# Multi-photon technology beats the odds amid the laws of photon physics

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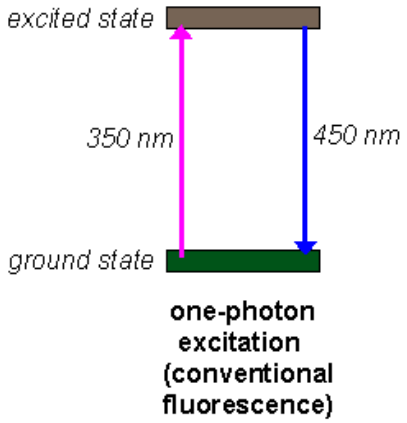
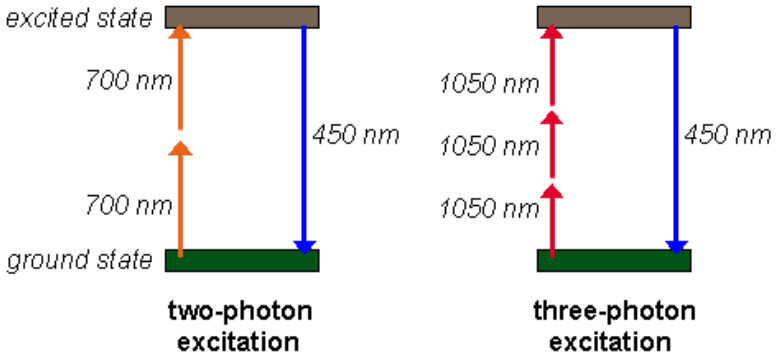
"In bright sun light, a molecule of Rhodamine B absorbs a photon through a one-photon absorption every second, a photon pair by two-photon absorption every 10 million years; no three-photon absorption is expected throughout the entire age of the universe." Denk and Svoboda, 1997 (1)

**Definitions**

When a fluorophore molecule absorbs a photon of light with sufficient energy, at a given wavelength, an electron from the ground state is boosted to an excited state. The subsequent drop back down to the ground-state results in the emission of another photon of light known as fluorescence




The fluorescence event can also be triggered by simultaneously absorbing the combined energy of two or three lower energy photons at double or triple the normal excitation wavelengths respectively. The term "multi-photon" refers to events involving two or more photons.



**Doing the trick**

Normally, the probability of having two or three photons hitting the fluorophore molecule, and be absorbed simultaneously, is almost nil. One could increase the odds by applying an extremely high laser flux to the sample, but risk destroying the sample before any appreciable two or three photon event could be detected. Cornell University physicist Watt Webb and colleagues, however, discovered a trick: to bombard the sample with extremely short laser pulses (femto-seconds) of near-infrared light (2, 3). Photon "doublets" and "triplets' could strike the focal point (and only at the focal point) over a brief enough time span to be considered simultaneous, and be absorbed, resulting in two and three photon excitation. Their work was first report 12 years ago as a conceptual breakthrough in two-photon excitation (2), which later extended to three-photon as well (3).

**Significance of multi-photon in live cell imaging**

Webb's work came at a time when confocal microscopy (another significant breakthrough in optical microscopy), was just undergoing an explosive expansion with new biological applications. Biologists were caught with the fascination of high resolution optical sectioning and 3-D reconstruction capabilities offered by the confocal technique. Not much attention was given to Webb's innovation, until his three-photon work was published in 1997 (3). The three-photon work was the first successful measurement of the neurotransmitter serotonin in secretory granules of live rat leukemia cells. Previously, serotonin could only be observed in fixed cells with UV illumination. Webb's team was able to induce three-photon UV excitation using near-infrared light. Suddenly, biologists realized the usefulness of this new technique in live cell imaging. It means:

- One could track protein molecules in live cells, without the need to use any harmful UV illumination, or to tag these molecules with fluorescent dyes (which could also be harmful to cells).
- One could view dynamic cellular events much longer and deeper into thick tissues (near-infrared light is less harmful and penetrates biological materials better, with less scattering).

# Main differences between confocal and multi-photon

Confocal Microscopy

- stringent excitation requirements: fluorophores with different spectral characteristics would each require a specific laser line for excitation.

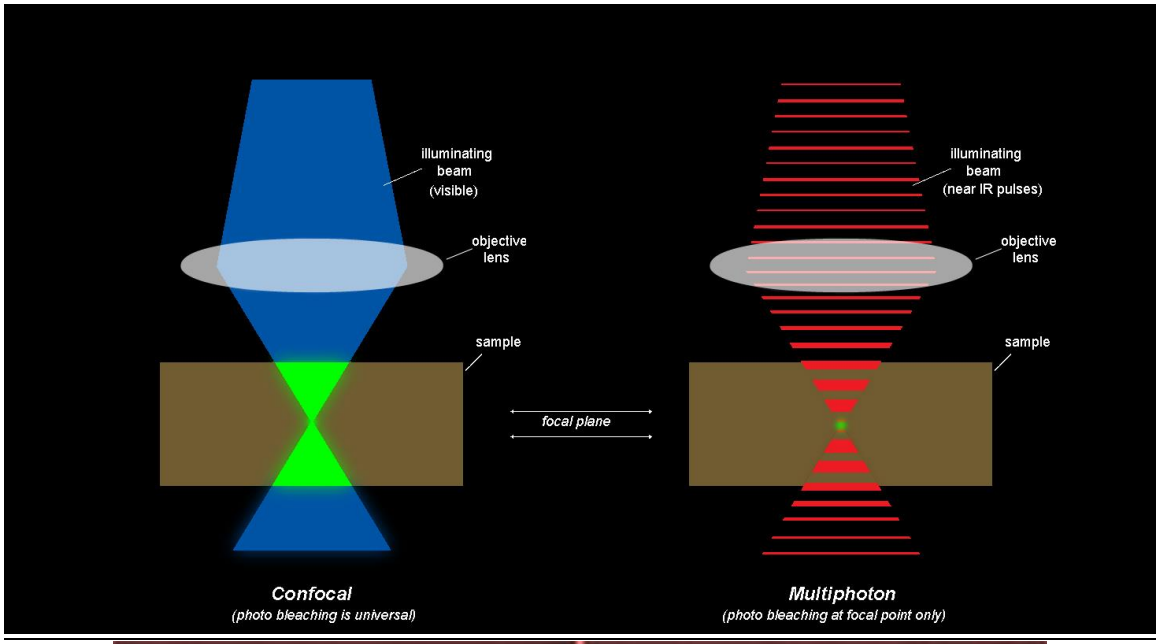
Multi-photon Microscopy

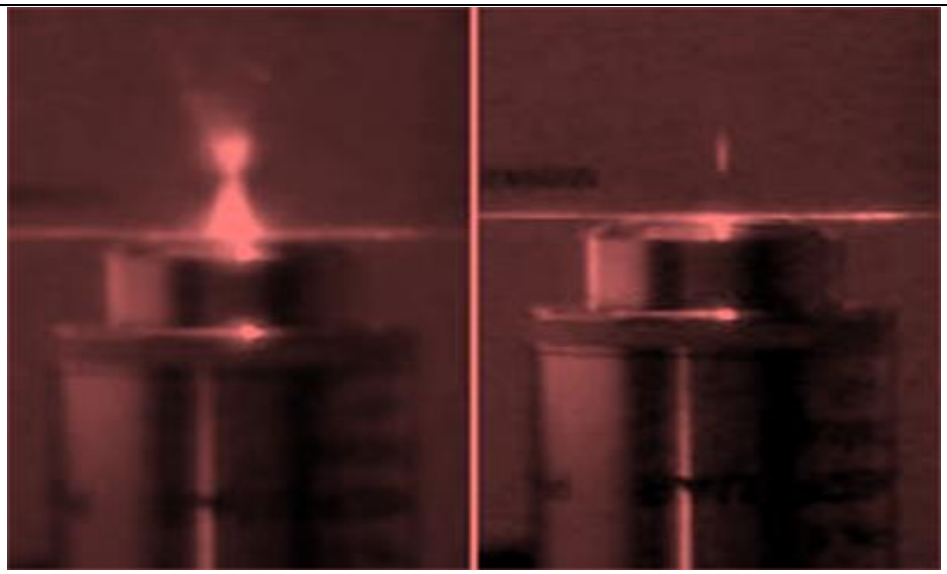
- excitation spectra are broad and forgiving. A single line of long enough wavelengths can simultaneously excite several fluorophores with different spectral requirements. eg. TRTIC, FITC and DAPI can be excited simultaneously with a single wavelength of 800 nm.*

*It is now known that the two-photon absorption spectra of most fluorophores are blue shifted (towards the shorter wavelengths) from their theoretical numbers (4)

- allows the whole specimen to be bombarded by higher energy, shorter wavelengths, excitation light. The whole specimen fluoresces. Photo-damage and photo bleaching of the whole specimen are universal.

- the excitation volume is highly localized (only at the focal point). Regions outside of this point, although get illuminated as well, have no fluorescence excitation or emission. Therefore photo-damage and photo bleaching are only limited to the focal point.

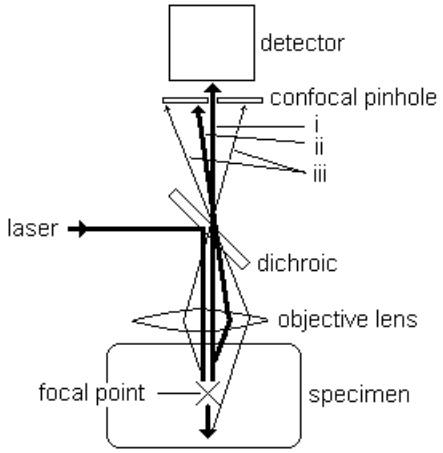


Confocal microscopy: Excitation occurs on the whole illuminated volume.

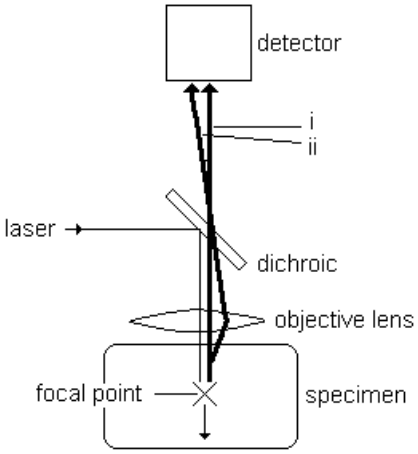
Multi-photon microscopy: only the focal point gets excitation.

- a pinhole in front of the detector is used to block off ‘out of focus light’ to achieve optical sectioning. Unfortunately, this pinhole also blocks off certain signals coming out from the focal plane but subsequently gets scattered in the sample.

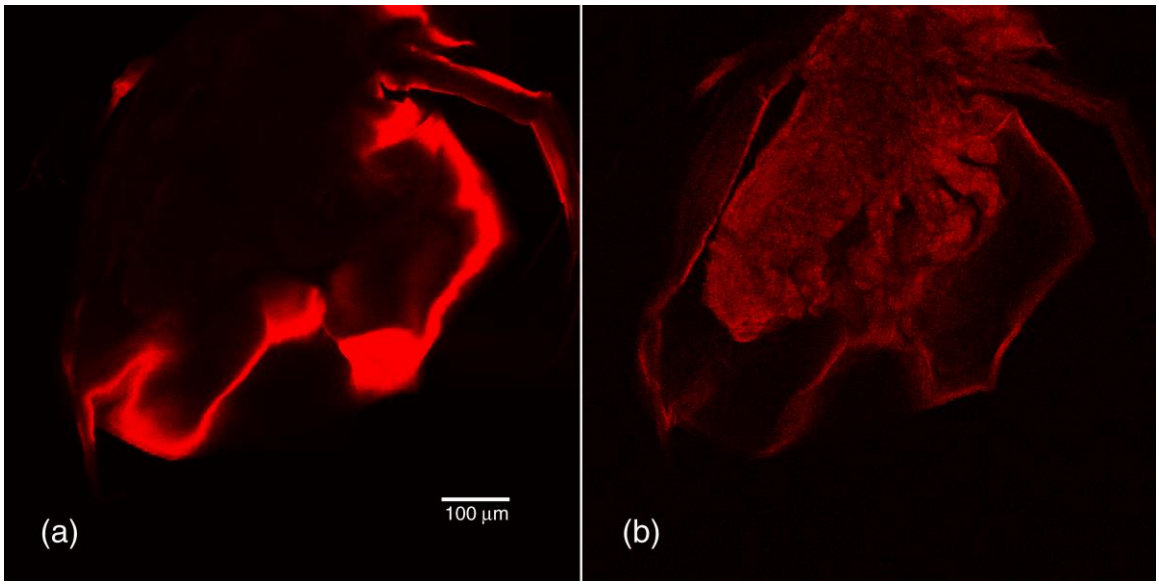



(diagram from Leung, 1997(5))
The confocal pinhole only allows un-scattered fluorescence emitting out from the focal point (i) to reach the detector. Fluorescence from out of focus regions (iii) as well as that emitting out from the focal point but subsequently gets scattered in the specimen (ii) are blocked off altogether.

- scanning the specimen along the plane of focus already produces an optical section. There is no need to place a pinhole in front of the detector and all signals coming out from the sample are collected, scattered or not, adding to the overall image contrast.



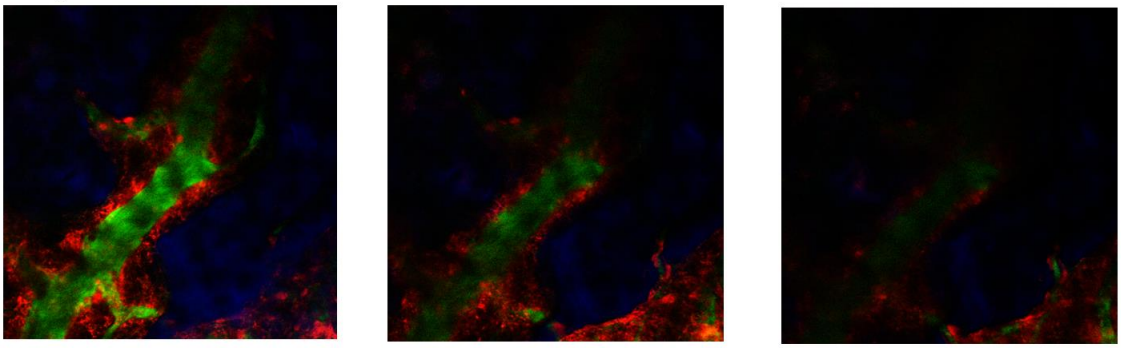

(diagram from Leung, 1997 (5))
Without the confocal pinhole, both un-scattered fluorescence (i) and scattered fluorescence (ii) emitting out from the focal point are being detected, increasing image contrast.



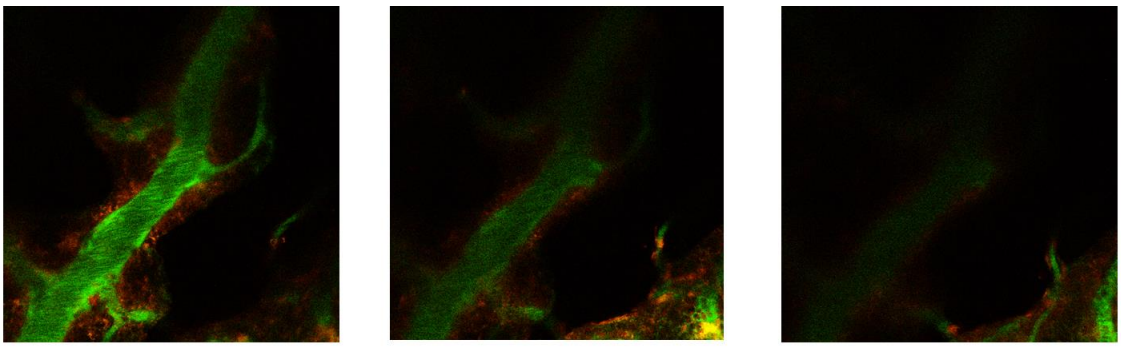

Imaging 150 µm deep into a water flea (*Daphnia sp.)*, eosin stained whole mount; emission wavelengths of ~600 nm. (a) Confocal mode, pinhole = 1.5mm; excitation wavelength = 568 nm. (b) Multi-photon mode, with an external detector, excitation wavelength = 800 nm.

## Comparison of two-photon and confocal imaging x-y

Two-photon excitation:



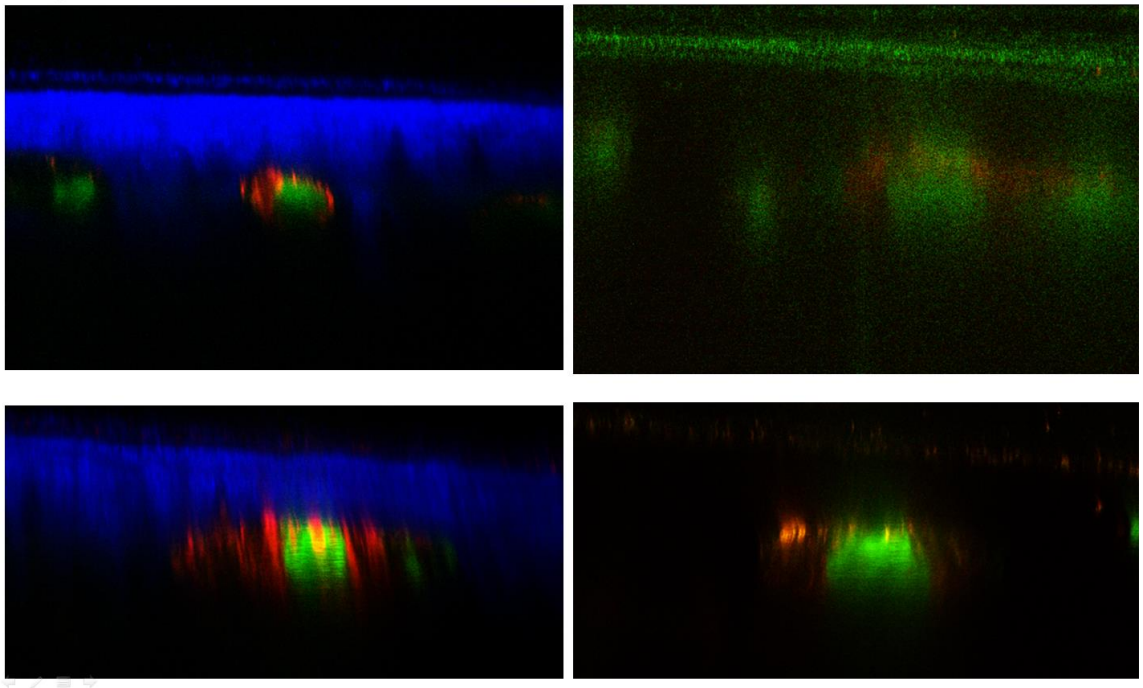
Confocal excitation:



## Comparison of two-photon and confocal imaging x-z

Two-photon excitation:

Confocal excitation:



**Areas of concern**

*Resolution*
Based on optical principles, multi-photon microscopy suffers from reduced theoretical resolution (using longer illuminating wavelengths) as compared to confocal microscopy. The difference is, however, submicron and not easily noticeable. The increased image contrast with better signal to noise ratio more than make up this shortfall.

*Photo-toxicity (the debate continues)*
Not much is known about the photochemistry in multi-photon excitation relative to cell viability. There is a general agreement that photo-toxicity is minimal and cells survive much longer under multi-photon imaging as compared to other imaging techniques. However, there is reason to believe that damage from the focal region alone could be severe. It has been reported that the photo-bleaching rate was indeed higher in two-photon cases than by single photons (6). Therefore, there is no benefit of imaging thin specimens with multi-photon. Samples containing dark pigments could also risk complete destruction (absorb too much infrared energy).

**What's next?**

Certainly, multi-photon excitation has left the physics lab and gone beyond the realm of photon physics. It offers many of the benefits that a confocal system has, and more. The small excitation volume and deep tissue penetration characteristics make multi-photon the ideal tool for studying the dynamics in living systems. New trends in molecular characterization using Fluorescence Resonance Energy Transfer (FRET) and Fluorescence Correlation Spectroscopy (FSC) will likely take advantage of the benefits offered by multi-photon. The imaging of second and third harmonic light emission (two- and three-photon events with a "twist"), may prove useful as a truly "non destructive" procedure, without photo bleaching. While some may vow about never going back to confocal, one should be reminded that multi-photon is not perfect. The technique is still in its infancy. As Webb himself put it, "There were a lot of unanswered questions and there may still be a long way to go." (4). However, multi-photon is gaining popularity rapidly. The next couple of years are expected to see an expansion of new applications. At the mean time, users should weight the pros and cons, based on their particular applications, to choose between confocal and multi-photon.

**References**

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4. Leung, H. (2001) Photonics West hosted the biggest multi-photon conference ever. Bulletin Micros. Society Canada 29(2):11-14.

5. Leung, H. (1997) Multi-photon excitation brings new high to fluorescence microscopy. Bulletin Micros. Society Canada 25(4):9-11

6. Leung, H. (2001) Confocal or multi-photon: Which one is better? Bulletin Micros. Society Canada 29(1):13-15.

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