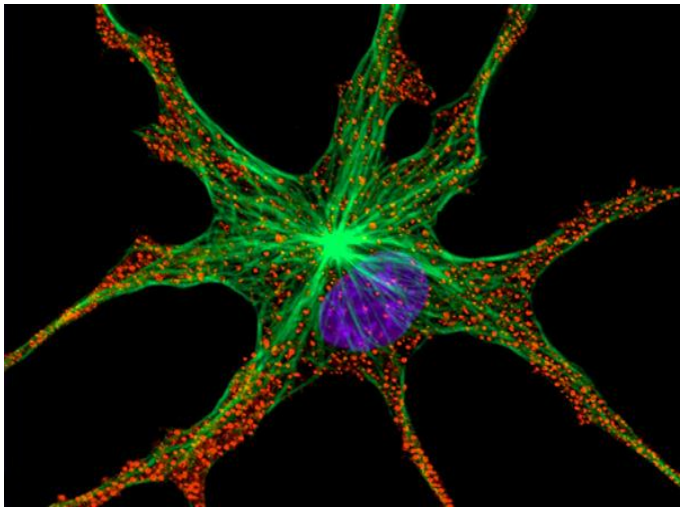
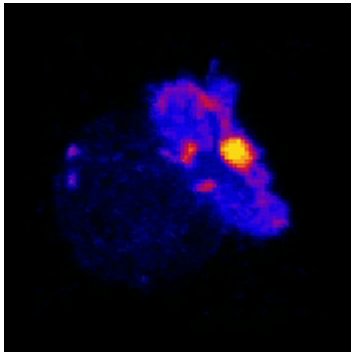
Imperial College London

Facility for Imaging by Light Microscopy
FILM
Observing Life As It Happens

Special Techniques 1

Mark Scott
FILM Facility





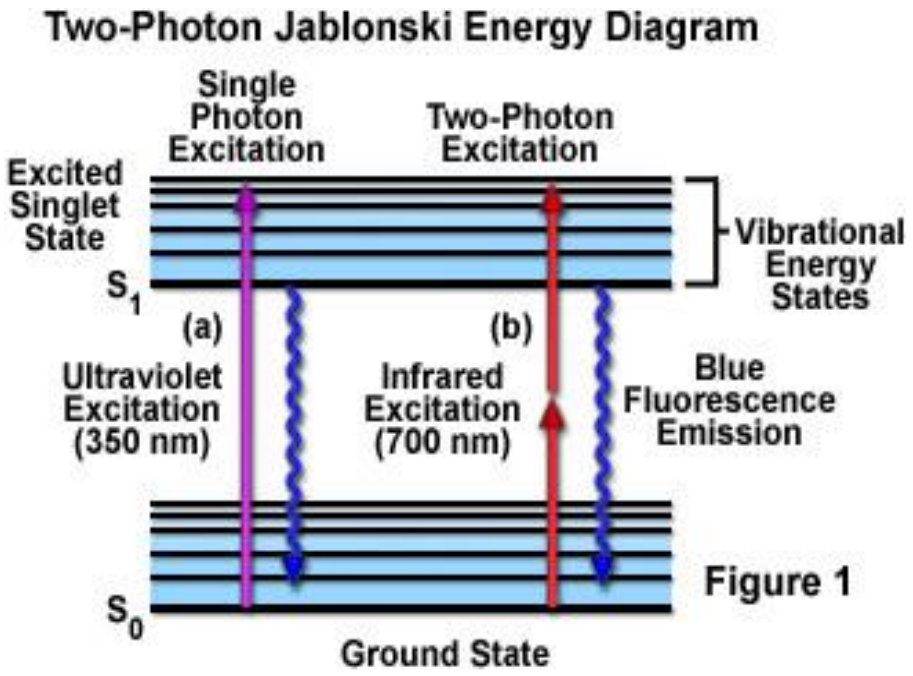
Special Techniques

- Multi-photon microscopy

- Second Harmonic Generation
- FRAP
- FRET
- FLIM
- In-vivo imaging

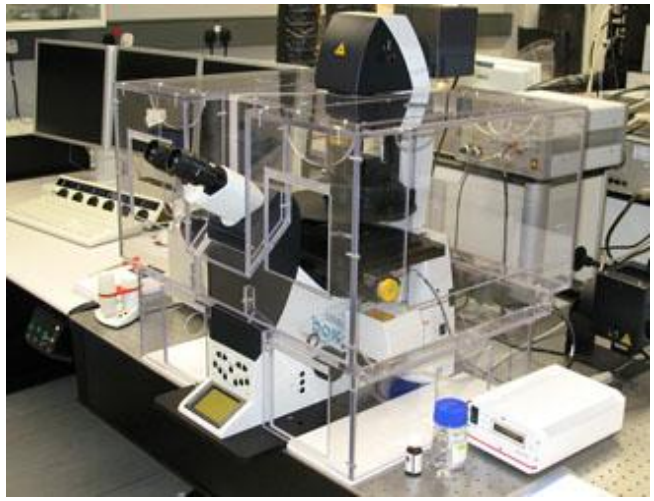
Two-photon Microscopy

- Alternative to confocal and deconvolution microscopy
- Two photons of half the energy combine
- Emission equivalent to being excited with a single photon laser

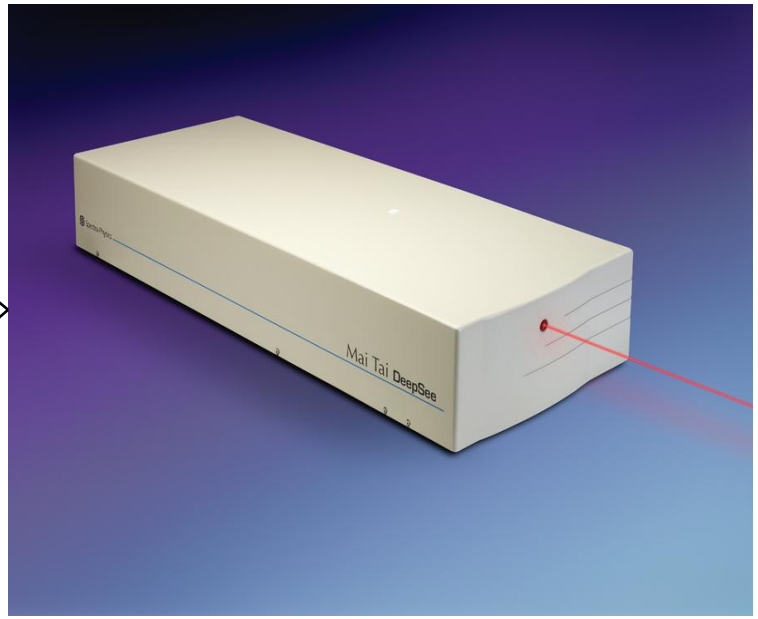


Two-photon Microscopy

Leica SP5 Upright



Leica SP5 Inverted



Newport Spectra-Physics Mai Tai Laser

690-1040nm

Two-photon Microscopy

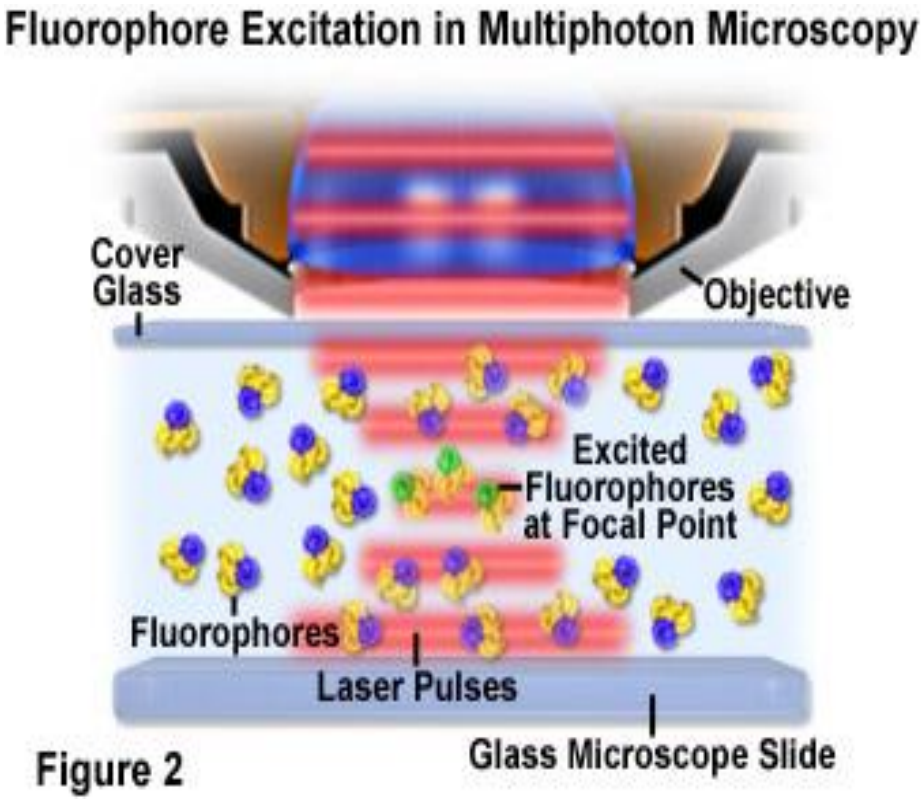


Figure 2

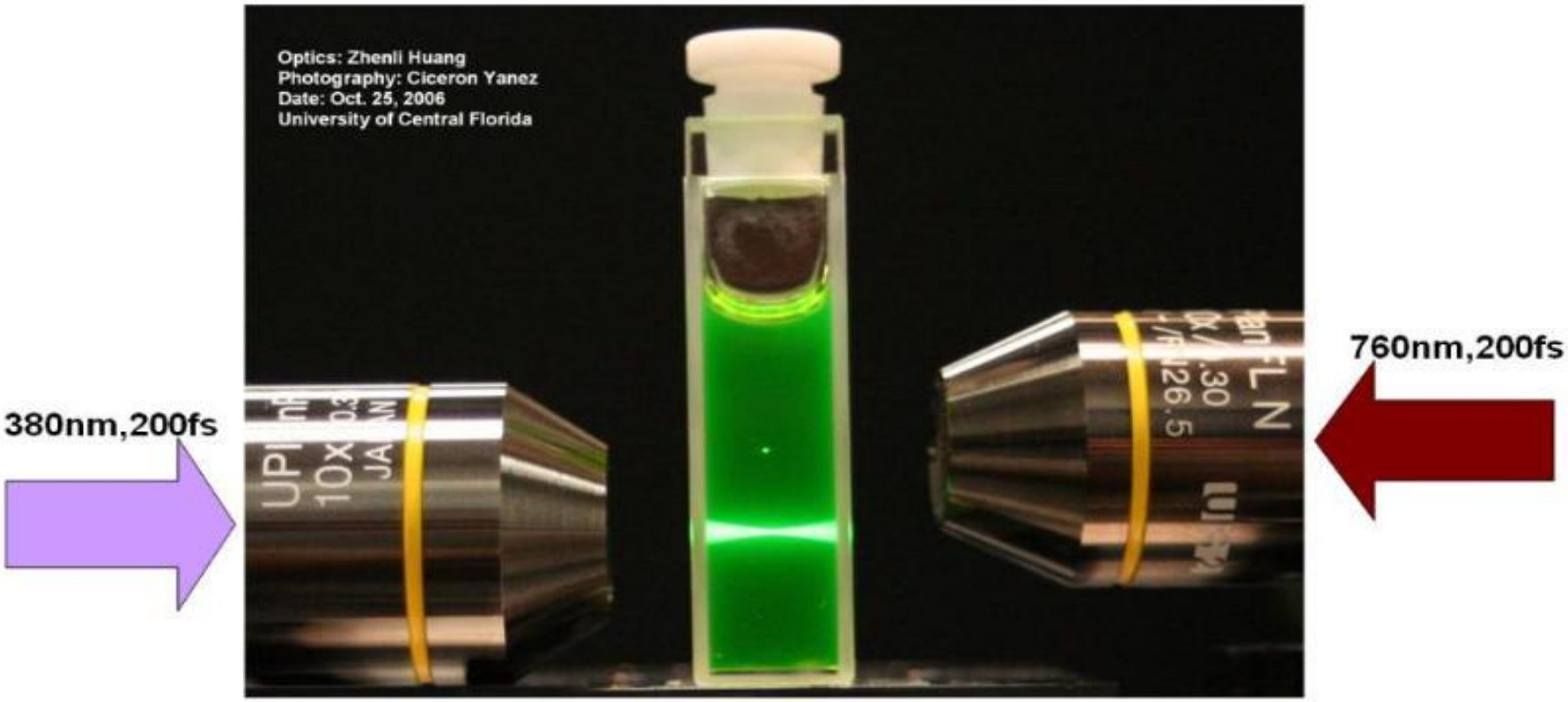
- Much higher energy required
  - ~1,000,000x greater
- Pulsed laser
- Femtosecond pulses
  - High power per pulse
  - Low average power
- Wave-form power output (2.7W peak)

Two-photon Microscopy

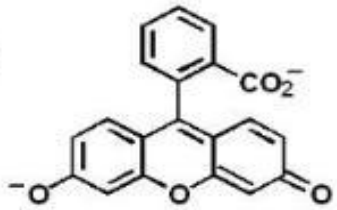
- No pinhole required (no out of focus light excited)
  - NDD detectors – higher sensitivity (less light path = less light lost)
- Less photo-bleaching/photo-toxicity (lack of excitation above and below the focal plane)
- Less scattering of light (Red vs Blue)
- Deeper penetration
- Protein uncaging – specialist application

- Image resolution/Thin specimens

Two-photon Microscopy



Fluorescein



Two-photon Stain Selection

- Spectral profile differs (peak may change)

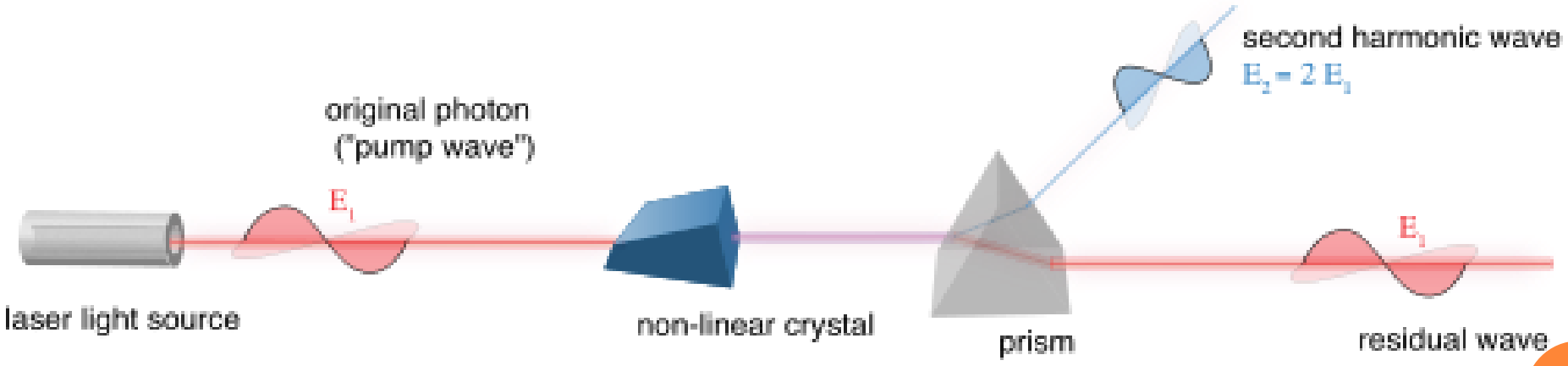
Dye	Single Photon Ex/Em	Two Photon Ex
GFP	488/507nm	860<960nm
DsRed	543/580nm	900<1064nm
DAPI	350/470nm	780>820nm
FITC	490/525nm	780>820nm
CY3	550/570nm	780nm
CY5	649/670nm	780>820nm

Three-photon Excitation

- Only 10x more power needed from 2-photon, not the million-fold increase going from 1-photon to 2-photon excitation.
- Requires 3 photons
  - ~1/3 of normal excitation
- 1020nm → 340nm
  - (510nm)

Second Harmonic Generation

- (Frequency Doubling)
- Photon's interact to form single photons (double the wavelength)
- Only non-centrosymmetric structures

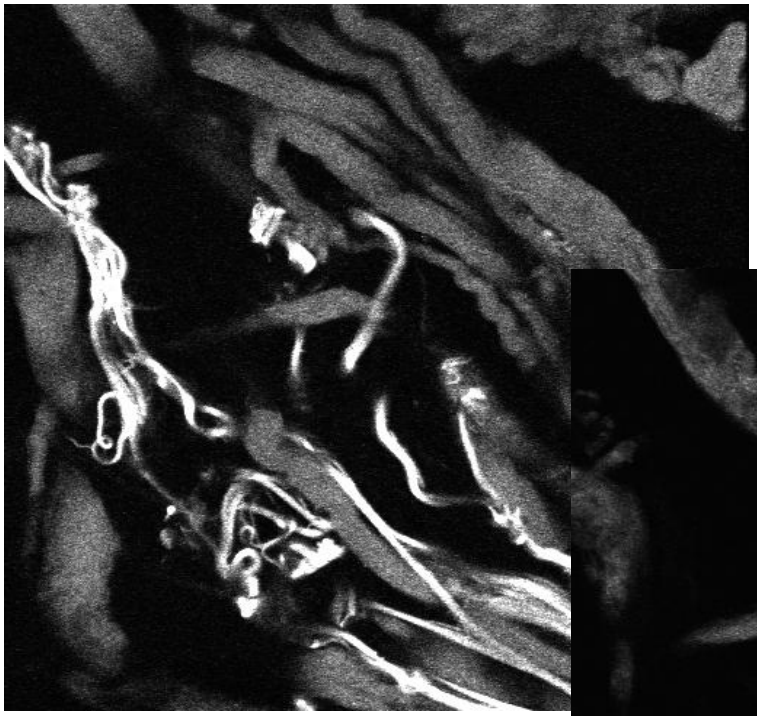


Second Harmonic Generation

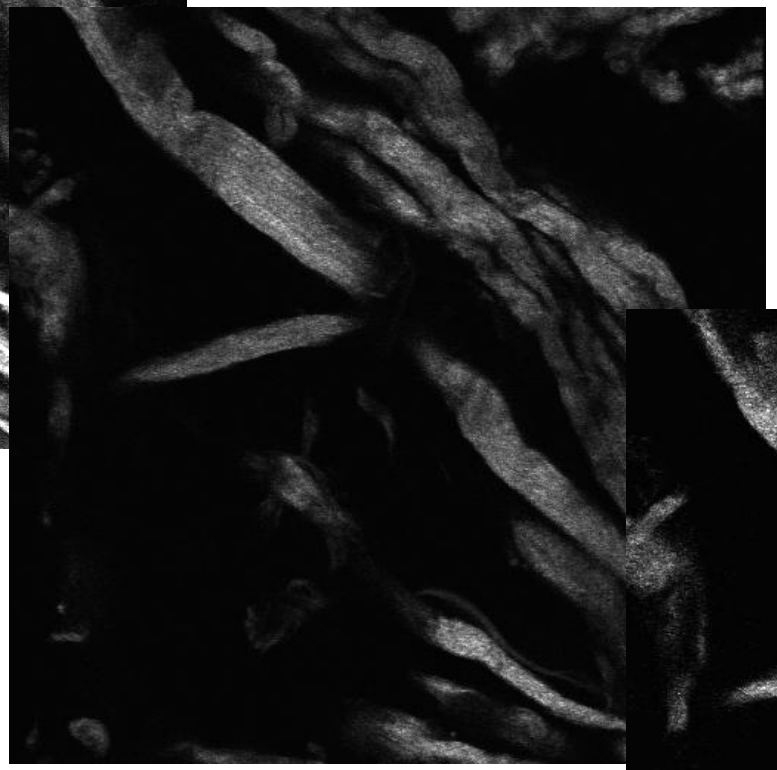
- Gives structural information without staining
- Useful for morphological information in whole tissue
  - Gain structural information of surrounding when tracking cells in-vivo without needing staining (live specimens)
- Can be combined with other microscopy techniques (Anisotropic imaging, Autofluorescence, Lifetime imaging)

Second Harmonic Generation

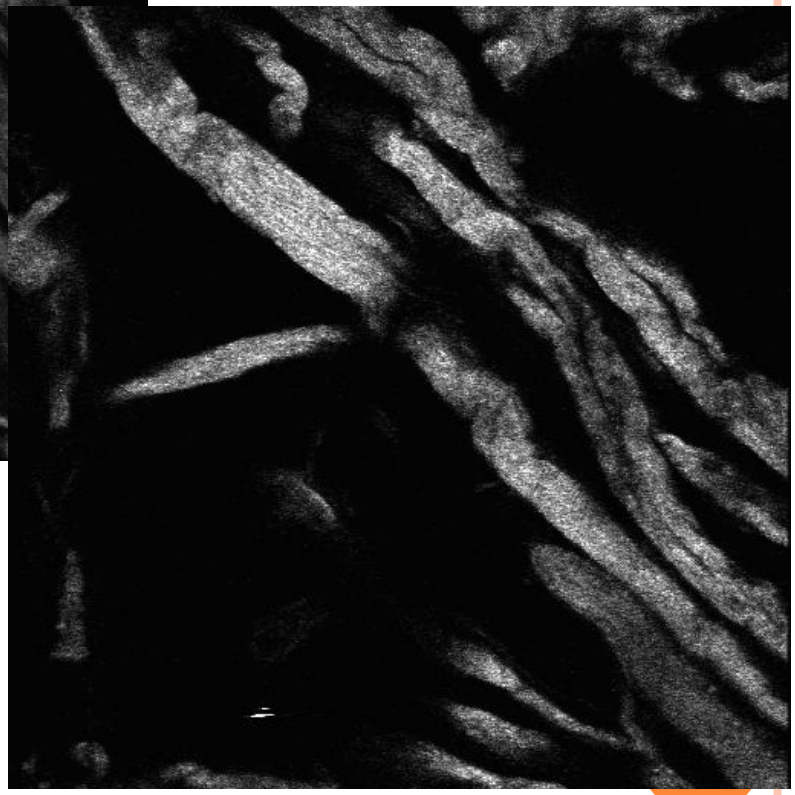
- Wavelength dependency for different structures
- Useful to distinguish between tissue composition without staining (with other techniques)
- Commonly imaged structures:
  - Collagen I/III(840/930nm)
  - Elastin (740nm)
  - NAD(P)H (680nm)



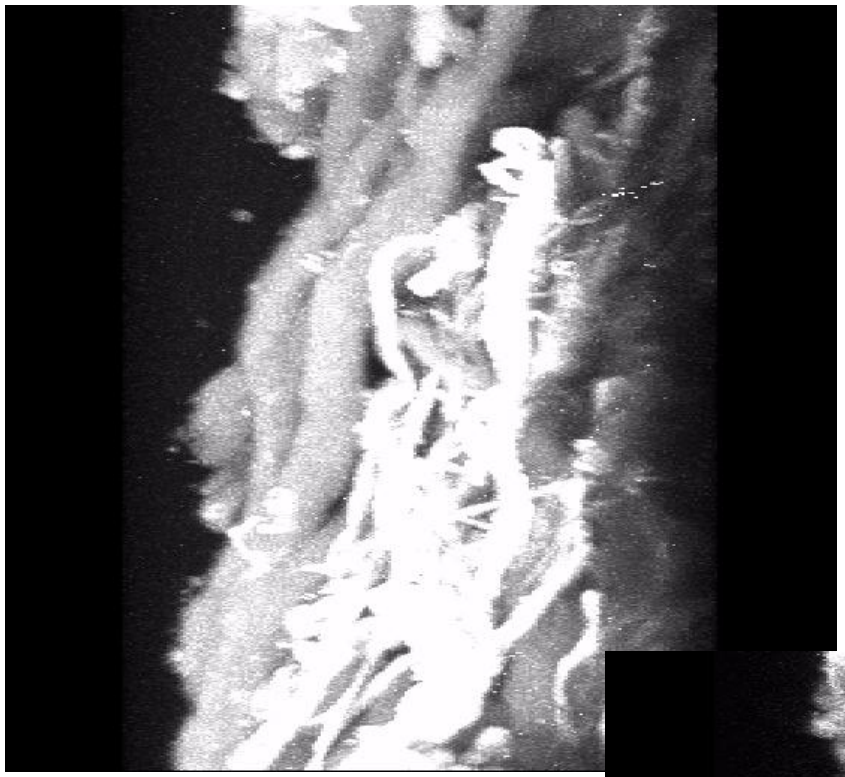
760nm



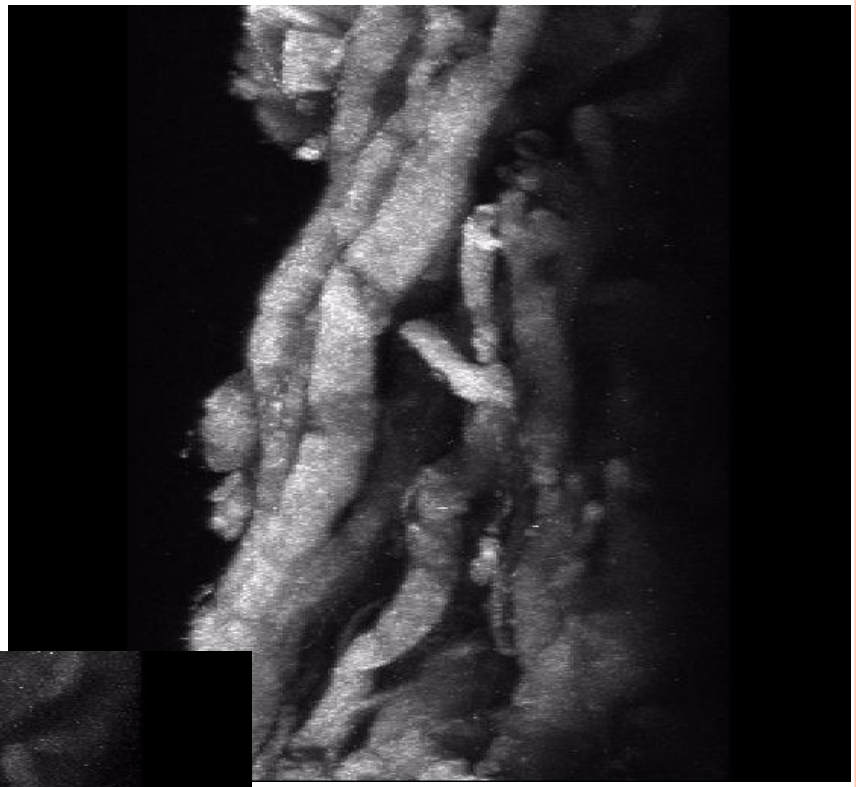
840nm



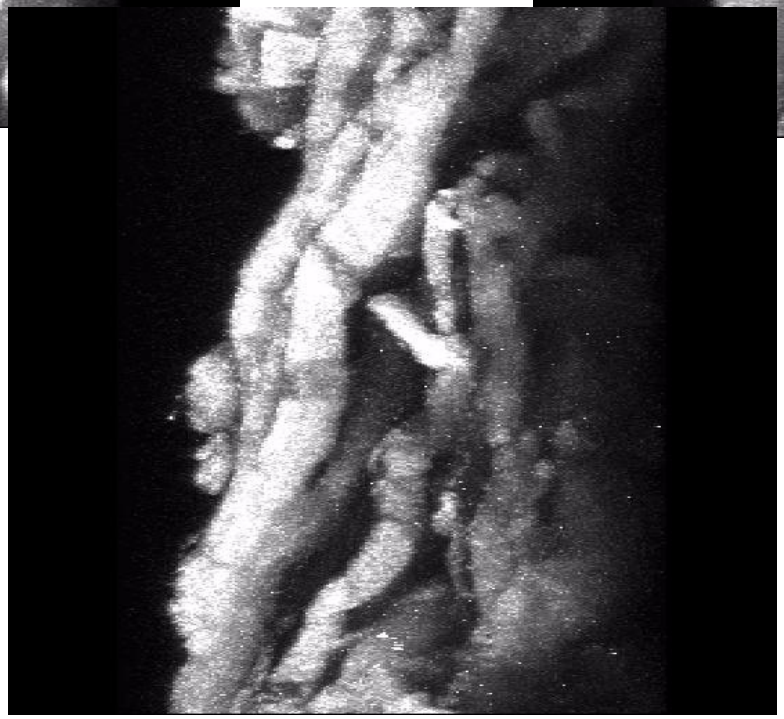
930nm



760nm

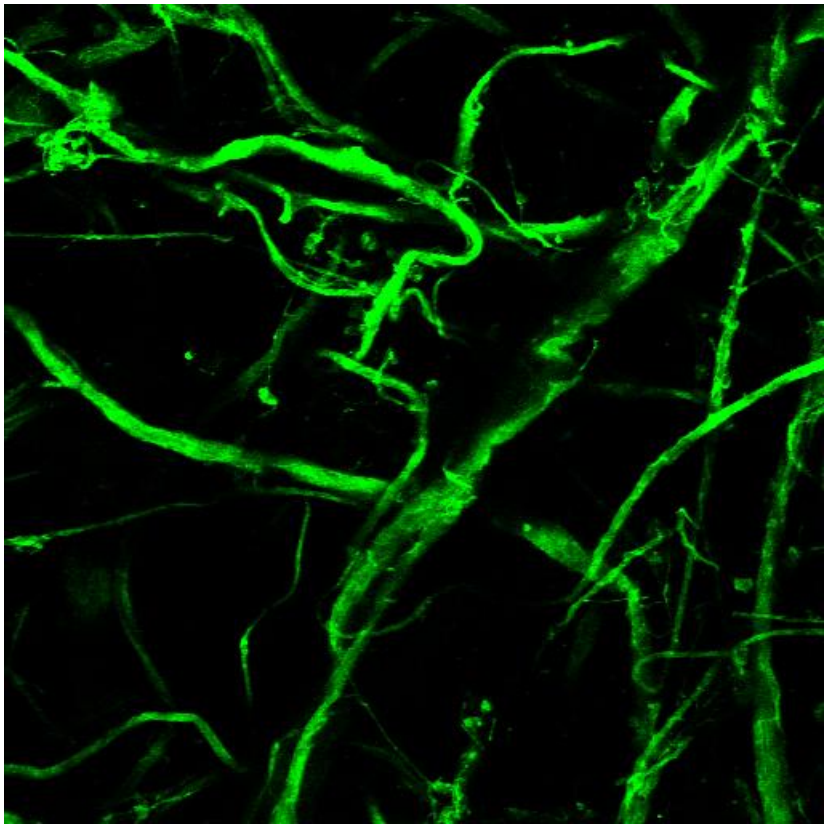


840nm

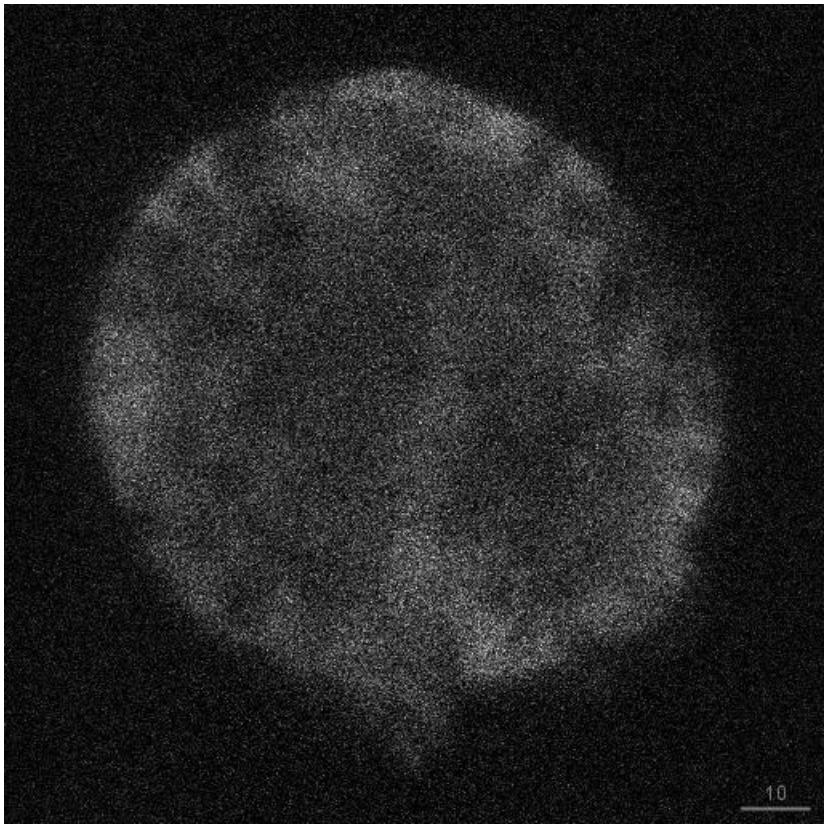


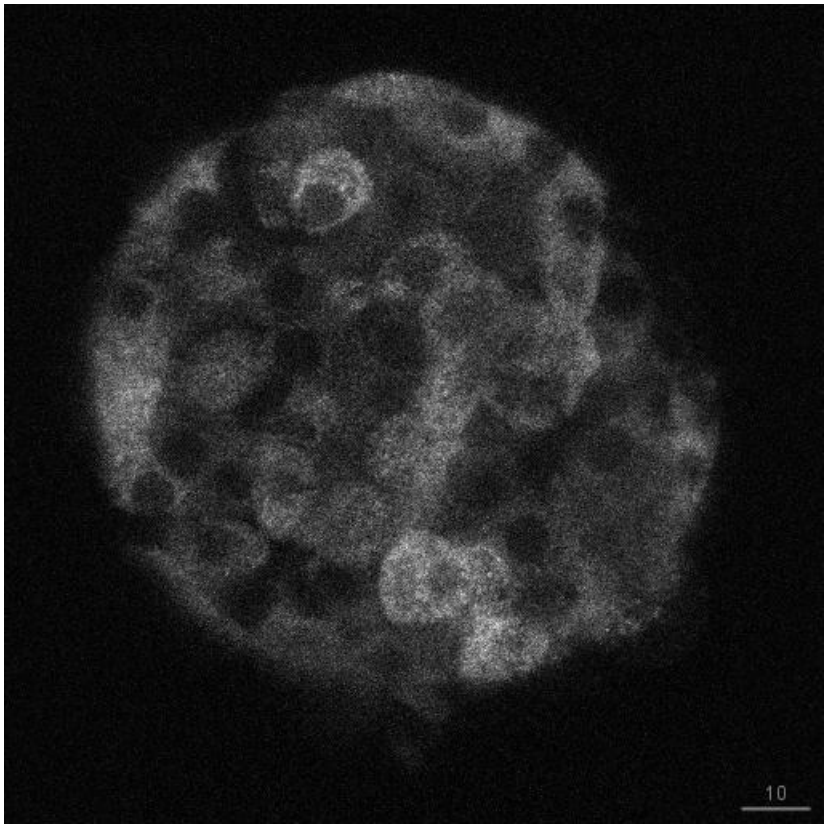
930nm

SHG 840nm



Collagen I antibody staining





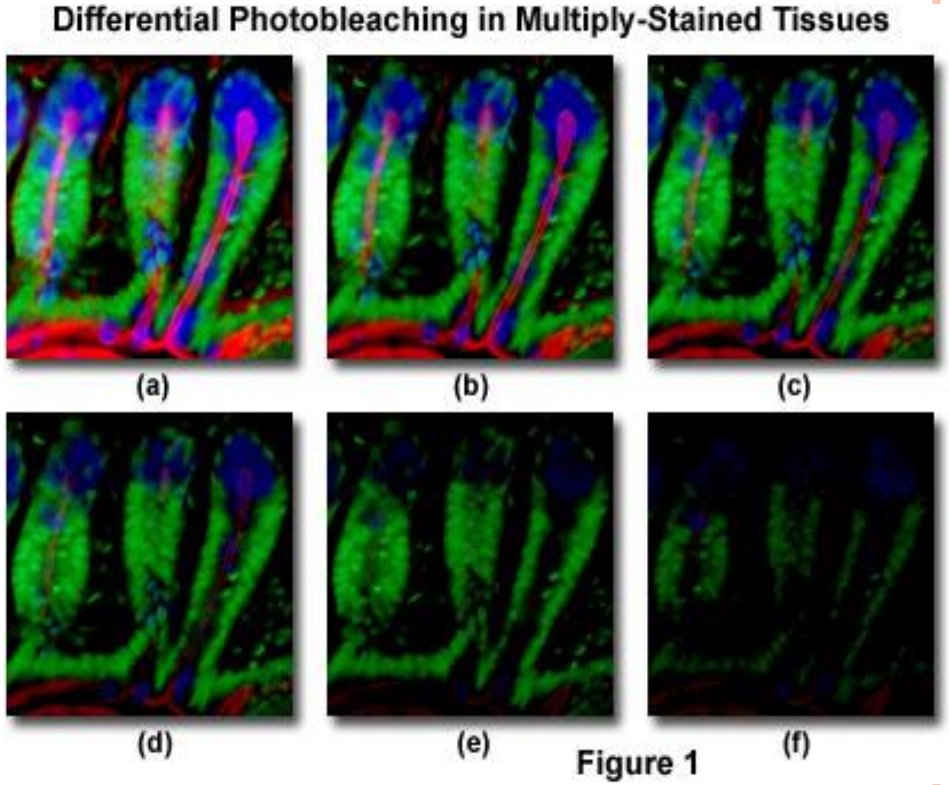
Pancreatic Islets showing two-photon SHG generation of intracellular NAD(P)H

Second Harmonic Dyes

- SHRIMPS (Second Harmonic Radiation Imaging Probes)
- Development of structurally significant molecules
- Specific wavelengths of SHG generation used to excite
- Structural interference/amplification
- Excellent possibilities for extreme long term imaging

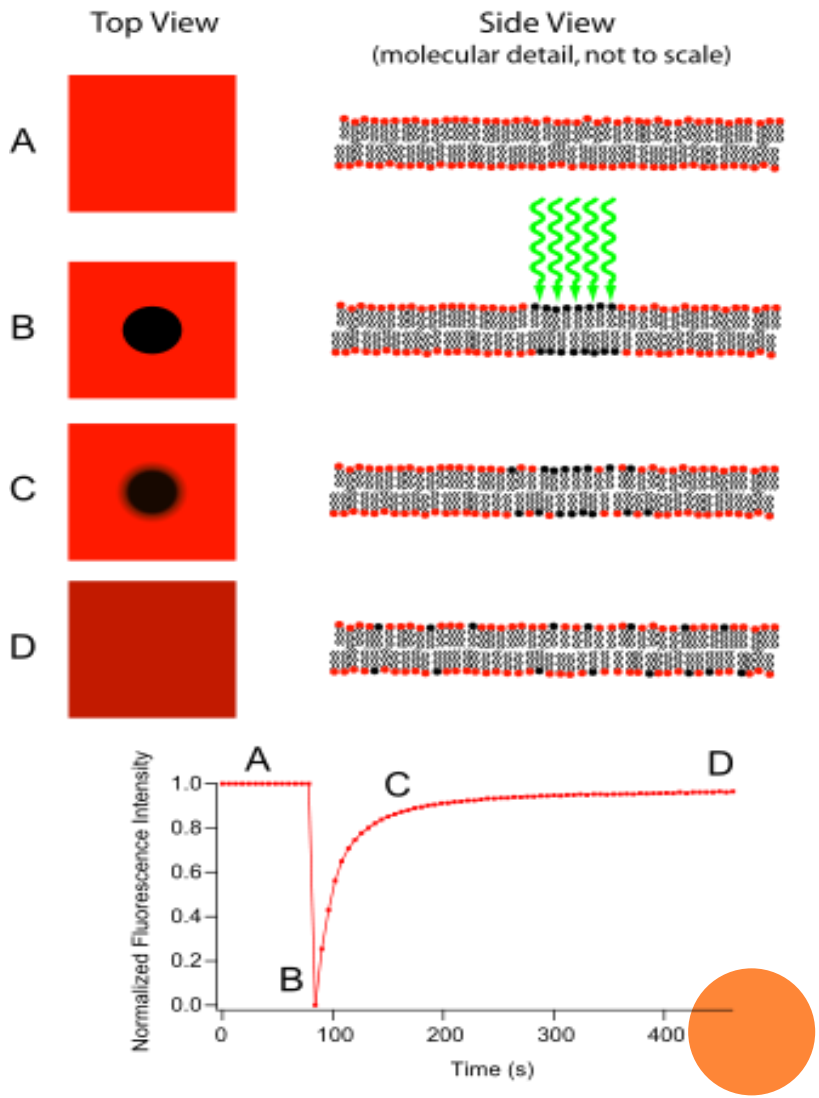
Photo-bleaching

- Photochemical destruction of fluorescent molecules
- Differs between fluorophores
- 2P excitation reduces this bleaching by limiting exposure



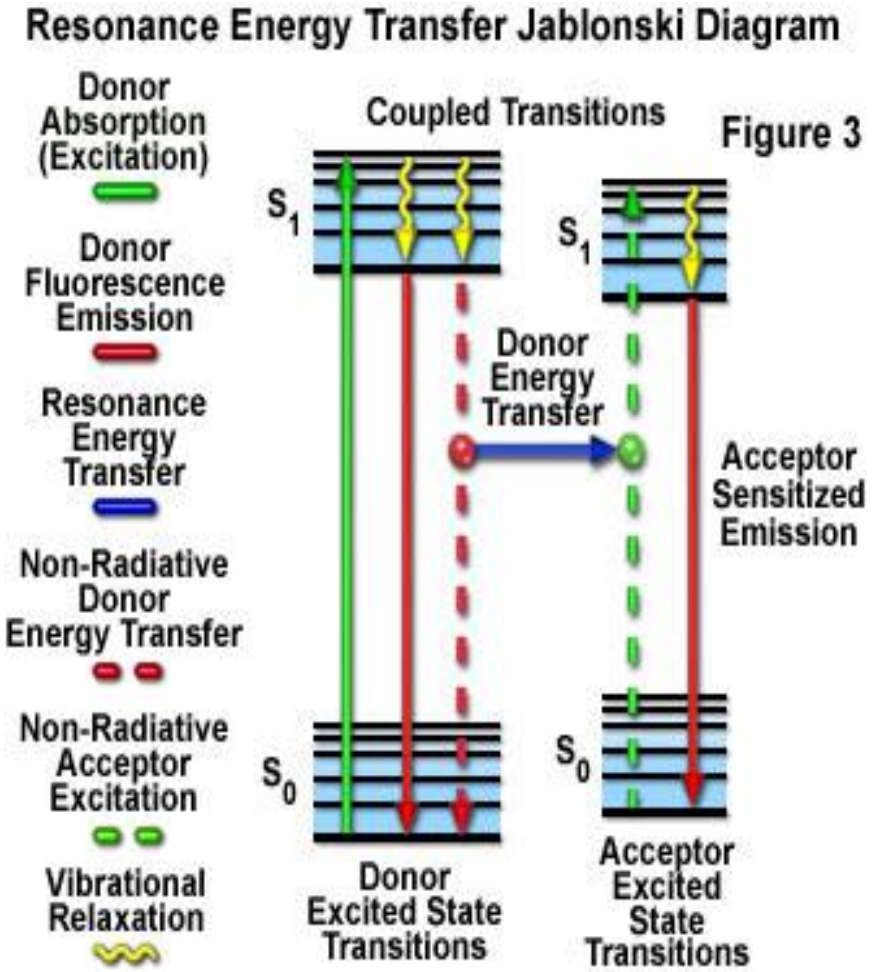
Fluorescence Recovery After Photo-bleaching

- FRAP
- Bleaching of specific areas
- Diffusion/transport experiments

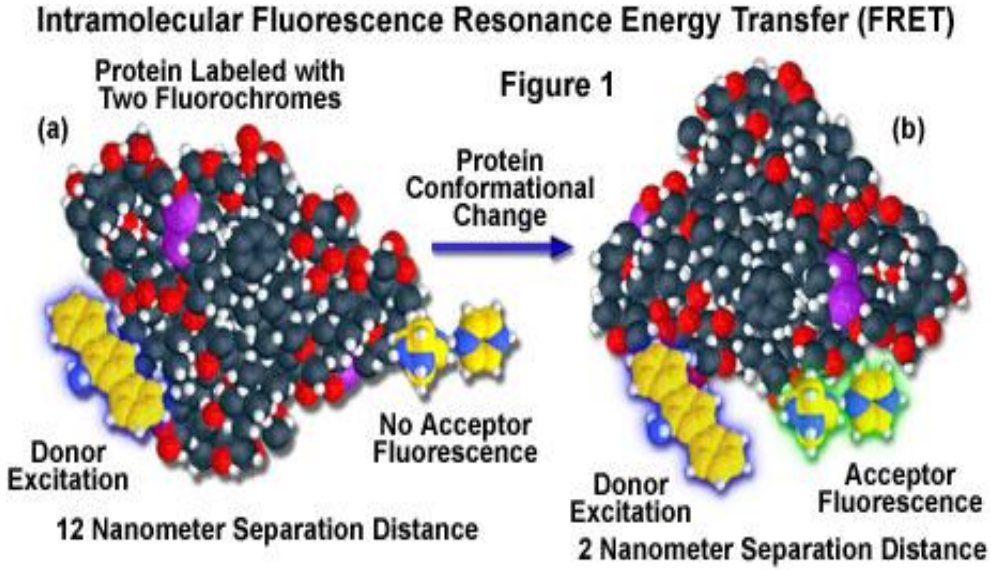


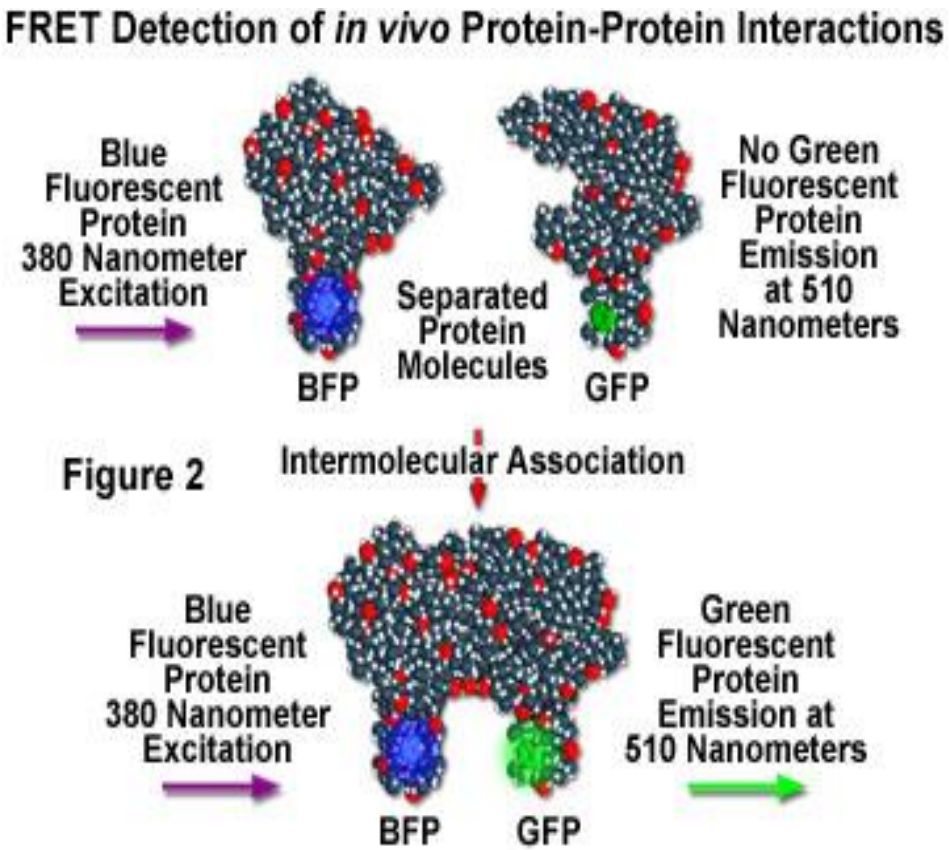
Forster Resonance Energy Transfer

- FRET
- Transfer of energy between fluorophores
- Typically under 15nm
- Quantitative measure of co-localisation
  - Only molecules within strict distances will FRET
  - FRET efficiency determined by distance between molecules



FRET


Intramolecular Fluorescence Resonance Energy Transfer (FRET)
Protein Labeled with Two Fluorochromes
Figure 1
(a)
(b)
Protein Conformational Change
Donor Excitation
No Acceptor Fluorescence
12 Nanometer Separation Distance
Donor Excitation
Acceptor Fluorescence
2 Nanometer Separation Distance


FRET Detection of *in vivo* Protein-Protein Interactions
Blue Fluorescent Protein 380 Nanometer Excitation
BFP
Separated Protein Molecules
GFP
No Green Fluorescent Protein Emission at 510 Nanometers
Figure 2
Intermolecular Association
Blue Fluorescent Protein 380 Nanometer Excitation
BFP
GFP
Green Fluorescent Protein Emission at 510 Nanometers

FRET

- Specific fluorophore pairings
  - Based on spectral overlap
    - Emission of Donor must overlap Excitation of Acceptor
  - FRET efficiency determines Forster distance

<u>Common Donor-Acceptor Pairs:</u>

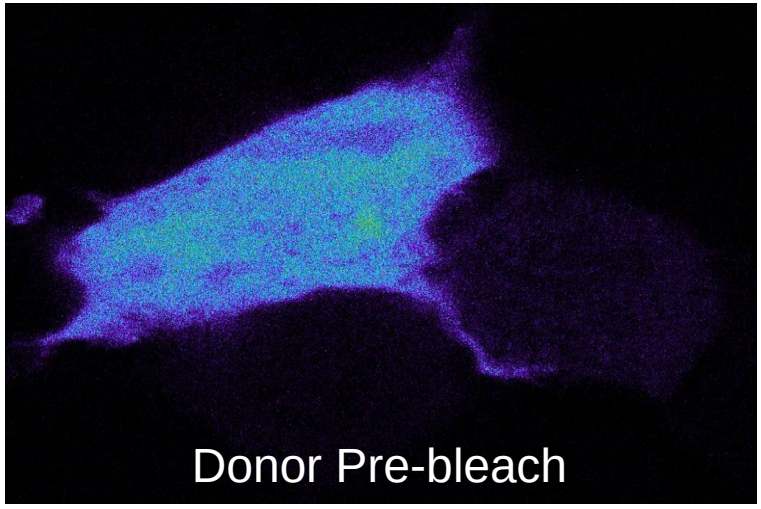
- FITC – Rhodamine (4.9nm)
- CY3 – CY5 (>5.0nm)
- PE – CY5 (7.2nm)
- CY5 – CY5.5 (>8.0nm)

FRET

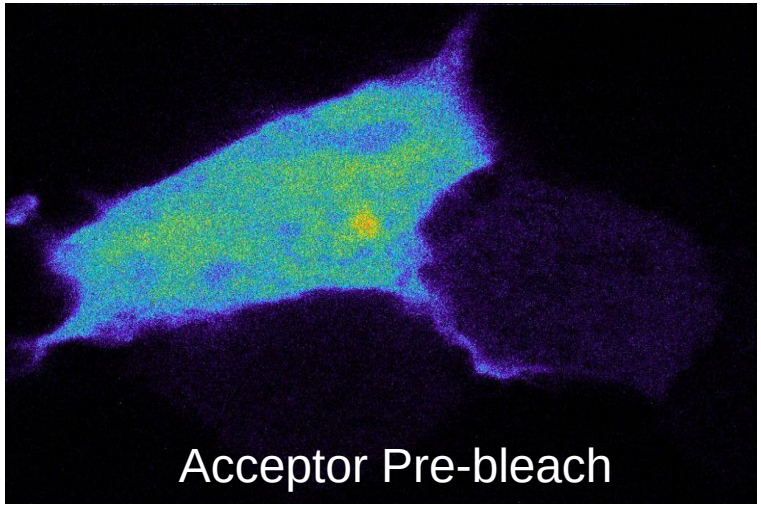
- Photobleaching effects the FRET transfer
- Ratio-metric analysis
- Acceptor bleached, Donor emission increases

- Two-photon bleaching more precise
  - Only bleaches in plane of focus so able to specify the 3D area of bleaching much more precisely

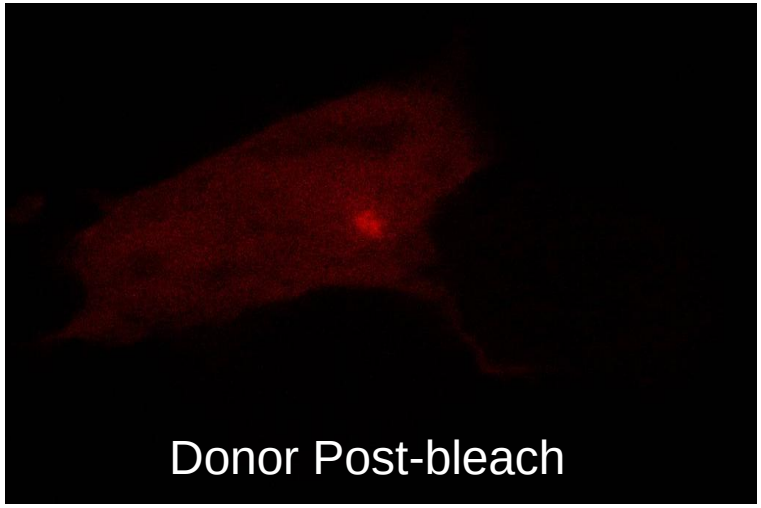
FRET



Donor Pre-bleach



Acceptor Pre-bleach

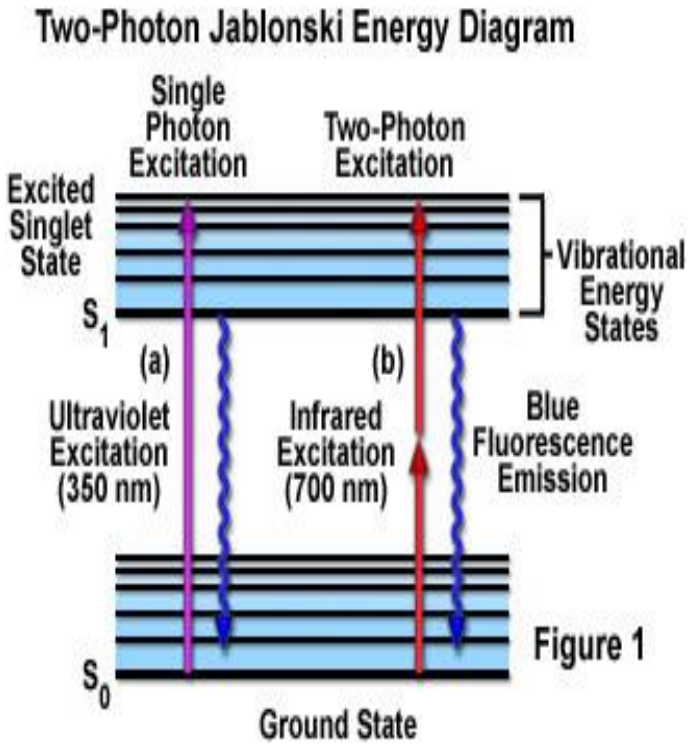


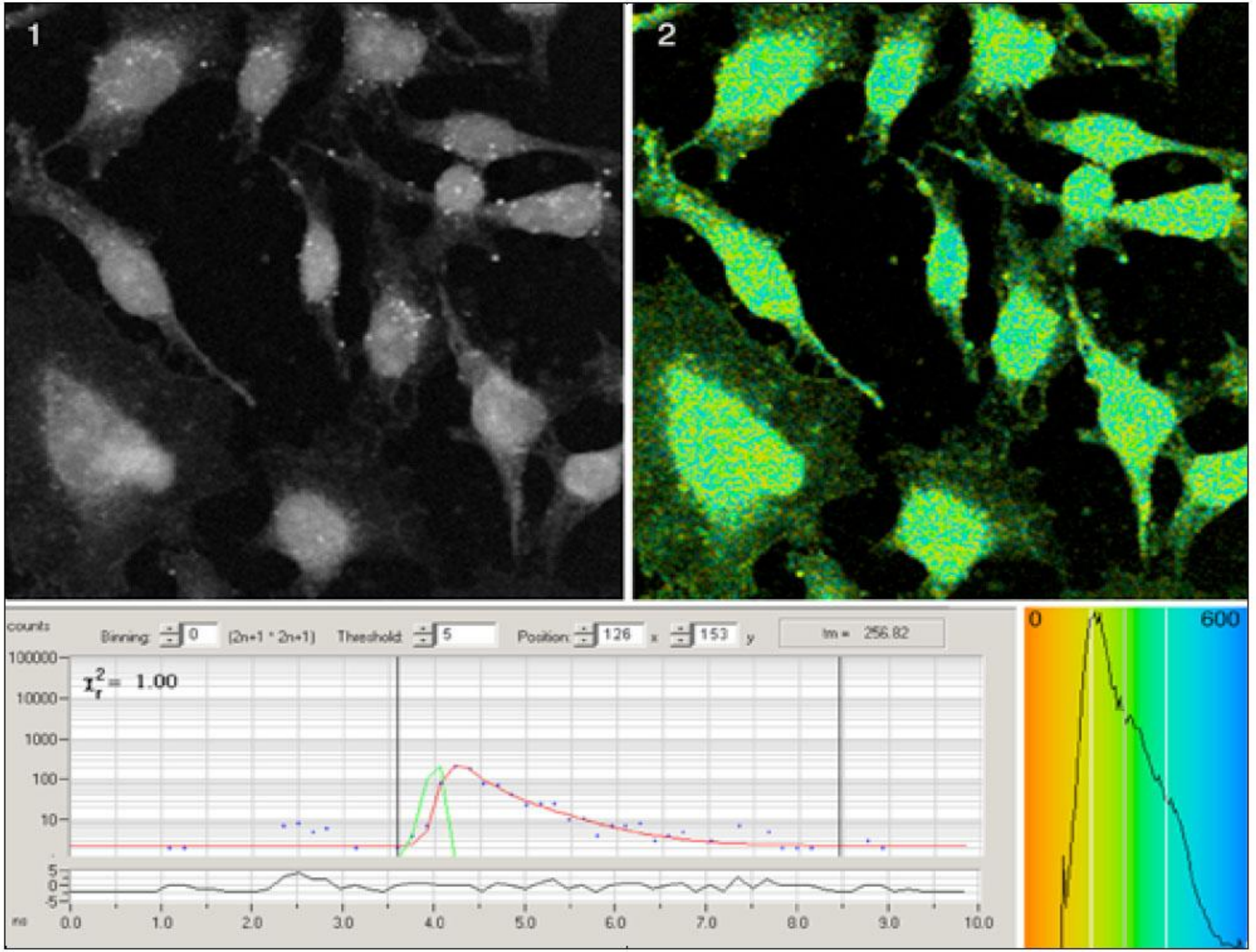
Donor Post-bleach

Acceptor Post-bleach

Fluorescence Lifetime Imaging Microscopy

- FLIM
- Pulsed mode-locked laser (2P – FILM Confocal 3 and 4)
- Measure of excitation decay




1
2
counts
Binning: 0
(2n+1 * 2n+1)
Threshold: 5
Position: 126 x 153 y
Im = 256.82
χ_r^2 = 1.00
100000
10000
1000
100
10
5
0
-5
ns
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
0
600

FLIM for FRET

- Lifetime of fluorophores effected by energy transfer
- Lifetime also changes with conditions of sample

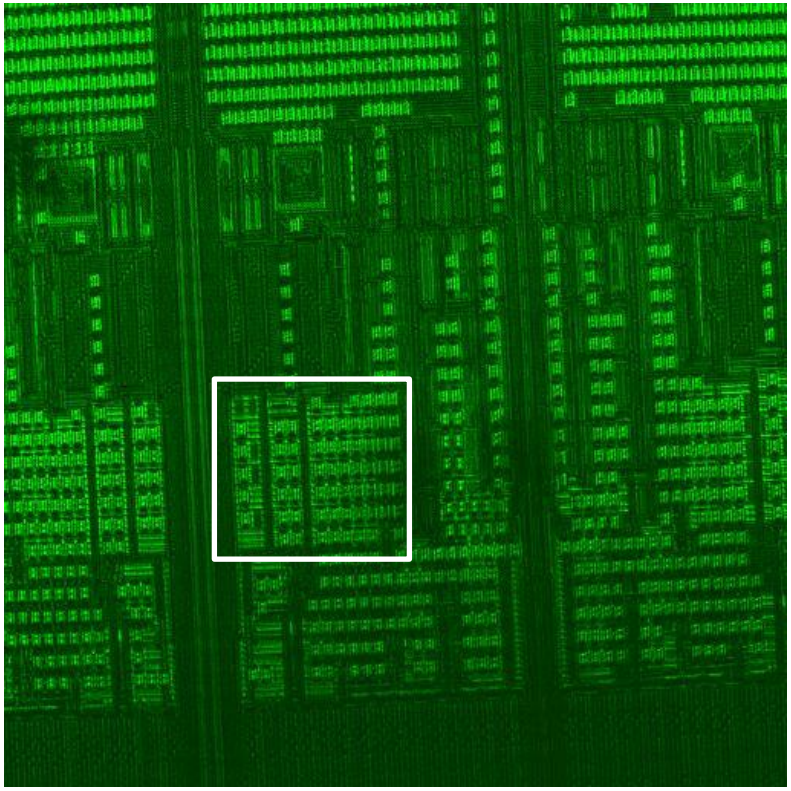
In-vivo Imaging

- Confocal 3
- Imaging whole tissue or animals
- Problematic due to light scatter, movement…

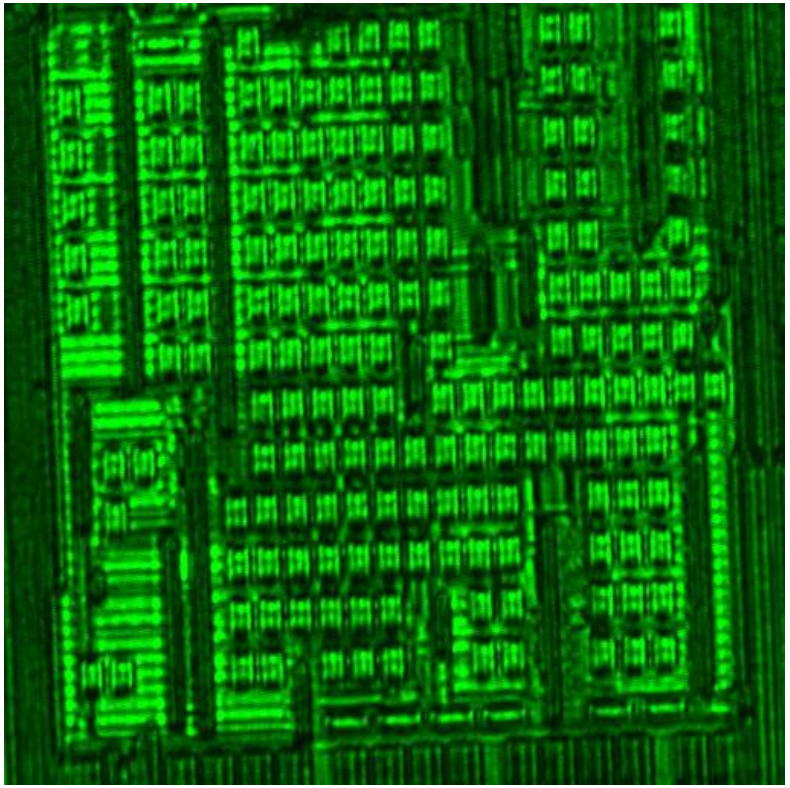
In-vivo Imaging

- High quality lens
  - 25x water immersion lens
  - 0.95 N.A.
- High confocal zoom possible
- Allows low power rapid scanning and high power area imaging

In-vivo Imaging



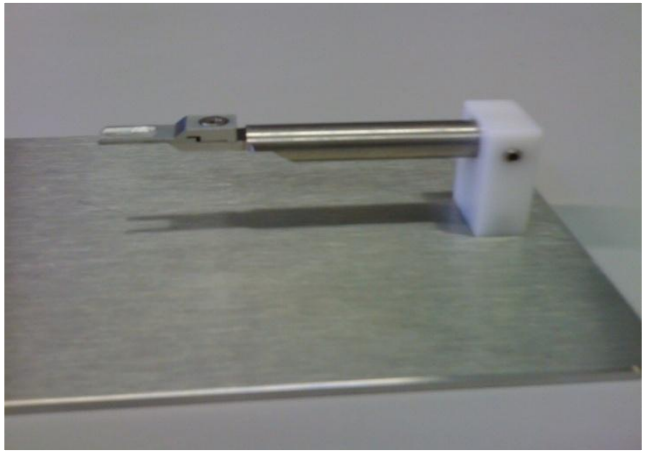
63x Objective – No zoom



25x Objective – 6x zoom

In-vivo Imaging

- Ensure specimen does not move
- Longer scan time possible
- Reproducibility increased



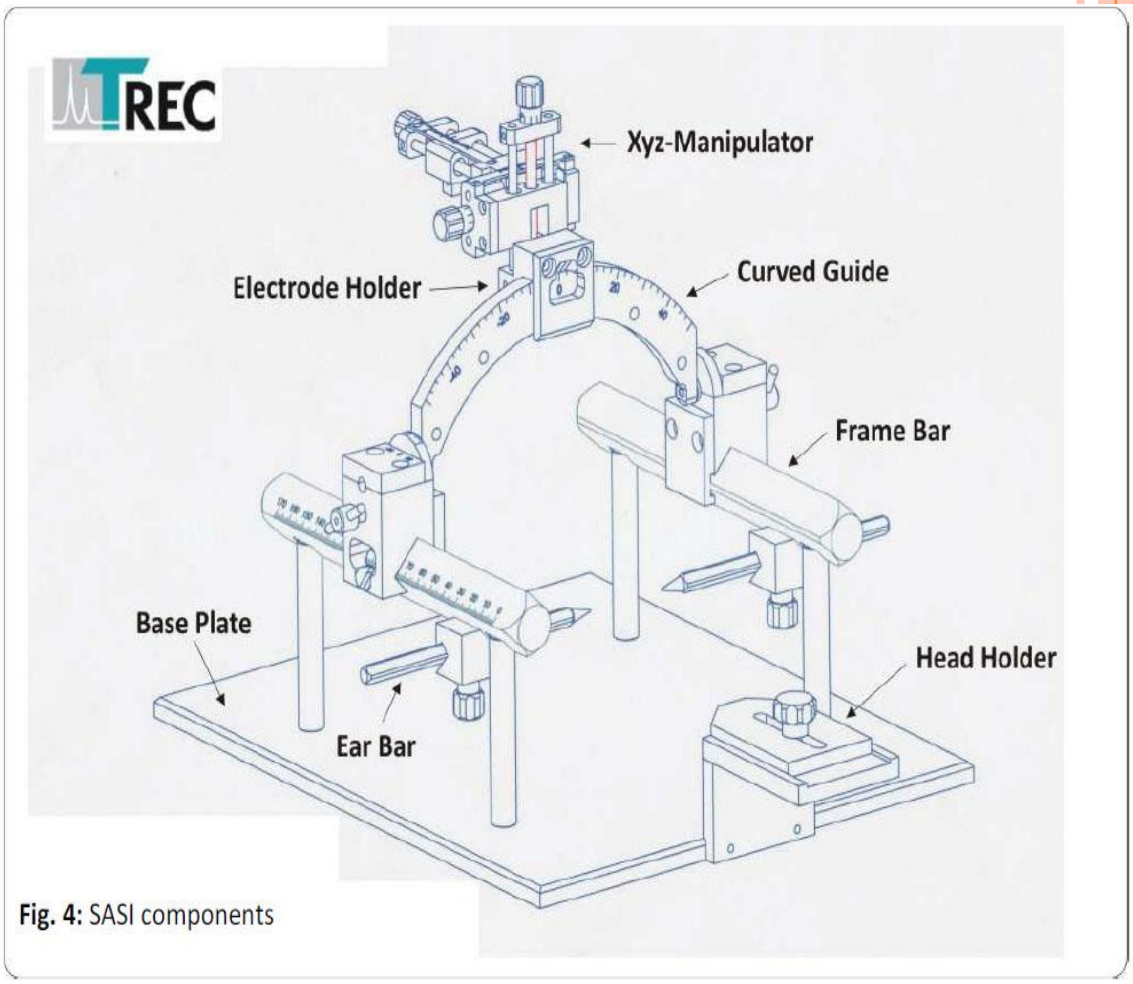
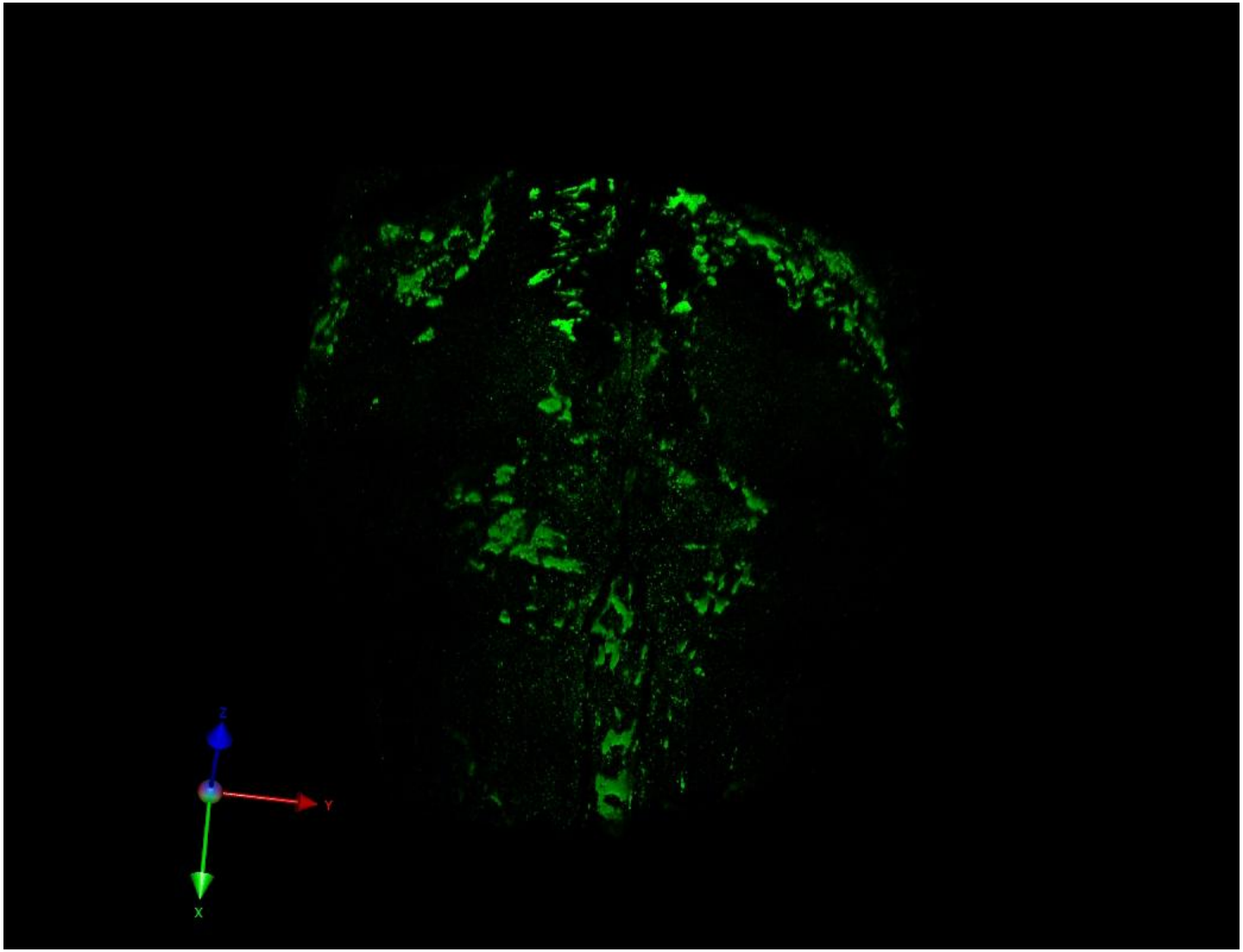


Fig. 4: SASI components

In-vivo Imaging

- 2P laser excitation – deeper penetration, less scatter, less damage…
- Steady specimen holder – increased scan time possible
- Multiple imaging techniques able to be combined

In-vivo Imaging



Thank You...