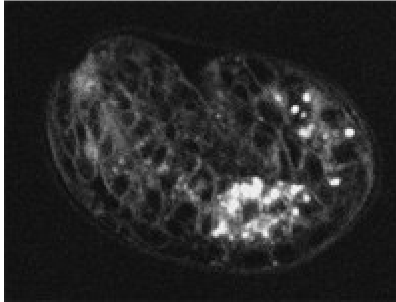
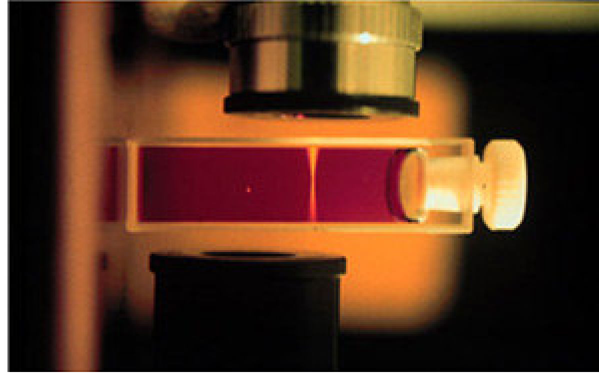
Multiple-photon excitation fluorescence microscopy

Multiple-photon excitation fluorescence microscopy is a technique that uses non-linear optical effects to achieve optical sectioning.



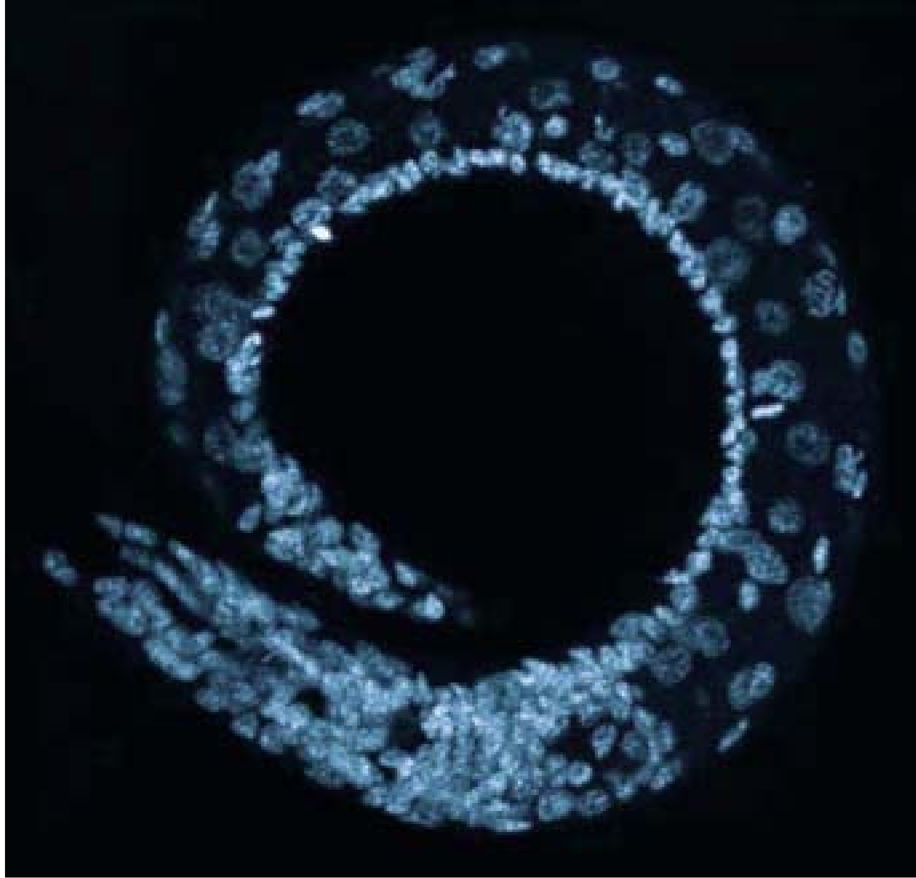
How it works: The sample is illuminated with a wavelength around twice the wavelength of the absorption peak of the fluorophore being used. For example, in the case of fluorescein which has an absorption peak around 500nm, 1000 nm excitation could be used. Essentially no excitation of the fluorophore will occur at this wavelength. However, if a high peak-power, pulsed laser is used (so that the mean power levels are moderate and do not damage the specimen), two-photon events will occur at the point of focus. At this point the photon density is sufficiently high that two photons can be absorbed by the fluorophore essentially simultaneously. This is equivalent to a single photon with an energy equal to the sum of the two that are absorbed. In this way, fluorophore excitation will only occur at the point of focus (where it is needed) thereby eliminating excitation of out-of-focus fluorophore and achieving optical sectioning.



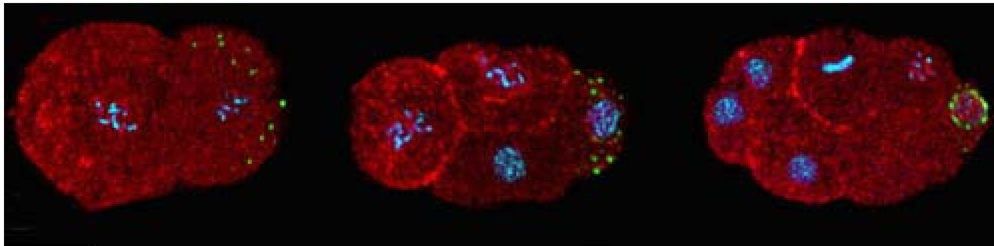
A cuvette of fluorescent dye excited by single photon excitation (right line) and multiphoton excitation (localized spot of fluorescence at left) illustrating that two photon excitation is confined to the focus of the excitation beam (courtesy of Brad Amos MRC, Cambridge).

2-Photon Animation

Three-photon excitation can also be used in certain circumstances. In this case three photons are absorbed simultaneously, effectively tripling the excitation energy. Using this technique, UV excited fluorophores may be imaged with IR excitation. Because excitation levels are dependent on the cube of the excitation power, resolution is improved (for the same excitation wavelength) compared to two photon excitation where there is a quadratic power dependence. It is possible to select fluorophores such that multiple labeled samples by can be imaged by combination of 2- and 3 photon excitation, using a single IR excitation source.



3-photon image of DAPI stained *C. elegans*



Combination 2-photon (red and green) and 3-photon (blue) image of C.elegans embryo

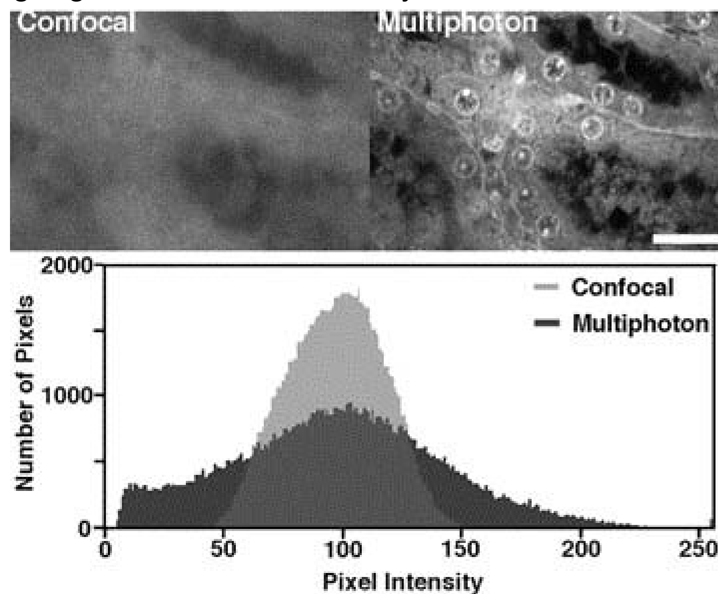
Advantages of multiphoton imaging:

Optical sections may be obtained from deeper within a tissue that can be achieved by confocal or wide-field imaging. There are three main reasons for this:

- the excitation source is not attenuated by absorption by fluorophore above the plane of focus
- longer excitation wavelengths suffer less scattering

- fluorescence signal is not degraded by scattering from within the sample as it is not imaged

When images of optical sections that are deep within a light-scattering sample are obtained using confocal microscopy, the fluorescence signal is attenuated by light scatter. Furthermore, some fluorescence originating from regions away from the point being instantaneously illuminated will be scattered such that this fluorescence will pass through the confocal pinhole thereby increasing background. Confocal imaging therefore suffers a deterioration in signal-to-background when obtaining images from deep within a sample. Multiphoton imaging is largely immune from these effects as little fluorescence is generated away from the point of illumination and all detected fluorescence photons may be used for imaging regardless of whether they have been scattered or not.



Images of acid fucsin stained monkey kidney taken at a depth of 60 µm by confocal (left) and multiphoton microscopy (right). Laser intensities were adjusted to produce the same mean photons per pixel. The confocal image shows a significant increase in local background resulting in a lower contrast image. However, the multiphoton image maintains contrast even at significant depths within a light scattering sample.(Reproduced from Figure 7 of Centonze,V.E and J.G.White. (1998) Biophysical J. 75:2015-2024)

Reduction in phototoxic effects

- Phototoxic effects can be problematic when imaging fluorescent probes *in vivo*. Non-radiative transitions of the fluorophore to the ground state can give rise to

highly reactive and destructive singlet oxegens. However, unlike confocal or wide-field fluorescence imaging, multiphoton imaging only excites fluorophore in the plane of the optical section being imaged. Therefore there is no fluorophore excitation in the bulk of the specimen. This considerably reduces the photo generation of toxic products.

- **A**ll the emitted photons from multi-photon excitation can be used for imaging (in principle) therefore no confocal blocking apertures or descanning optics have to be used allowing for a simpler, more light efficient optical design.
- **U**V flourophores may be excited using a lens that is not corrected for UV as these wavelengths never have to pass through the lens.

Limitations of multi-photon excitation

- **S**lightly lower resolution with a given fluorophore when compared to confocal imaging. This loss in resolution can be eliminated by the use of a confocal aperture at the expense of a loss in signal.
- **T**hermal damage can occur in a specimen if it contains chromophores that absorb the excitation wavelengths, such as the pigment melanin.
- **O**nly works with fluorescence imaging.
- **C**urrently rather expensive.