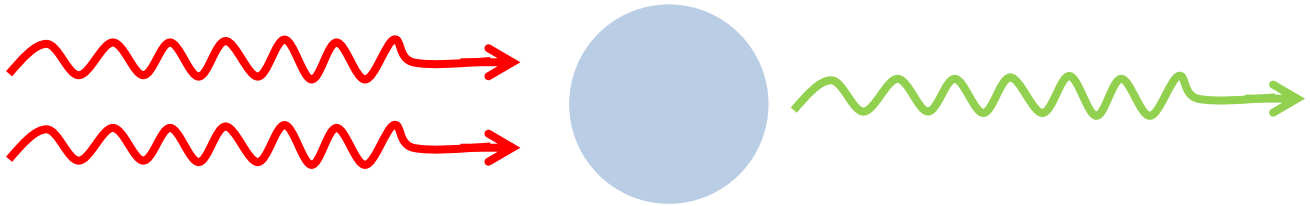
Lecture 2

Two-photon microscopy

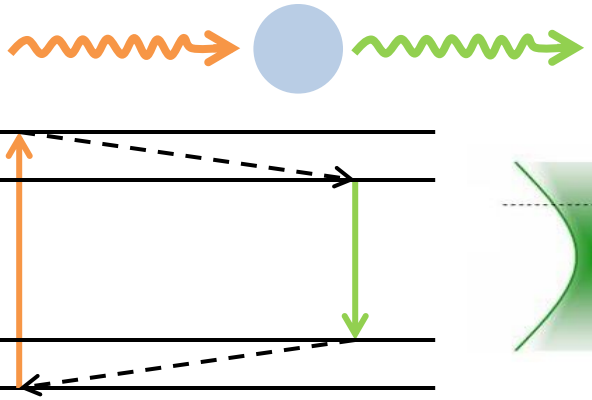


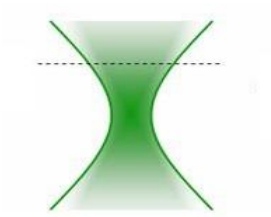
Outline

- Why two-photon?
- The principles of two-photon microscopy
- A close look at two-photon setup instrumentation
- Phosphorescence lifetime imaging with two-photon microscopy

Why two-photon?

Single-photon excitation





1990: combining the idea of two-photon absorption with the use of a laser scanner

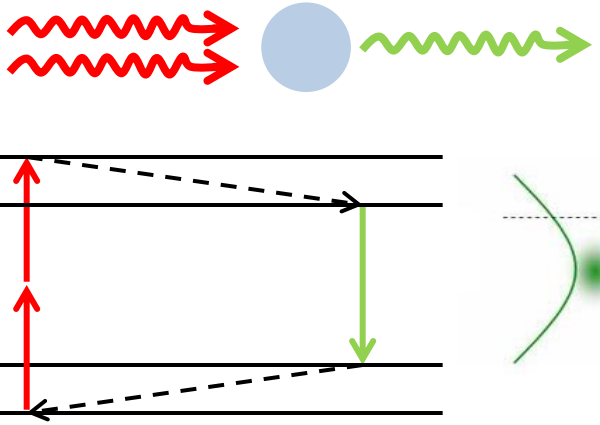
High degree of spatial confinement

Reduced background signal

Lower energy excitation laser (near IR or IR)

Lower scattering and absorption in tissue

Two-photon excitation



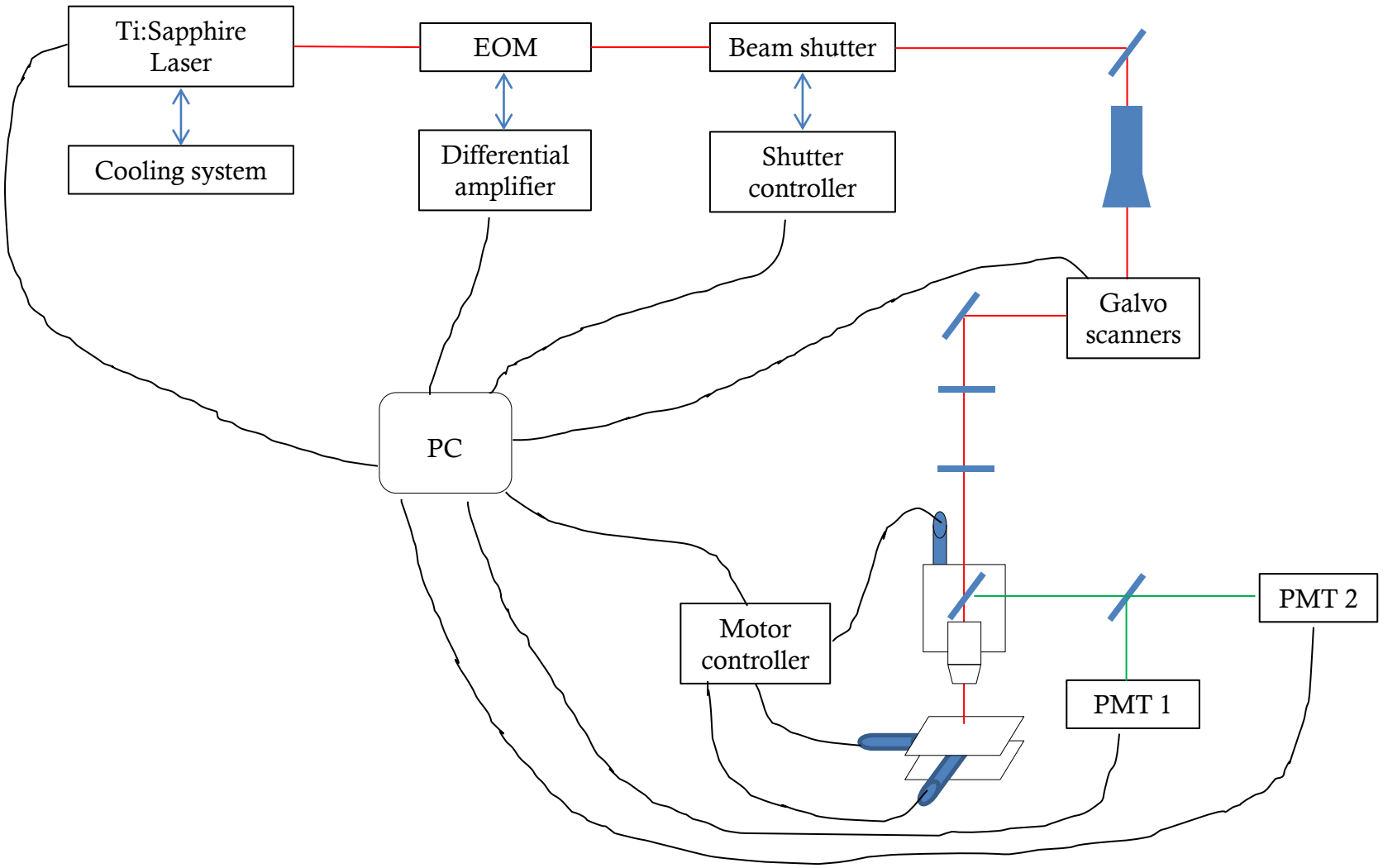
3D imaging

Higher spatial resolution

Reduced risk of photodamage (phototoxicity)

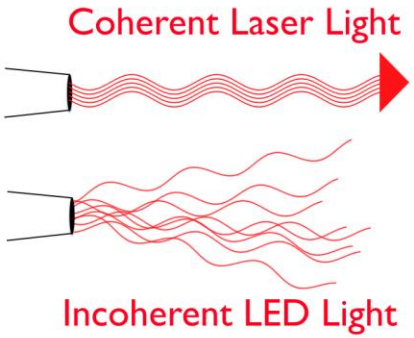
pO_2 measurements deeper in the tissue

Two-photon setup



Laser source

- Laser versus other sources of light:



  - Spatial coherence: focus to a tight spot; narrow beam over great distances
  - Temporal coherence: light with a very narrow spectrum

- Two-photon absorption
  - Extremely low probability, high photon density and flux

- Continuous wave lasers versus pulsed lasers:
  - Continuous-wave laser: high average laser power
  - Pulsed lasers: minimized average power deposition in the specimen(higher peak power but lower duty cycle)

Laser source

Spectra-Physics Mai Tai® Ti:Sapphire oscillator (HP 5274) Price: $114,000

Lasing medium: Ti:sapphire (Ti:Al_2O_3)

High output power (average power 2.5 W)

Short output pulse width (less than 100 fs) to provide high peak power

Wide tuning range (690-1040 nm)

High repitition rate (80 MHz)

Polarization (>500:1 horizontal)

Tight beam diameter (< 1.2 mm)

Low beam divergence (< 1.2 mrad)

No drift in wavelengh

Minimal average power fluctuations

Laser cooling system

Solid state cooling system, ThermoRack 401

Price: $4,500

Vibration free

Precise temperature control (±0.05° C)

Enough cooling capacity (315 to 420 Watts)

Instantaneous response to load changes

Energy Efficiency

Coolant/Process fluid: Koolance (27% propylene glycol / water mix)
or 27-50% ethylene glycol / water mix

Electro-optic modulator (EOM)

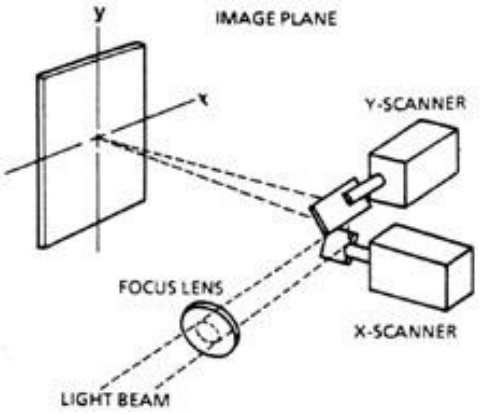
- Electro-optic effect: a change in the optical properties (refractive index) of a material in response to a DC or low-frequency electric field
- Nonlinear optical material, such as electro-optic crystals and organic polymers
- Modulation of phase, frequency, amplitude, or polarization of the beam

- **ConOptics modulator, Model 350-80LA + Model 302RM Driver**
- Aperture 3.5mm
- Wavelength ranges: 700-1100 nm

Price: $11,000

Galvanometer mirror system

- **Dual-Axis Small Beam Galvo/Mirror Assembly** Price: $2,000
- Fast response (300 μs small angle response time)
- High-precision optical mirror position detection
- Silver coating protection
- Wide operating wavelength range (400 -2000 nm)
- Wide scan angle (12.5°)
- High resolution (0.0008°)
- High motor and position sensor linearity (99.9%)
- Optimized size, shape, and inertia of the mirrors for maximized performance in the presence of large angular accelerations



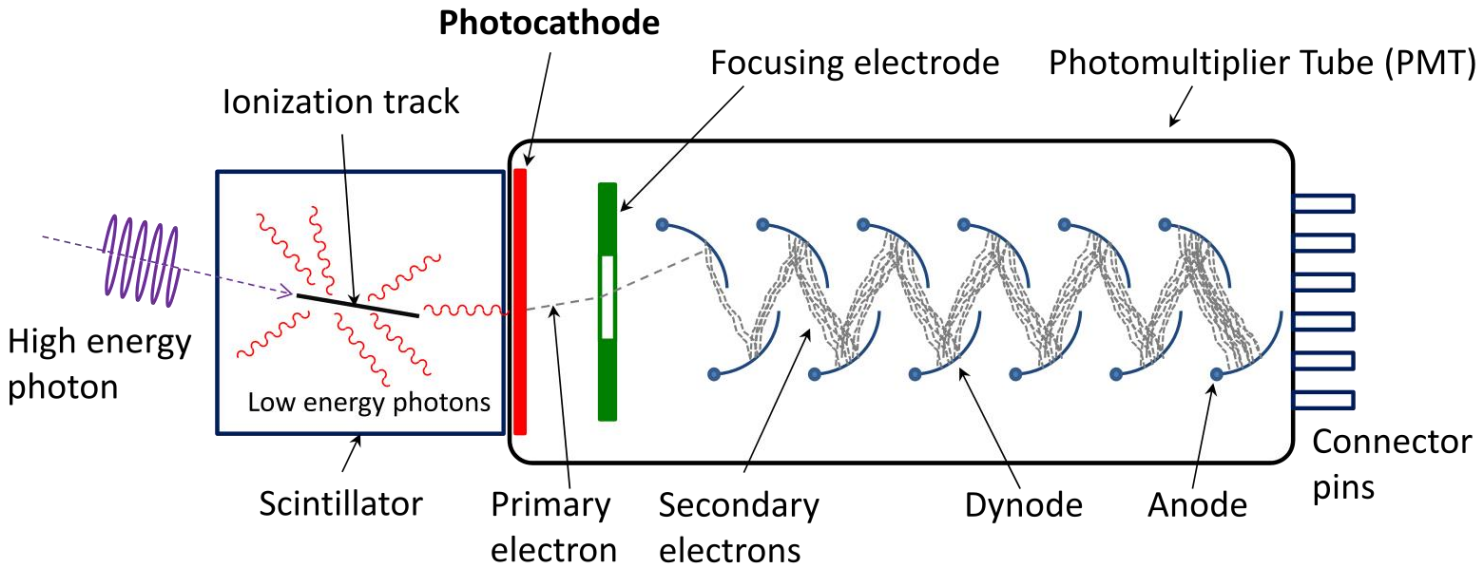
Objective

Price: $7,000

- **20X objective, Olympus XLUMPLFLN-W**
- Designed for multiphoton imaging
- Water immersion
- High numerical aperture (NA=1)
  - a measure of the acceptance angle
  - capture photons scattered through deep tissue
- Long working distances (2 mm)
  - access to deep tissue
- Excellent transmission in UV to NIR wavelengths (400 - 900 nm)

Photomultiplier tube (PMT)

- A class of vacuum tubes, extremely sensitive to light
- Multiply the current produced by incident light by as much as 100 million times
- Multiple dynode stages; exponentially-increasing number of electrons



- High gain, low noise, ultra-fast response, large area of collection, detection of individual photons

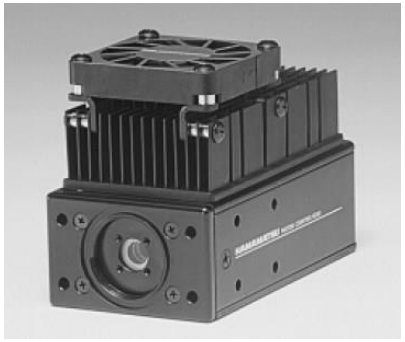
Photomultiplier tube (PMT)

- **R3896, Hamamatsu Photonics**
- Large photocathode area (8x24 mm)
- Wide sensitivity range (185-900 nm)
- Fast response (rise time 2.2 ns)

Price: $900

- **H7422, Hamamatsu Photonics**
- Wide sensitivity range (300-720 nm)
- Large photocathode area (5 mm diameter)
- Higher cathode and anode radiant sensitivity
- Faster response (rise time 1ns)
- Higher SNR (cooler to reduce the thermal noise)

Price: $3,400



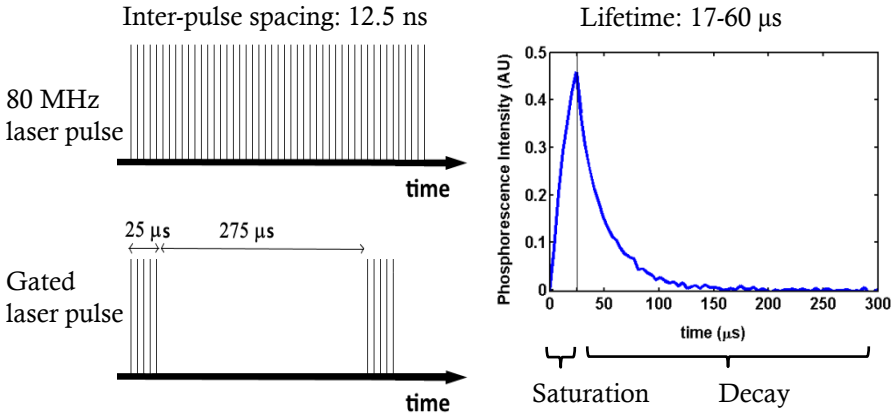
Data acquisition (DAQ) devices

- **NI USB 6353 (National Instruments)** Price: $2,500
- Digital maximum clock rate (sampling rate): 1 MHz
- Counter max source frequency: 100 MHz

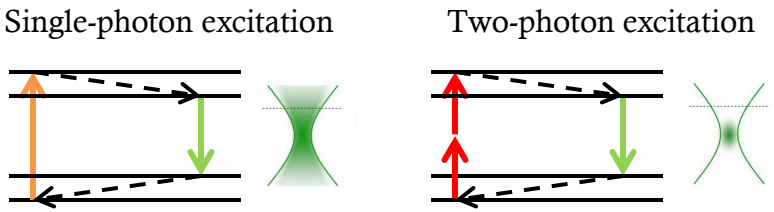
Phosphorescence lifetime imaging with two-photon microscopy

Phosphorescence lifetime imaging

Based on oxygen-dependent quenching of phosphorescence



Two-photon microscopy

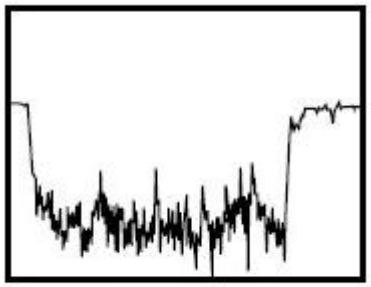


Sakadžić et al. (2010) Nature Methods, 7, 755.

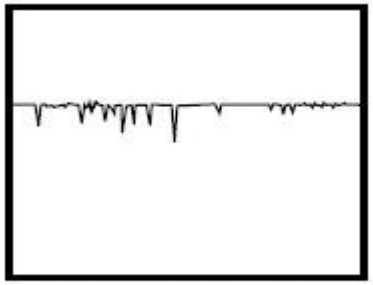
Lifetime imaging setup

- Synchronization with EOM
  - Gated laser pulse and phosphorescence decay detection
  - DAQ device timer/counter
  - Trigger

- Detection of phosphorescence decays
  - Analog and digital (photon counting) modes



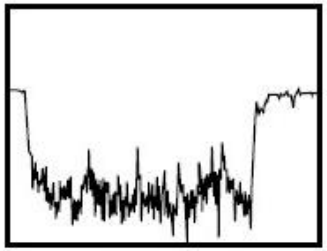
(a) HIGH



(c) VERY LOW

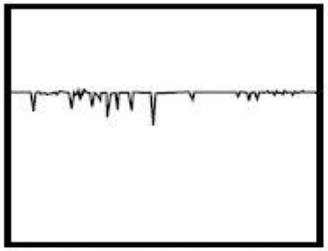
Lifetime imaging setup

- Analog mode
  - Output signal is the mean of the signals (including the AC components)



(a) HIGH

- Photon counting mode
  - Detection of individual pulses
  - Pulse height discriminator (separation of signal pulses from noise pulses)
  - High-precision measurement
  - Higher SNR
  - Exceptionally effective in detecting low level light



(c) VERY LOW