fluorophore per pulse is given by:

\[
n_a \approx \frac{p_0^2 \delta}{\tau_p f_p^2} \left( \frac{(NA)^2}{2\hbar c \lambda} \right)^2
\]

\( \tau_p \): the pulse duration.
\( \delta \): the fluorophore's two-photon absorption at wavelength.
\( p_0 \): the average laser intensity.
\( f_p \): the laser's repetition rate.
\( NA \): the numerical aperture of the focusing objective.
TPM System

Optical Components

Mode-locked Ti-Sapphire laser

X-Y scanning Mirror and beam expander

Dichroic mirror

Z-scan

Objective lens

Photon Sensor

TPM system diagram
Advantages and Limitations of Two Photon Fluorescence Microscopy

• Advantages
  – Deep Sectioning
    • Absence of out-of-focus absorption.
    • The infrared excitation light suffers less scattering.
  – Lack of out-of-focus absorption increase image contrast.
  – Reduce the volume of photodamage and photonbleaching.
  – Most fluorophores function fairly well with two-photon excitation illumination.

• Limitations
  – Image Resolution is not better than confocal microscope for the longer excitation wavelengths.
  – The photodamage at focal point is severe.
Components setup at Room 206
Two-Photon Microscope Instrumentations

• Instrumentations:
  – Microscope Model:
    • Olympus IX71 Research Inverted System Microscope
    • Olympus BX51 Research System Microscope
  – Exciting Laser Source:
    • Titanium Sapphire Laser (Tsunami, Spectra Physics), λ = 700~1000nm, Power ~ 800mW
  – Objectives:
    • U Plan Apochromat objective 20X
    • U Plan Apochromat objective 20XO
    • U Plan Apochromat objective 40XOI
    • Plan Apochromat objective 60XO
    • U Plan Apochromat water Immersion objective 60XW
    • Long working distance UM Semi Apochromat water Immersion objective 40XW
    • Long working distance UM Semi Apochromat water Immersion objective 60XW
• **Specification:**
  – Image Scanning Resolution: max 512x512 pixels.
  – Z-Scanning Range: range: 400 microns, resolution 1.25nm.
  – Images acquire speed: 0.3 frame/sec with 256x256 pixels.
  – Detection: PMT Gain=1x10⁶, Dark Count=80S⁻¹.

• **Techniques offers:**
  – Optical section of live or slide based specimens
  – Two-photon excitation fluorescence imaging and deep tissue imaging
Applications

• Optical section of live or slide based specimens
• Two-photon excitation fluorescence imaging and deep tissue imaging, \textit{in vivo} imaging.
• Two-photon Imaging of NAD(P)H
• Uncaging - Three-dimensionally resolved photorelease of caged compounds.
References

• http://www.microscopyu.com/
• http://www.olympusmicro.com/
Applications

- Two-Photon Polymerizations
  - Use photosensitive materials to fabricate complicated three-dimensional (3D) microstructures.

SEM micrometer-scale image of (a)Venus fabricated by 2PP. (b)photonic crystal structure fabricated by 2PP. (J. Serbin, et al., Laser Zentrum Hannover e.V., Hollerithallee 8, D-30419 Hannover, Germany)