Intravital multiphoton microscopy
Principles and challenges

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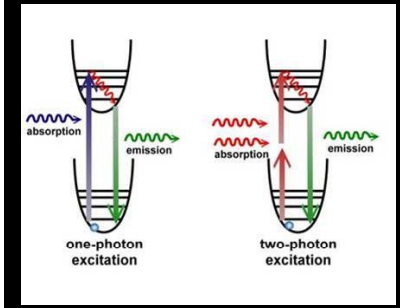
Indiana Center for Biological Microscopy
Indiana University Medical Center

Multiphoton fluorescence excitation
Requires an enormous density of photons



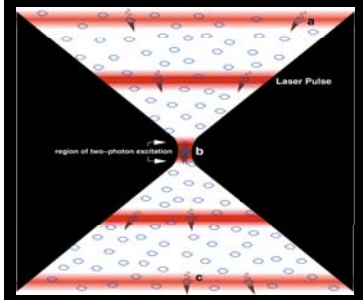
Biophotonics Imaging Lab, Univ. Illinois Urbana-Champaign

Fluorescence can be stimulated by the absorption of one photon of a particular energy level.

Fluorescence can also be stimulated by the simultaneous absorption of multiple, low-energy photons

"Simultaneous" means within ~ 10 attoseconds (10^{-17} seconds)
Two-photon fluorescence excitation requires an enormous flux of photons

Multiphoton fluorescence excitation
Requires an enormous density of photons



Dave Piston

Detectible two-photon fluorescence excitation requires peak power on the order of 200 GW/cm^2

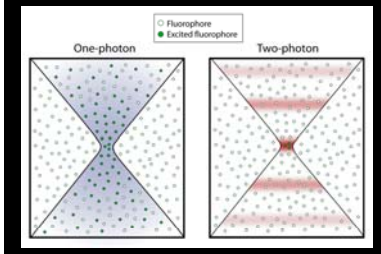
~ 300,000x the surface of the sun

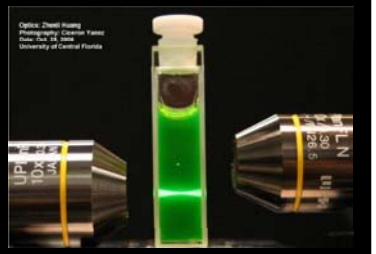
For multiphoton microscopy, this power is provided by focusing powerful pulsed lasers through high NA objective lenses.

Photon density decreases with the 4th power of distance from the lens focus

- Two-photon absorption occurs ONLY in the sub-femtoliter volume at the focus

Multiphoton fluorescence excitation
is restricted to the focus of a lens for microscopy

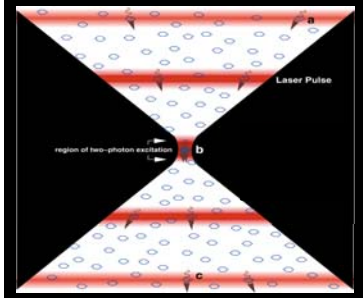




Multiphoton microscopy provides the capability to stimulate fluorescence in a specific sub-femtoliter volume in a sample -

High resolution, 3D microscopy

Multiphoton fluorescence excitation using pulsed (mode-locked) lasers

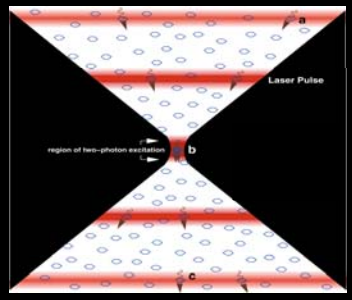


Dave Piston

Pulsing laser illumination into ~100 femtosecond pulses at a rate of 80 MHz provides peak power sufficient for multiphoton excitation, but average power low enough to minimize damage.

(e.g. 28 mW average provides 200 GW/cm^2 peak with 0.8 NA objective)

Misconceptions about multiphoton fluorescence excitation – Pulsing the laser limits the volume of excitation

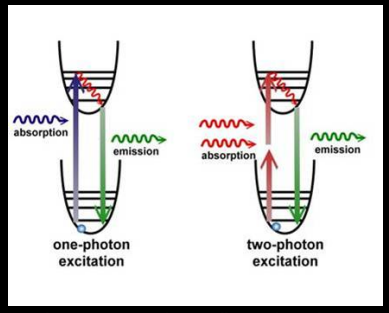


Dave Piston

Pulses are actually ~ 45 microns long and ~ 4 meters apart

To be accurate, the pulses as displayed here should be ~ 5 miles apart

Misconceptions about multiphoton fluorescence excitation - Two photon excitation occurs when a second photon is absorbed by an already-excited fluorophore



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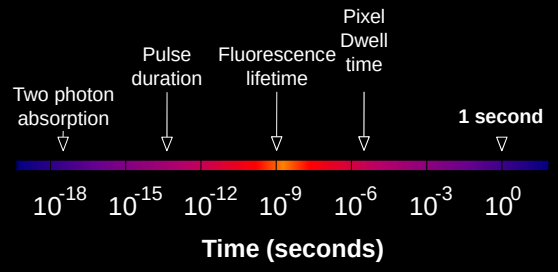
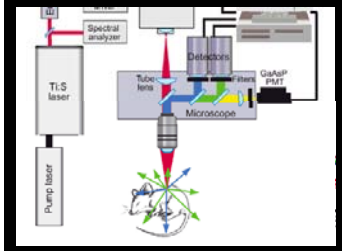
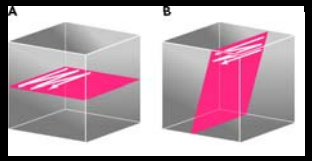


- Fluorescence lifetimes are ~ 1 billion times the length of the virtual intermediate state
- At 80 MHz, the laser pulses are spaced ~ 3 fluorescence lifetimes apart (serendipitous)
- Although illuminated only 1/80,000 of the time , a fluorophore will still be illuminated ~320 times during a 4 microsecond pixel dwell time

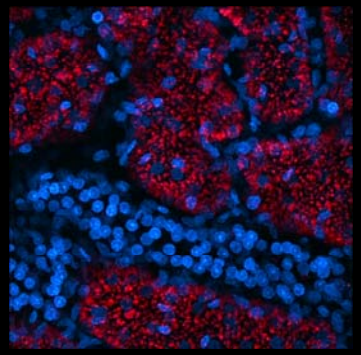
Making an image using multi-photon microscopy – point scanning



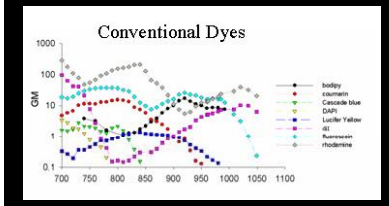
Zipfel et al., 2003

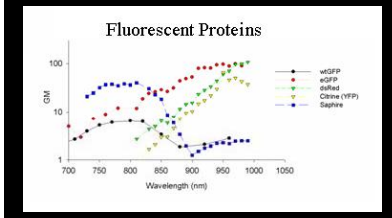


Göbel and Helmchen 2007

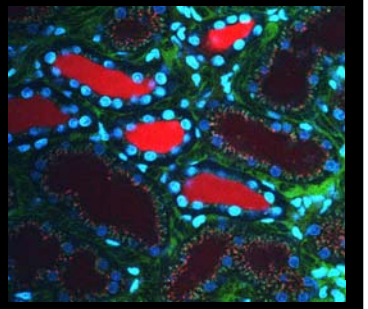


Two-photon action cross sections





Developmental Resource for Biophysical Imaging Opto-electronics, Cornell
(Watt Webb and Warren Zipfel)



10 kD Rhodamine Dextran,
500 kD Fluorescein Dextrn
Hoechst 33342

Dunn et al 2002

Fun facts about multiphoton microscopy

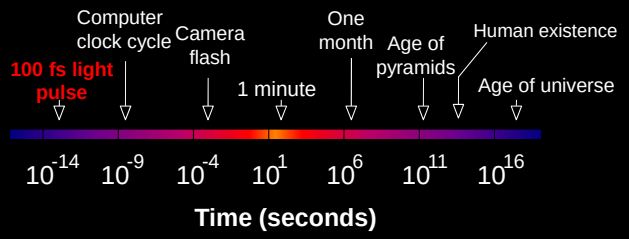
How frequently does multiphoton fluorescence excitation occur outside of a microscope?

Calculations made by Winfried Denk indicate that a molecule of rhodamine B exposed to direct sunlight will experience:

- A one-photon absorption around once per second.
- A two photon absorption once every 10,000 years.
- A three-photon absorption . . . never in the history of the universe.

Fun facts about multiphoton microscopy

Multiphoton fluorscence excitation typically utilizes mode-locked lasers producing pulses ~ 100 femtoseconds in duration

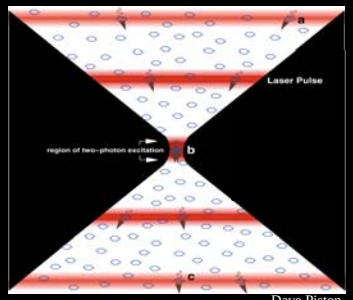


10 fs is to 1 minute as 1 minute is to the age of the universe.

Rick Trebino, University of Georgia

Fun facts about multiphoton microscopy

When the laser is on, it's mostly off.



Dave Piston

The sample is illuminated with 100 femtosecond pulses at a rate of 80 million times per second.
Only 0.001% of the time
For sense of scale, this duty cycle is equivalent to a one second pulse occurring once a day.

Two photon microscope designs

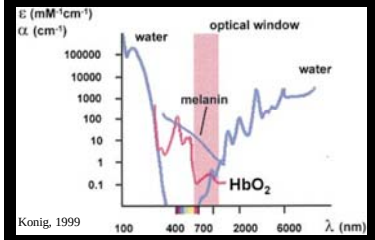
Why multiphoton microscopy?

Complex

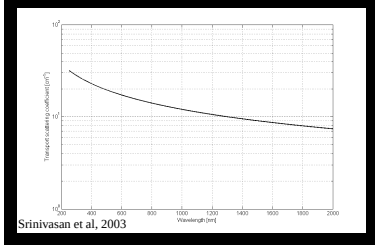
Expensive

Poorer resolution - 0.3 by 0.9 microns versus .21 by .75 microns

Why multiphoton microscopy?



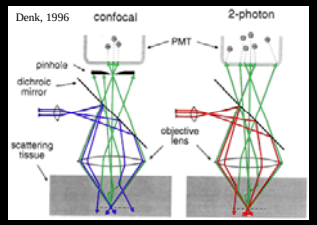
Konig, 1999

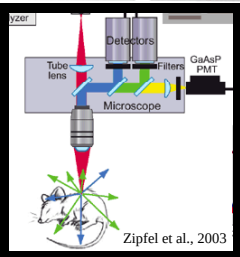


Srinivasan et al, 2003

- IR light penetrates deeper, with less damage

Why multiphoton microscopy?

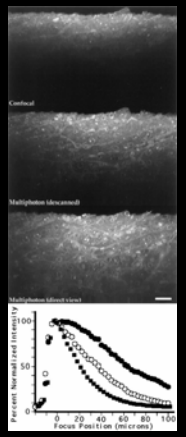




Zipfel et al., 2003

- IR light penetrates deeper, with less damage
- Optical sectioning without an emission aperture - less loss to scattering

Why multiphoton microscopy?



Centonze and White, 1998

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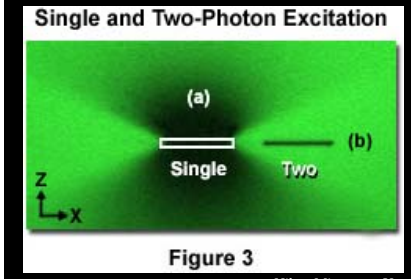


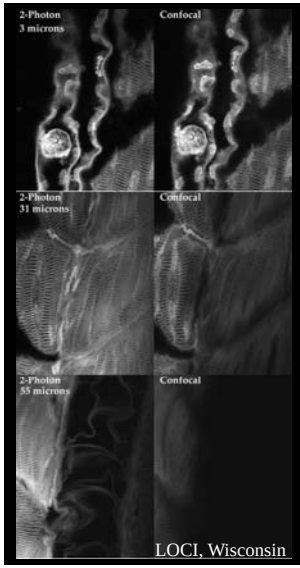
Nikon MicroscopyU

- IR light penetrates deeper, with less damage
- Optical sectioning without an emission aperture - less loss to scattering
- Photodamage only in the focal plane

Misconceptions about multiphoton fluorescence excitation - Multiphoton microscopy causes less photobleaching

Since only the focal plane is excited, image volumes can be collected without cumulative photodamage and photobleaching. Reimplanted hamster embryos develop normally after being imaged every 15 minutes for 24 hours.

However, the rate of photobleaching increases disproportionately with illumination, increasing with the third or even fourth power at high levels. Cellular toxicity increases abruptly at power levels above around 10 mW.



Why multi-photon microscopy? Deep-tissue imaging at high resolution

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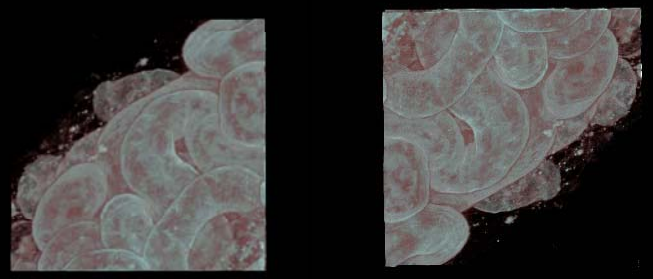
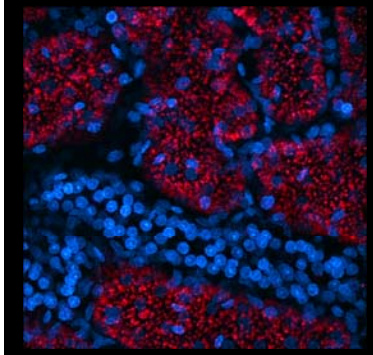


120 micron thick volume of mouse embryonic kidney - Carrie Phillips, Nephrology

Why multi-photon microscopy? Intravital microscopy



- Intravital microscopy provides submicron resolution and nanomolar sensitivity in under a second.
- Multiphoton microscopy has extended the reach of intravital microscopy to the scale of tissues and the functional components of organs.

Applications of intravital multiphoton microscopy in biomedical research

Brain function and pathology
- Neural development and activity - Winfried Denk, Karel Svoboda
- Neural activity and blood flow in epilepsy - Gyorgy Buzsaki
- Alzheimer's disease - Brian Bacskai, Brad Hyman
- Astrocyte-neuron signaling - Jan Nedergard
- Vascular function in the brain - David Kleinfeld

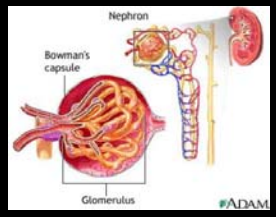
Tumor biology
- Tumor cell dynamics and metastasis - John Condelis, John Segal
- Angiogenesis/vascular function, gene expression - Rakesh Jain

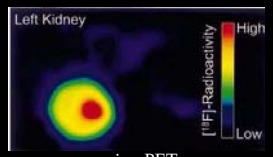
Immunology
- T-cell interactions and dynamics- Michael Dustin, Ullrich Von Andrian, Michael Cahalan
- Immune surveillance in the brain - Fritjof Helmchen, W.B. Gan

Liver function
- Mitochondria and liver injury - John Lemasters
- In vivo analysis of hepatobiliary transport - H.S Lee

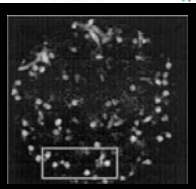
Kidney function
- Pathology and treatment of renal ischemia - Bruce Molitoris
- Pathobiology of diabetes - Katherine Kelly
- Mechanisms of renal septic injury - Pierre Dagher
- Microvascular function in renal injury - Tim Sutton
- Regulation of glomerular function - Janos Peti-Peterdi

In vivo imaging of the kidney

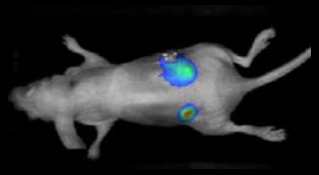




microPET
Johnstrom et al. 2002. Clin. Sci

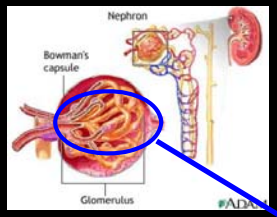


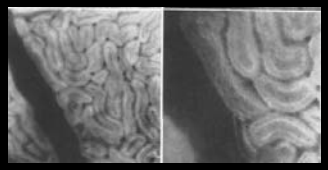
microCT
Toyota et al. 2004. Kid. Int.



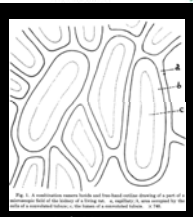
Bioluminescence
Que, Kajizel and Löwik, LUMC, Netherlands

Intravital microscopy of the kidney

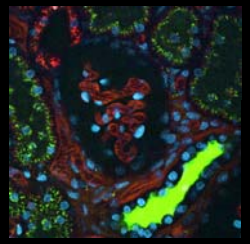




Wide-field microscopy
Steinhausen et al., 1963.



Wide field microscopy
Edwards and Marshall, 1924



Multiphoton microscopy

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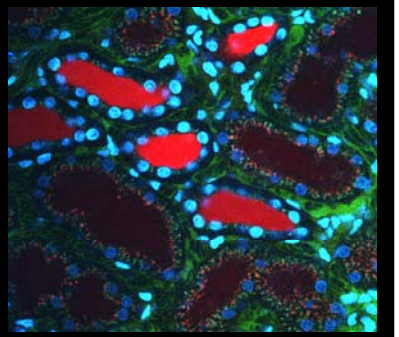
Dynamic intravital microscopy of the kidney

Ruben Sandoval and Bruce Molitoris

Intravital microscopy of multiple kidney functions via intravenous injection of fluorescent probes

Simultaneous imaging of a rat injected with fluorescent dextrans and Hoechst 33342 reveals multiple processes

- glomerular filtration
- proximal tubule endocytosis
- tubular solute concentration
- tubular flow
- capillary blood flow
- vascular permeability
- apoptosis
- tubular sloughing

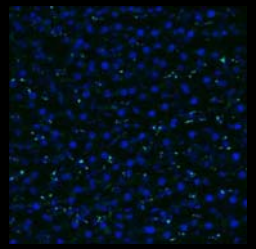


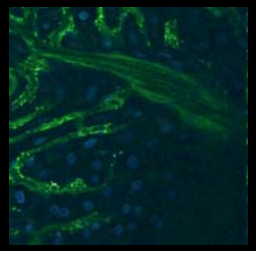
Dunn et al., 2002. AJP Cell. 283:C905-C916

Challenges of intravital microscopy

- Motion artifacts from respiration and heartbeat
- Slow image capture rate
- Limited depth at which useful images can be collected
- Discrimination of different fluors
- Introduction of fluorescent indicators

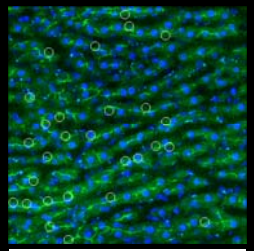
Importance of eliminating motion artifacts in intravital microscopy

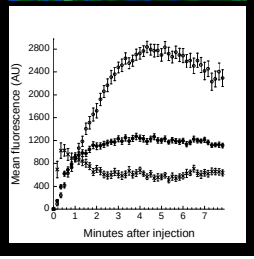




- Motion artifacts can obscure structures

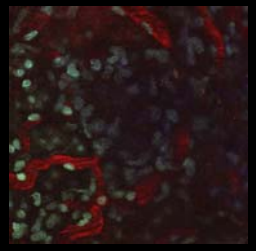
Importance of eliminating motion artifacts in intravital microscopy

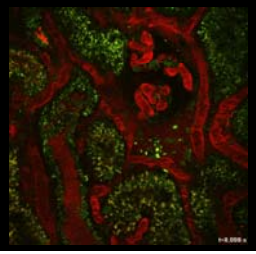




- Motion artifacts can obscure structures
- Motion artifacts can confound quantitation

Importance of eliminating motion artifacts in intravital microscopy

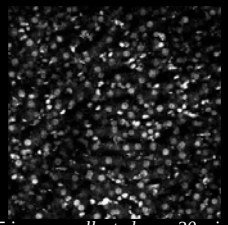




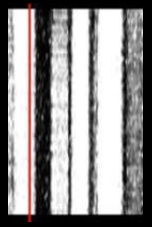
- Motion artifacts can obscure structures
- Motion artifacts can confound quantitation
- Motion artifacts complicate characterizing structural changes

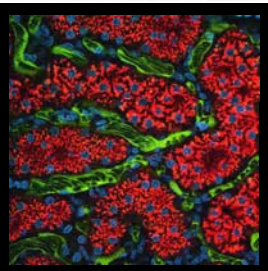
Kang, Toma, Sipos, McCulloch and Peti-Peterdi. 2006. AJP Renal

Reducing motion artifacts in intravital microscopy - Immobilize the organ



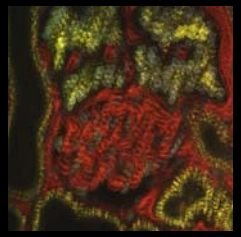
75 images collected over 30 min



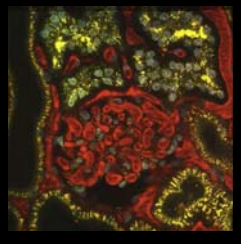


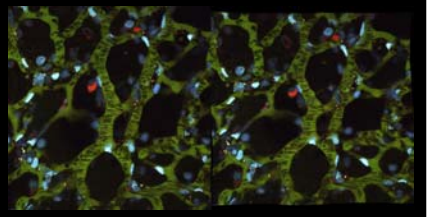
Reducing motion artifacts in intravital microscopy - Non-rigid image registration

Before



After





Before *After*

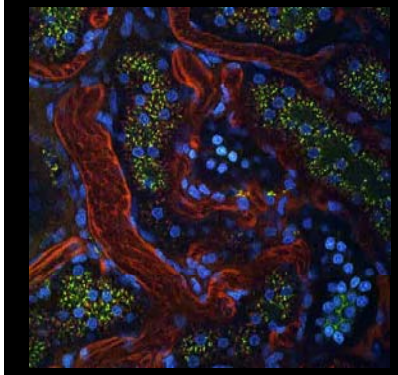
Presson, R., Brown, M., Sandoval, R., Dunn, K., Lorenz, K., Delp, E., Salama, P., Molitoris, B. and Petrache, I. Two-photon imaging within the murine thorax without respiratory and cardiac motion artifact. Am. J. Path. In press

Lorenz, K., Salama, P., Dunn, K. and E. Delp. Digital correction of motion artifacts in microscopy image sequences collected from living animals using rigid and non-rigid registration. Submitted

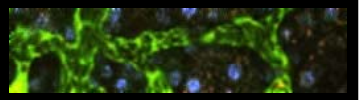
Challenges of intravital microscopy

- Motion artifacts from respiration and heartbeat
- Slow image capture rate

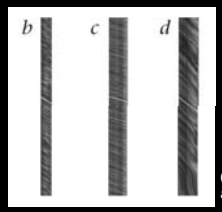
Single point scanning with a galvonometer One 512 by 512 frame per second



512 lines at 1 frame per second



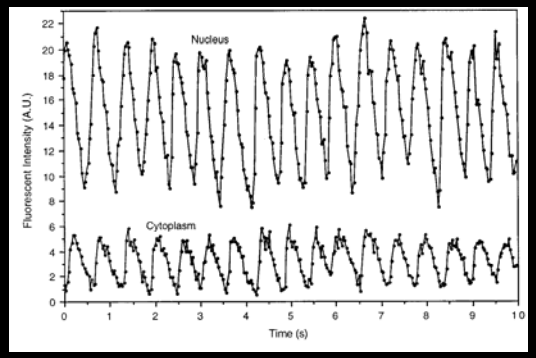
128 lines at 4 frames per second



(Brown et al., 2001)

1 line at 512 lines per second

Single point scanning with a resonant scanner
Calcium transients measured at 30 fps



Fan et al., 1999. Biophys. Journal 76:2412

Speed limits for multi-photon microscopy

30-fold higher frame rates require 30-fold shorter pixel dwell times which then requires 30 fold better signal. For many applications increasing the signal will require increasing the excitation.

- In many applications of multiphoton microscopy, power is already limiting – you may not be able to extract more signal with more power - especially since 30 fold increase requires 900 fold more power
- In many applications, multiphoton imaging is done at power levels very close to fluorophore saturation – you may not get proportional increases in signal
- Photobleaching increases at greater than the cube of power – disproportionately more photobleaching than signal with more power

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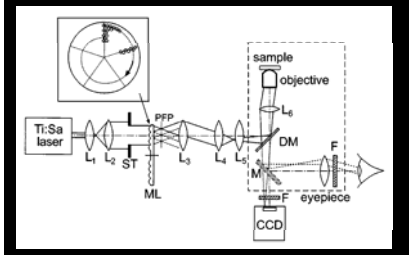
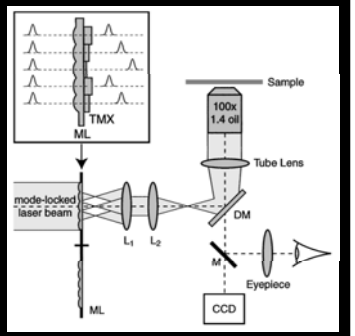
BUT, FOR SYSTEMS WITH SUFFICIENT SIGNAL, RESONANT SCANNERS ARE UNIQUELY CAPABLE OF QUANTIFYING DYNAMICS IN VIVO -

TRY THE THORLABS MULTIPHOTON SYSTEM AND THE NIKON AND LEICA CONFOCAL SYSTEMS

Multi-point scanning multiphoton microscopy



Bewensdorf et al., 1998. Optics Letters 23:655



Andresen et al., 2001. Optics Letters 26:75

Andresen system incorporates temporal delay between adjacent foci, avoiding overlapping excitation in out-of-focus planes

Speed limits for multi-photon microscopy

- Multi-focal systems require that power be divided among multiple excitation points - again, this presumes sufficient laser power
- Imaging onto a CCD results in scattered emissions being attributed to the wrong position in the image, increasing background and reducing resolution
- The system would thus be of limited utility for imaging deep into tissues, the conditions that justify multiphoton microscopy in the first place

Challenges of intravital microscopy

- Motion artifacts from respiration and heartbeat
- Slow image capture rate
- Limited depth at which useful images can be collected

Multiphoton microscopy
Attenuation of signal with depth in the kidney

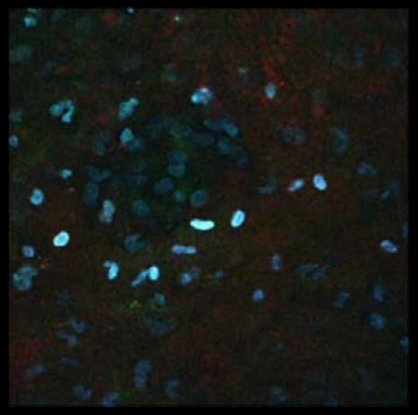
Glomeruli of most rats, mice are around 400 microns from the surface of the kidney

Cortico-medullary boundary is around 2 mm from the surface of the kidney

70 microns

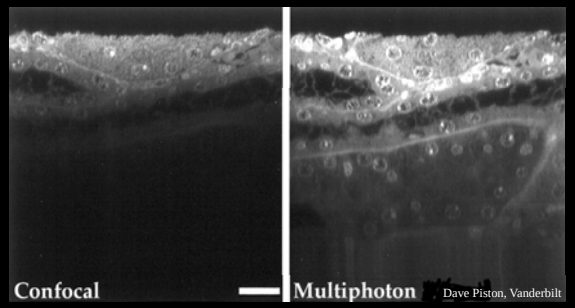
With a 60X NA 1.2 water immersion objective, signal attenuation prevents imaging deeper than around 100 microns in kidney of living rats

Multiphoton microscopy
Attenuation of signal with depth in the kidney



Ruben Sandoval

Multiphoton microscopy
Sources of signal attenuation of signal with depth



- Light absorption
- Light scattering
- Spherical aberration

Multiphoton microscopy
Light absorption as a source of signal attenuation with depth

Model light extinction as - $I_z = I_0 e^{-az}$

Where

I_z = intensity of light at depth z

a = the absorption coefficient

For fluorescence excitation

Near infrared light, a = .05 to 2 cm^{-1} in biological tissues

So transmission attenuated by 50% in 0.35 - 14 cm

But excitation is proportional to I^2,

Excitation is attenuated by 50% in .17 - 7 cm

- Excitation reduced by 50% in ~ 1700 to 70,000 microns - not a big deal

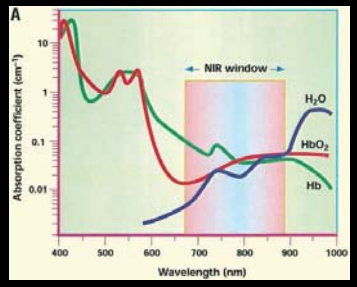
For collection of fluorescence emissions

For 550 nm light, a = 4 to 20 cm^{-1} in biological tissues

so transmission attenuated to 50% in 350-1730 microns

Most investigators agree, absorption is seldom a significant issue in biological tissues, except in skin (melanin) and under blood vessels (hemoglobin)

Multiphoton microscopy
Absorption of light by blood (hemoglobin)



For fluorescence excitation

a = 4 cm^{-1} at 780, - excitation attenuated to 50% at 860 microns

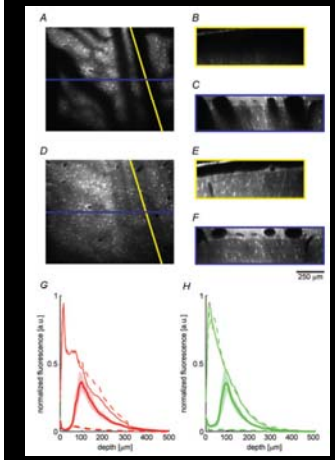
would lose < 1% over 10 micron capillary

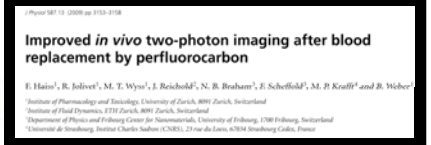
For collection of fluorescence emissions

a = 250 cm^{-1} at 550, transmission attenuated to 50% at 28 microns

would lose ~20% under a 10 micron capillary

Multiphoton microscopy
Absorption of light by blood (hemoglobin)




Improved *in vivo* two-photon imaging after blood replacement by perfluorocarbon

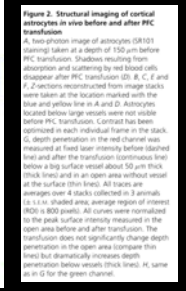


Figure 2. Structural imaging of cortical astrocytes *in vivo* before and after PFC transfusion

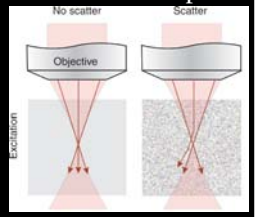
Multiphoton microscopy
Light scattering as a source of signal attenuation with depth

Model light extinction as - $I_z = I_0 e^{-sz}$

Where

I_z = intensity of light at depth z

s = the scattering coefficient



For fluorescence excitation

Near infrared light, s = 5 - 100 cm^{-1} in biological tissues

So transmission attenuated by 50% in 70 to 1400 microns

But excitation is proportional to I^2,

Excitation attenuated by 50% in 34 to 700 microns

Multiphoton microscopy
Light scattering as a source of signal attenuation with depth

Model light extinction as - $I_z = I_0 e^{-sz}$

Where

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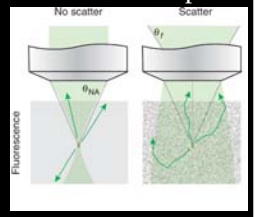
s = the scattering coefficient



For fluorescence excitation

Near infrared light, s = 5 - 100 cm^{-1} in biological tissues

So transmission attenuated by 50% in 70 to 1400 microns

But excitation is proportional to I^2,

Excitation attenuated by 50% in 34 to 700 microns

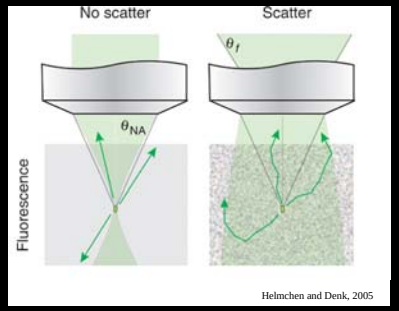
For collection of fluorescence emissions

For 550 nm light, s = 100 - 500 in biological tissues

So transmission attenuated by 50% in 15 - 70 microns

but even scattered emissions collected with non-descanned detectors

Multiphoton microscopy
Light scattering as a source of signal attenuation with depth

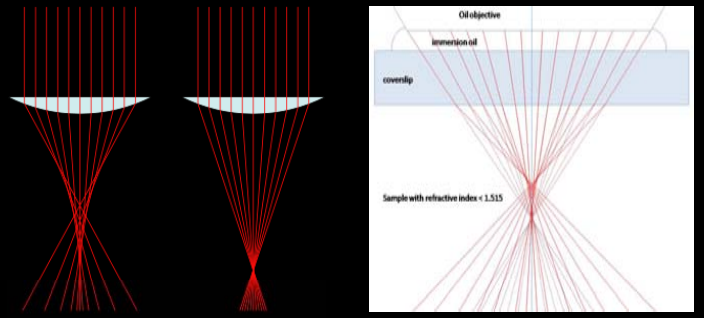


In most biological tissues there are effectively no ballistic emissions at 200 microns depth

Consequently, fluorescence arises from a diffuse region whose diameter is 1.5X the focal depth

Under these conditions, the collection angle is larger than the NA

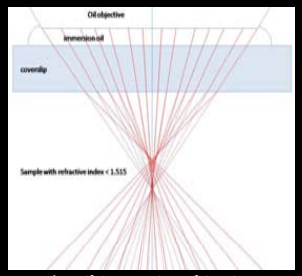
Multiphoton microscopy
Refractive index mismatch and spherical aberration



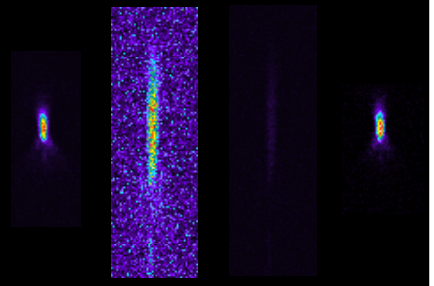
A lens with spherical aberration focuses axial rays and peripheral rays to different points.
Spherical aberration commonly results from refractive index mismatch.

Since the average refractive index of the kidney is ~1.4, we incur spherical aberration with either water immersion or oil immersion objectives

Multiphoton microscopy
Spherical aberration and signal attenuation with depth



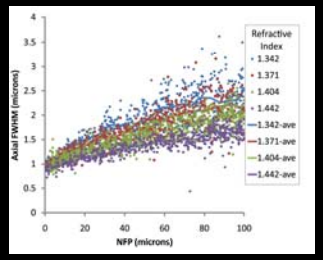
An oil-immersion objective imaging into an aqueous medium



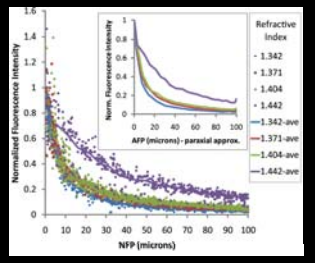
1.34 at surface | 1.34 at ~100um | 1.34 at ~100um (no contrast stretch) | 1.51 at ~75um

Since fluorescence is quadratically related to illumination, broadening of the focus by spherical aberration seriously decreases multi-photon fluorescence excitation

Multiphoton microscopy
Spherical aberration and signal attenuation with depth



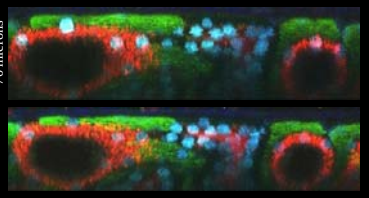
Effect of refractive index mismatch on size of the point spread function



Effect of refractive index mismatch on fluorescence intensity

Young, Pamela, Clendenon, S., Byars, J., Decca, R. and **K. Dunn.** 2011. The effects of spherical aberration on multiphoton fluorescence excitation microscopy. J. Microscopy.

Minimizing spherical aberration in living kidney – oil or water immersion objectives?

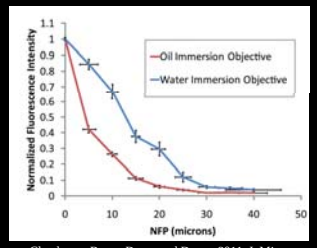


60X – Oil immersion

60X – Water immersion

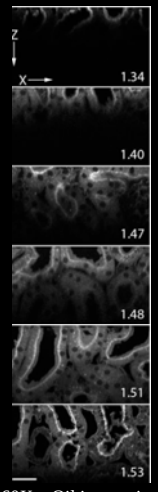
Ruben Sandoval

Fluorescent beads in fixed kidney mounted in PBS

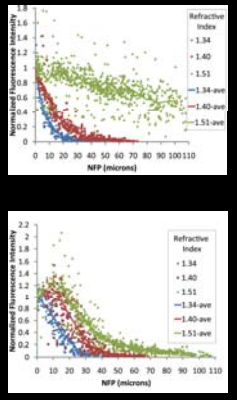


Young, Clendenon, Byars, Decca and Dunn. 2011. J. Microscopy

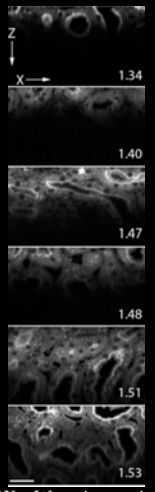
Predominant source of signal attenuation in kidney tissues?



60X – Oil immersion

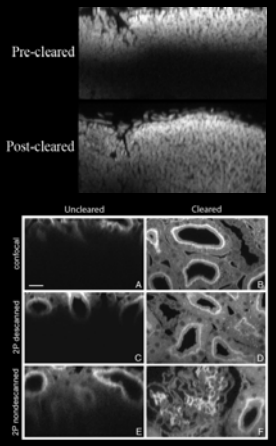


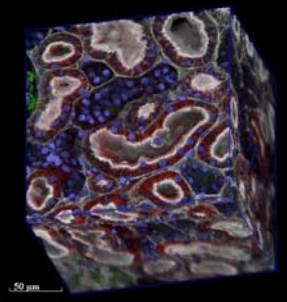
Young, Clendenon, Byars and Dunn. 2011. The effects of refractive index heterogeneity within kidney tissue on multiphoton fluorescence excitation microscopy. J. Microscopy.



60X – Water immersion

Tissue clearing - Elimination of refractive index mismatch and scattering

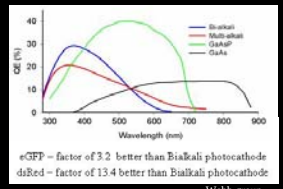




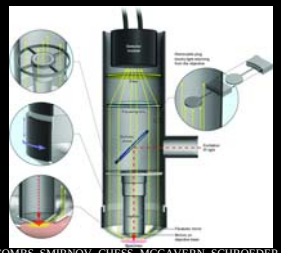
Clendenon, Ferkowicz, Young, and Dunn. Deep Tissue Fluorescent Imaging in Scattering Specimens Using Confocal Microscopy. Microscopy and Microanalysis. In press.

Limited optical reach in the living kidney Potential solutions

- Improve fluorescence collection



eGFP – factor of 3.2 better than Bialkali photocathode
dsRed – factor of 13.4 better than Bialkali photocathode
Webb group

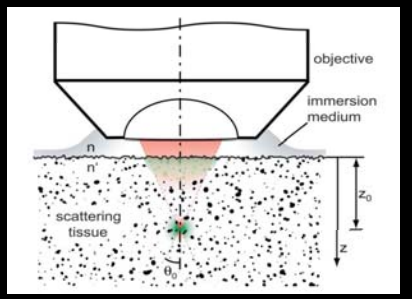


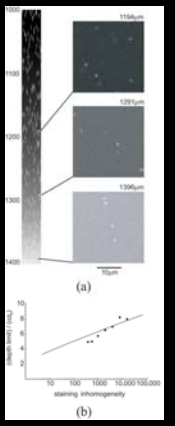
COMBS, SMIRNOV, CHESS, MCGAVERN, SCHROEDER, RILEY, KANG, LUGAR-HAMMER, GANDJBAKHCHE, KNUTSON, and BALABAN (2011), Journal of Microscopy

Limited optical reach in the living kidney Potential solutions

- Improve fluorescence collection
- Jack up the laser? Up to a point . . .

Fundamental imaging depth limit in two photon microscopy

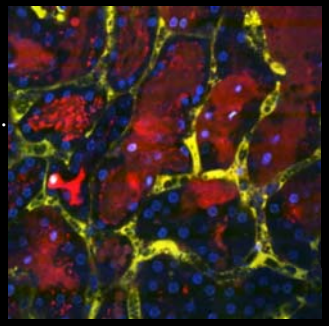




Imaging depth increases with labeling inhomogeneity, with decreasing pulse-width and with decreasing scattering anisotropy, but is ultimately limited by near-surface fluorescence - Theer and Denk, 2006

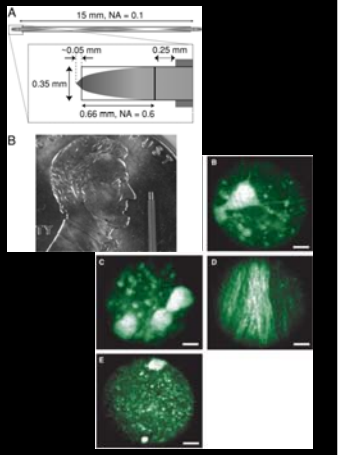
Limited optical reach in the living kidney
Potential solutions

- Improve fluorescence collection
- Jack up the laser? Up to a point . . .
- Invade the kidney - "cut down"



Limited optical reach in the living kidney
Potential solutions

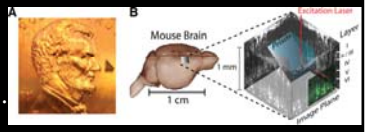
- Improve fluorescence collection
- Jack up the laser? Up to a point . . .
- Invade the kidney - "cut down", GRIN lens

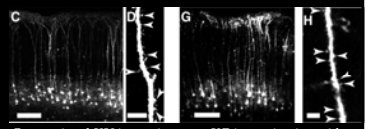


Levene et al., 2004, J. Neurophysiol

Limited optical reach in the living kidney
Potential solutions

- Improve fluorescence collection
- Jack up the laser? Up to a point . . .
- Invade the kidney - "cut down", GRIN lens, micro-prisms





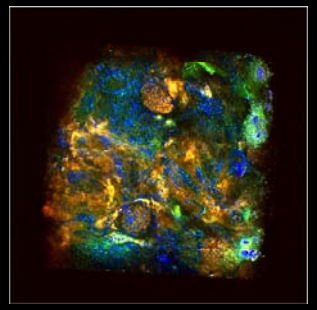
Conventional XY image in brain slice

XZ image in vivo with prism

Chia and Levene. 2009. Microprisms for in vivo multilayer cortical imaging. J. Neurophysio.

Limited optical reach in the living kidney
Potential solutions

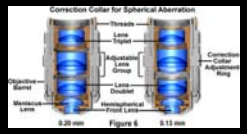
- Improve fluorescence collection
- Jack up the laser? Up to a point . . .
- Invade the kidney - "cut down", GRIN lens, micro-prisms

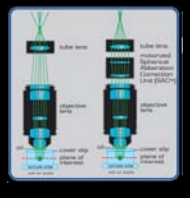


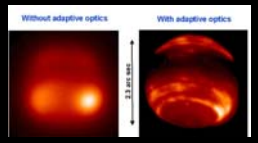
Gosia Kamocka and George Rhodes

Limited optical reach in the living kidney
Potential solutions

- Improve fluorescence collection
- Jack up the laser? Up to a point . . .
- Invade the kidney - "cut down", GRIN lens, micro-prisms
- Correct for aberrations - correction collars, adaptive optics

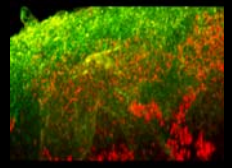


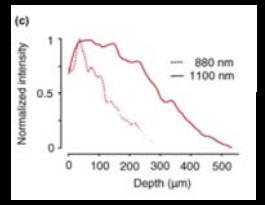




Limited optical reach in the living kidney
Potential solutions

- Improve fluorescence collection
- Jack up the laser? Up to a point . . .
- Invade the kidney - "cut down", GRIN lens, mini-prisms
- Correct for aberrations - correction collars, adaptive optics
- Longer wavelengths of light



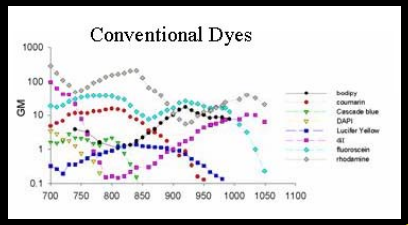


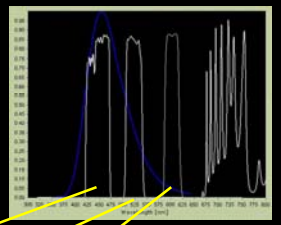
Andresen et al., 2009. Infrared multiphoton microscopy: subcellular-resolved deep tissue imaging. Curr. Opin. Biotech.

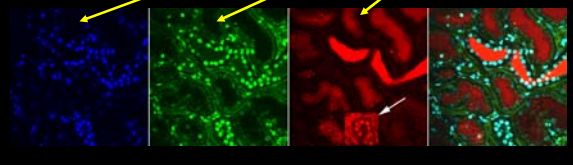
Challenges of intravital microscopy

- Motion artifacts from respiration and heartbeat
- Slow image capture rate
- Limited depth at which useful images can be collected
- Discrimination of different fluors

Exciting (and distinguishing) multiple fluors in multi-photon microscopy



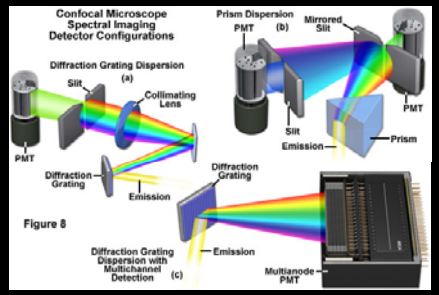




Fluorescence crosstalk correction

- Linear unmixing via spectral detectors

  Effect of scattered light?



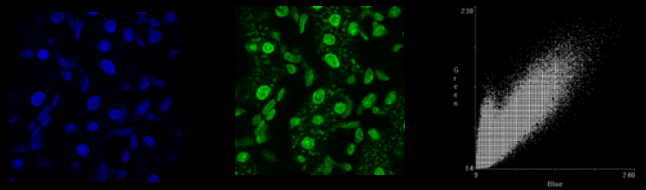
Fluorescence crosstalk correction

- Linear unmixing via spectral detectors

  Effect of scattered light?

- Subtracting a fixed percentage of one channel from another

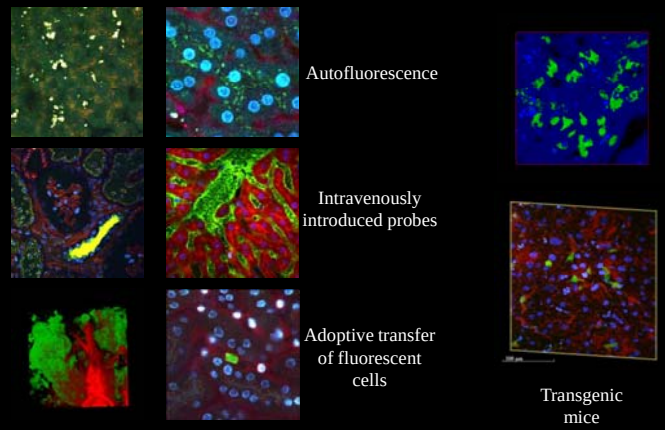
  Quantitative?



Challenges of intravital microscopy

- Motion artifacts from respiration and heartbeat
- Slow image capture rate
- Limited depth at which useful images can be collected
- Discrimination of different fluors
- Introduction of fluorescent probes

Sources of fluorescence contrast in intravital multiphoton microscopy



Further information

Websites

http://www.drbio.cornell.edu/ - Watt Webb's laboratory

http://www.microscopyu.com/ - a great general microscopy education website

http://www.aecom.yu.edu/aif/intravital_imaging/introduction.htm - John Condeelis's site

http://www.loci.wisc.edu - Laboratory for Optical and Computational Instrumentation

Reviews

Girkin, J. 2003. Optical physics enables advances in multiphoton imaging. J. Phys. D: Appl. Phys. 36:R250-R258.

Helmchen, F. and W. Denk. 2005. Deep tissue two-photon microscopy. Nature Methods 2:932-940

Zipfel, W., R. Williams and W. Webb. 2003. Nonlinear magic: multiphoton microscopy in the biosciences. Nature Biotech. 21:1369-1377.