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Development of a Two-Photon Laser Scanning Microscope

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Background

One- and Two-Photon Fluorescence

Electronic transitions occur when a molecule absorbs a photon with energy E=hν equal to the difference in energy levels, called single photon excitation (SPE). Proposed by Maria Göppert-Mayer in 1931 and experimentally verified in 1961, it is possible for two photons each with half the energy of the transition to both be absorbed in a single quantum event, called two-photon excitation (TPE).[1-3] After being excited to S_1, the electron relaxes and emits a photon of a different energy. This is called fluorescence.

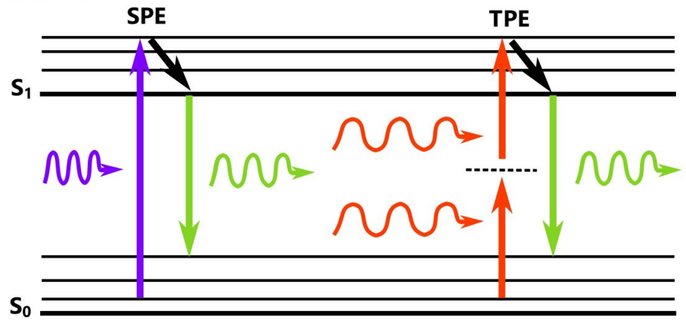

Figure 1. Jablonski energy diagram showing equivalent electronic transitions by single photon excitation and two-photon excitation.

The probability of absorption is measured by the cross section and is dependent on the temporal and spatial densities of photons. The TPE cross section for a molecule is typically many orders of magnitude smaller than that for SPE, so high laser intensities are necessary to obtain frequent TPE.[3]

Laser Scanning Fluorescence Microscopy

Laser scanning microscopes (LSM) scan a sample with a laser. The beam is directed down through a light microscope to excite fluorophores in a sample on the stage, shown in figure 3. Emitted photons are then collected and used by a computer to generate an image, mapping the fluorophores in the sample. TPE microscopy was patented in 1990 by Winifred Denk and James Strickler.[4]

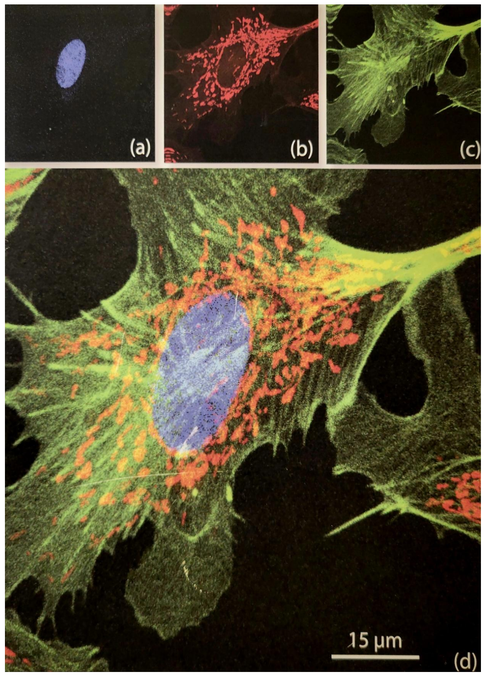

Figure 2. Bovine artery endothelial cells imaged by a similar far-field TPE LSM, courtesy of Derek Nowak.

Both SPE and TPE allow for resolution beyond the diffraction limit of light, $\lambda/2$, making it a valuable imaging technique. TPE is preferable to SPE for imaging biological samples due to high penetration of near-IR as well as lower excitation volume and photon energy, causing less damage to delicate samples. The high intensity necessary for TPE is achieved by using a mode locked (pulsed) laser so the peak intensity is high but the average intensity is low, saving resources and the sample.[2]

Methods

Research question: How can a Molecular Dynamics Sarastro 2000 confocal laser scanning microscope (CLSM) be cheaply converted into a system capable of two-photon imaging?

Optics

The main alteration to the physical system was the implementation of a Vitesse Ti:sapphire 800 nm pulsed laser. The laser beam is directed up from the table to the optical compartment above the light microscope. Many components of the original optical compartment could be used for TPE. The detector box consists of a barrier filter to block ambient light, particularly 800 nm light from the laser, as well as a secondary dichroic and other barrier filters to isolate different wavelengths of light produced by different transitions. The scan mirrors were completely replaced with a two-axis galvanometer (galvo) scan head.

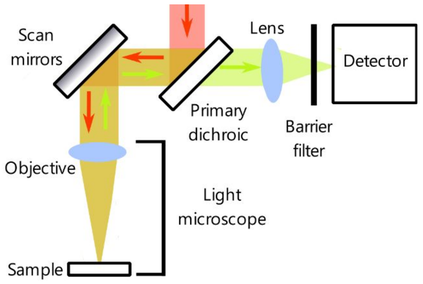

Figure 3. Simplified partial schematic of the scanning and detection portion of the TPE laser scanning microscope (side view).

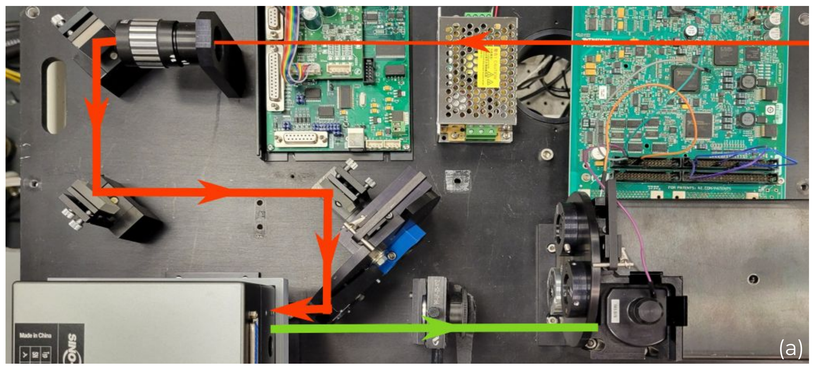

Figure 4. a) Top view of the current setup of the optical compartment and b) schematic representation of the same.

Electronics

The major electronic components of the TPE LSM are the computer, the control board for the galvo, the galvo, the PMTs that convert emitted light from the sample to an electrical signal, and a data acquisition board (DAQ) that interfaces with the computer.

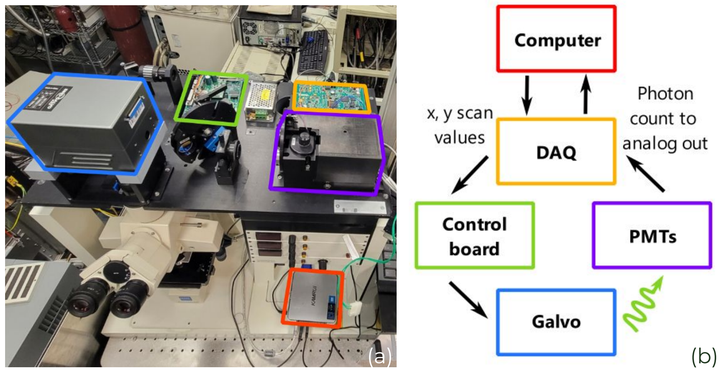

Figure 5. a) The TPE LSM system with major components highlighted and b) block diagram of their connections.

These components were chosen with use of particular in-house software in mind. The software is compatible with analog input and output, and digital compatibility would add an unnecessary layer of complexity to operation, so extra steps had to be taken to implement an all-analog system, including ordering a custom analog two-galvo scan head and control board. Though we plan to start with the fast x-scanning galvo and the slow y-scanning galvo to map out a two-dimensional raster scan, the scan system and control board include capability for x, y, z, and rotary axis control, allowing for the possibility of three-dimensional image compilation.

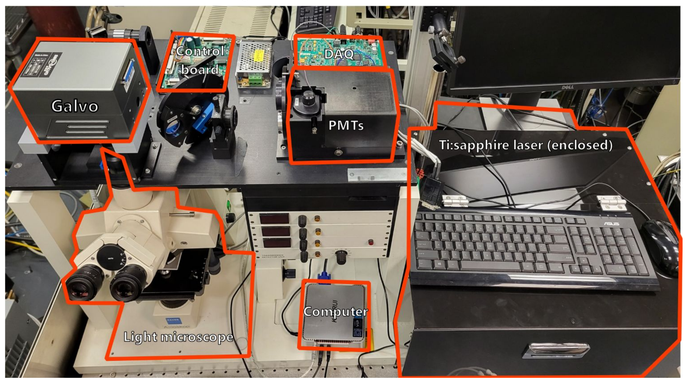

Figure 6. Complete setup as it currently stands with major components labeled.

Future Work

The electronics are currently being assembled. Though not strictly necessary due to the high power of the Vitesse, for efficiency, the path will be changed to bypass mirrors in the optical compartment since each mirror causes a significant loss of power. A camera can be mounted on the light microscope in order to also display the visual appearance of the sample in comparison to the fluorophore mapping. Future implementation of z-axis control for three-dimensional imaging is possible.

Sources

[1]M. G. Mayer, "Über die wahrscheinlichkeit des zusammenwirkens zweier lichtquanten in einem elementarakt," Die Naturwissenschaften 17, 932 (1929), english translation by Barry R. Masters.
[2]D. W. Piston, T. J. Fellers, and M. W. Davidson, "Multiphoton microscopy: Fundamentals and applications in multiphoton excitation microscopy,".
[3]B. R. Masters, "The origins of Maria Göppert's dissertation on two-photon quantum transitions at Göttingen's Institutes of Physics 1920-1933," in *Traditions and transformations in the history of quantum physics: HQ-3, Third International Conference on the History of Quantum Physics, Berlin, June 28-July 2, 2010*, 5, edited by C. L. Shaul Katzir and J. Renn (Edition Open Access, Berlin, 2013) Chap. 8, pp. 209–230.
[4]W. Denk, J. H. Strickler, and W. W. Webb, "Two-photon laser scanning fluorescence microscopy," Science 248, 73–76 (1990).

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