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international centre for theoretical physics

SMR 1495 - 3

WINTER COLLEGE ON BIOPHOTONICS:
Optical Imaging and Manipulation of Molecules and Cells
(10 - 21 February 2003)

Fluorescence Lifetime Imaging

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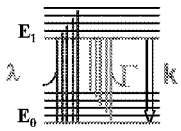
Head of Photonics Group, Physics Department,
Imperial College London, U.K.

These are preliminary lecture notes, intended only for distribution to participants.

Imaging tissue with fluorescence

Aim: to detect or image different types of tissue or states of tissue using optical radiation to achieve ***contrast***



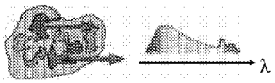
Intensity ~ f{η}, $\eta = \Gamma/(\Gamma+k)$

Wavelength, $\lambda \sim hc/(E_1-E_0)$

Lifetime, $\tau = 1/(\Gamma+k)$

Problems: heterogeneity, scattering and background fluorescence

Difficult to make absolute intensity measurements ⟹



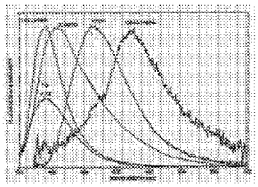
Solution: **image** I(λ) or I(τ) – ***relative*** *measurements*

Laser-induced fluorescence (LIF) imaging of biological tissue

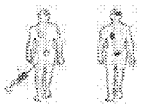
Fluorescence contrast

Most biological samples exhibit "autofluorescence"

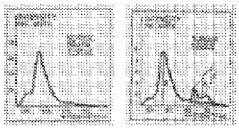
Challenge is to find an unambiguous contrast between target and background, *e.g. normal and malignant tissue*



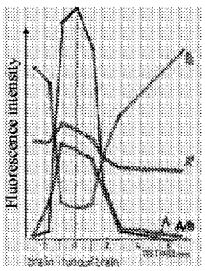
Tissue differences may be enhanced using a contrast agent, *e.g. HPD, ALA-5-induced Protoporphryin IX*



Wavelength-ratiometric laser-induced fluorescence imaging of brain tissue



Wavelength ratiometric fluorescence measurements reduce the impact of scattering, since this is assumed to be constant with wavelength – or can be fitted to an assumed model



Experimental data from Lund, Svanberg et al.

Spectrally-resolved fluorescence imaging

How to acquire spectral data cube (x, y, λ)?

Scanning microscopy ⇒ *serial pixel acquisition (single channel detection)*
⇒ *slow image frame rate*
⇒ *can use sophisticated detectors*

e.g. spectrograph, spectrometer, FTS with PMT, APD detectors

Wide-field (x,y) imaging ⇒ *parallel pixel acquisition (many channels)*
⇒ *high speed image acquisition*
⇒ *λ requires one dimension ⇒ need to scan?*

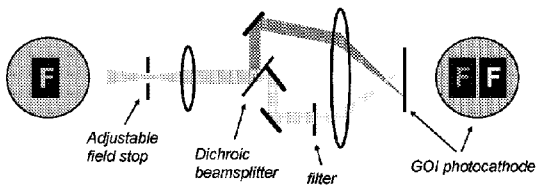
- acquire x, λ, scan y: e.g. spectrograph with CCD detectors

- acquire x, y, scan t: e.g. filter wheel, electro-optic filter with CCD detectors

- acquire x, y, scan z: e.g. FTS with CCD detectors

Wide-field multispectral fluorescence imaging

Wide-field imaging ⇒ ***parallel pixel acquisition***
⇒ ***high speed data acquisition***

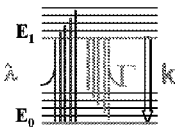


Multi-spectral imager **provides 2-channel (*up to 8*) spectral resolution - preserves whole-field 2D/3D imaging**

(Dichroic beamsplitter may be replaced by a polarizing beamsplitter to provide whole-field images of time-resolved polarization anisotropy)

Imaging tissue with fluorescence

Aim: to detect or image different types of tissue or states of tissue using optical radiation to achieve ***contrast***



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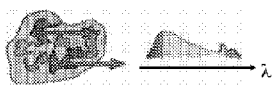
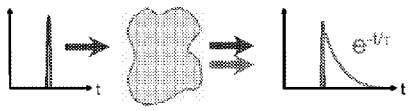


Solution: **image** I(λ) or I(τ) – ***relative*** *measurements*

How to measure fluorescence lifetime?

Time-domain measurement

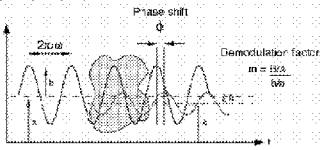


Frequency-domain measurement

$\tan \phi = \omega \tau_p$

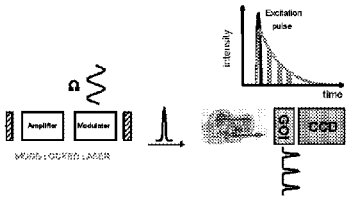
$m = [1 + \omega^2 \tau_m^2]^{-1/2}$

For single exponential decay: $\tau_p = \tau_m = \tau$

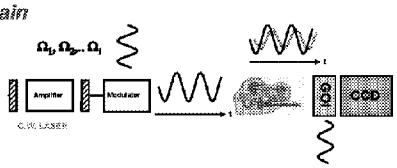


Fluorescence lifetime imaging technology

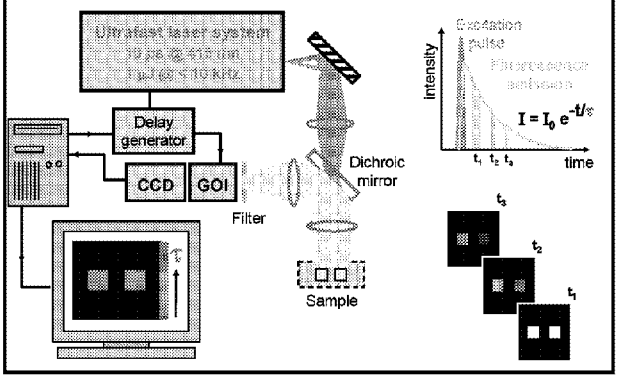
Time domain



Frequency domain



Fluorescence lifetime imaging (FLIM)



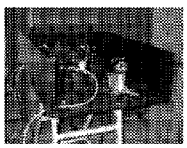
Ultrafast laser sources for FLIM

Ti:sapphire femtosecond laser (Spectra-Physics)

100 fs @ 415 nm, 190 pJ @ 80 MHz

Diode-pumped ultrafast Cr:LiSAF laser oscillator and amplifier system (FLOAT)

10 ps @ 415 nm, 1 µJ @ single shot, 1Hz-20 kHz

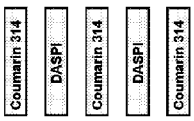


Blue diode laser (PicoQuant)

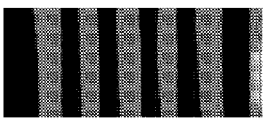
FWHM < 30 ps @ 400 nm,
Average Power = 1.1 mW,
Repetition rate up to 40 MHz

FLIM of dye samples: chemically specific imaging

Dye samples:

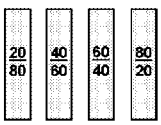


Fluorescence lifetime map:

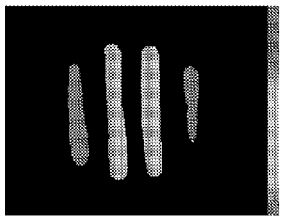


FLIM of fluorophore environment (viscosity)

DASPI solvent: $\frac{\text{ethanol}}{\text{glycerol}}$:



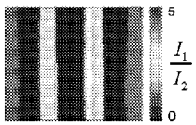
Fluorescence lifetime map:



Quantitative whole-field FLIM of [fluorophore] ratio

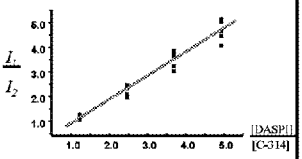
Pipettes with dye mixtures

[DASPI] / [C-314] = 1.2 2.5 3.7 4.9



Two component decay:

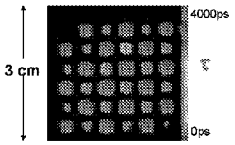
$$I(t) = I_1 e^{\frac{-t}{\tau_1}} + I_2 e^{\frac{-t}{\tau_2}}$$



Quantitative high-speed assays
e.g. bio-chips, HTS, DNA sequencing...

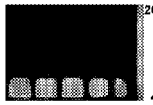
Macroscopic multi-well-plate imaging – for assays

Chemically specific imaging



Coumarin 314
DASPI

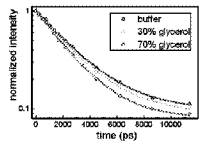
Influence of the fluorophore environment (viscosity)



DASPI + ethanol / glycerol

100/0 80/20 60/40 40/60 20/80

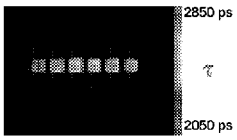
E-GFP lifetime depends on local refractive index

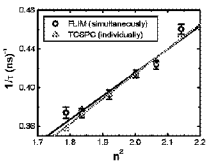


E-GFP in glycerol/buffer solution

% glycerol ⟶ decreasing

n ⟶ decreasing

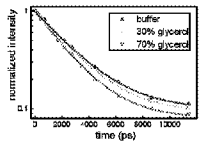


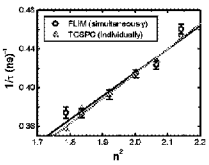


Strickler-Berg-plot

Application to cell imaging?

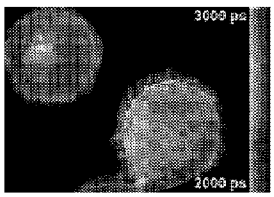
E-GFP lifetime depends on local refractive index





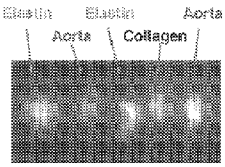
Strickler-Berg-plot

EGFP-tagged MHC protein at B-cell surface



Origin of fluorescence lifetime heterogeneity?

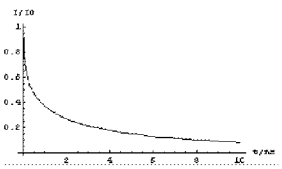
FLIM of biological (rat) tissue proteins



Fluorescence intensity images:

Fluorescence lifetime images:

Analysis of complex fluorescence decay profiles



single $I(t) = \alpha \exp\left(-\frac{t}{\tau}\right)$

multiple $I(t) = \sum_{i=1}^{j} \alpha_i \exp\left(-\frac{t}{\tau_i}\right)$

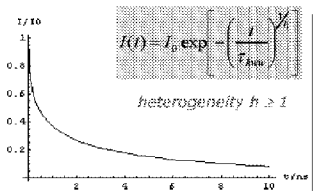
⇒ continuous lifetime distribution, $j \to \infty$ $I(t) = \int_0^\infty \exp(-\frac{t}{\tau}) \rho(\tau) d\tau$

Stretched exponential function

$h \geq 1$

$$I(t) = I_0 \exp\left[-\left(\frac{t}{\tau_{KWW}}\right)^h\right]$$

Stretched Exponential Function



$$I(t) = I_0 \exp\left[-\left(\frac{t}{\tau_{Decay}}\right)^h\right] \Longleftrightarrow I(t) = \int_0^\infty \exp(-\frac{t}{\tau})\rho(\tau)\,d\tau$$

continuous lifetime distribution

$$\langle\tau\rangle = h \cdot \tau_{Decay} \cdot \Gamma(h)$$

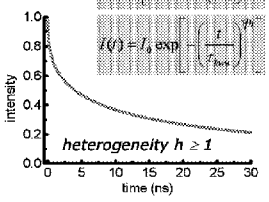
- 1863: F. Kohlrausch describes structural relaxation in glassy fibre.
- 1970: G. Williams & D.C. Watts describe dielectric relaxation in polymers.
- since then: applied to earthquakes, galactic light emissions, economics, biological extinction, MRI, etc.

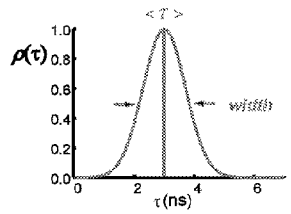
Stretched Exponential Function

$$I(t) = \int \rho(\tau)\exp\left[-\frac{t}{\tau}\right]d\tau$$

$$I(t) = I_0 \exp\left[-\left(\frac{t}{\tau_{Decay}}\right)^h\right]$$

Continuous lifetime distribution $\rho(\tau)$

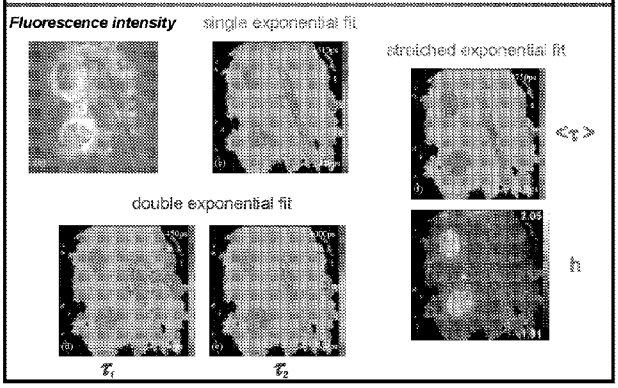




Fitting parameters

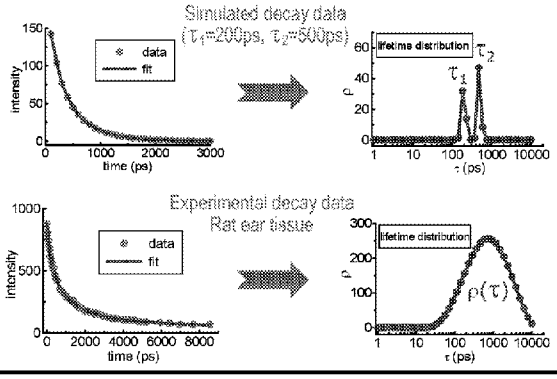
- average lifetime $\langle\tau\rangle = h \cdot \tau_{Decay} \cdot \Gamma(h)$
- heterogeneity $h \sim width$

Complex fluorescence decay profiles in rat ear (x10)



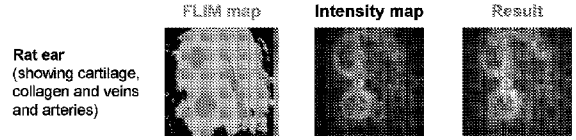
Laplace transformation: I(t) ⟹ τ_3, ρ(τ)

CONTIN algorithm by S.W. Provencher: Comput. Phys. Commun. 27, 213 (1982).



How to represent lifetime, heterogeneity and intensity information

H,S,L colour plot: H,S encode *lifetime*, L encodes *intensity*



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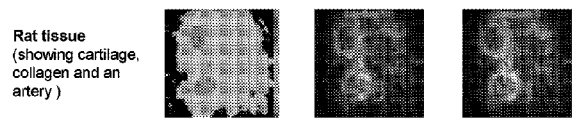
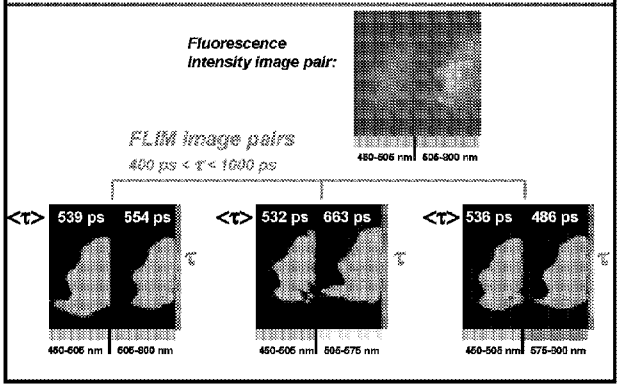
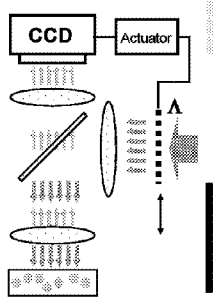


Spectrally-resolved FLIM of rat heart tissue



light

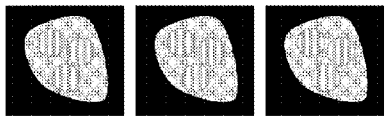


Method of obtaining optical sectioning by using structured light in a conventional microscope

Opt. Lett. 22 (1997) 1905

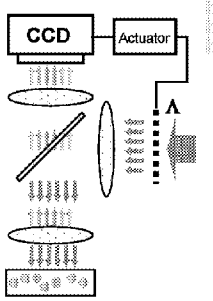
Only *zero spatial frequency* does not attenuate with defocus

⇒ spatially modulate whole-field image using grid



+Λ/3 +2Λ/3

light



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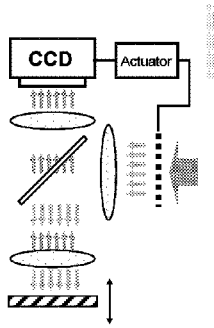
⇒ spatially modulate whole-field image using grid

Sectioned image *Conventional image*

light



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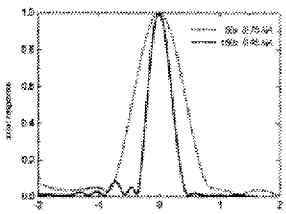


Measured axial response

3-D FLIM of 15 μm fluorescent microspheres

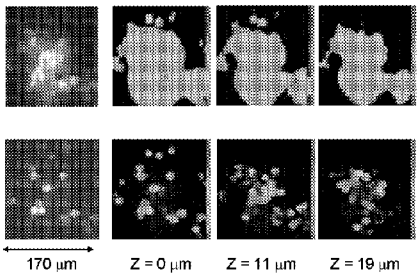
(8 bit ICCD)

Intensity images

FLIM images (0.2 - 7 ns)

Conventional Images:

Sectioned images:

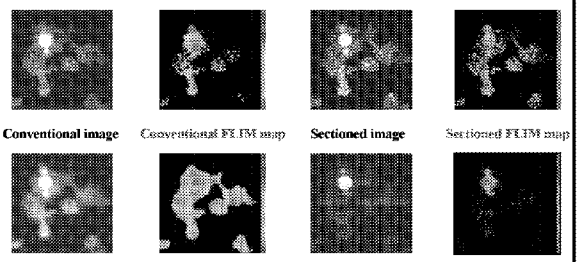


170 μm Z = 0 μm Z = 11 μm Z = 19 μm

3-D FLIM of 4.5 and 15 μm fluorescent microspheres

(12-bit CCD)

Image plane 1, z = 0 μm



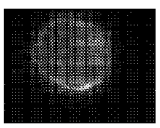
Conventional image Conventional FLIM map Sectioned image Sectioned FLIM map

Image plane 2, z = 4 μm

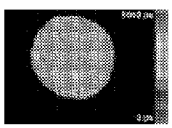
Wide-field optical sectioning

FLIM of EGFP in cell membrane

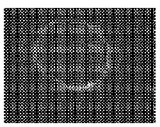
Conventional intensity image



Conventional FLIM map



Sectioned intensity image (Δz~ 1.4 μm)

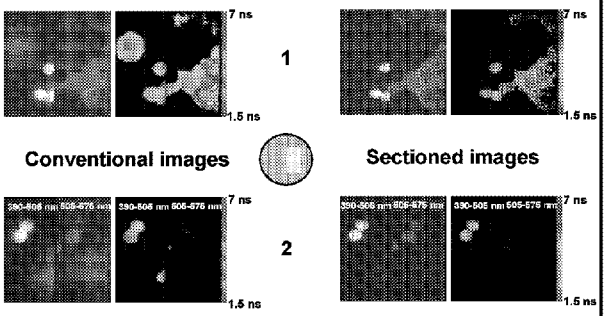


Sectioned FLIM map

Optical sectioning by structured illumination

Mark Neil et al, Opt. Lett. 22 (1997) 1905

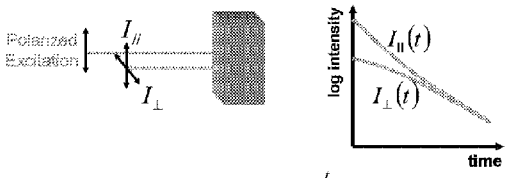
5-D fluorescence imaging of 4.5 μm & 15 μm beads



Simultaneous lifetime, spectral and depth information

Time resolved fluorescence anisotropy imaging

Anisotropy is defined by $r(t) = \frac{I_{//}(t) - I_{\perp}(t)}{I_{//}(t) + 2I_{\perp}(t)}$



For a spherical molecule : $r(t) = r_0 e^{(-\frac{t}{\theta})}$

with rotational correlation time $\theta = \frac{\eta V}{RT}$ viscosity

Polarization-resolved FLIM

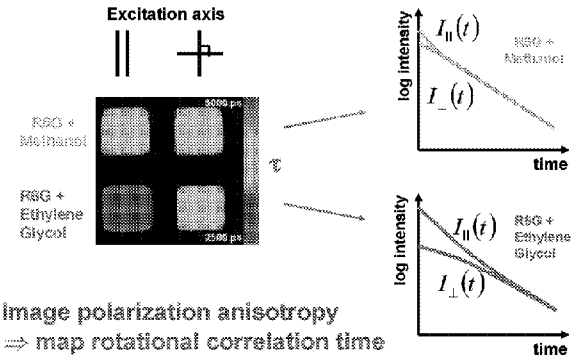
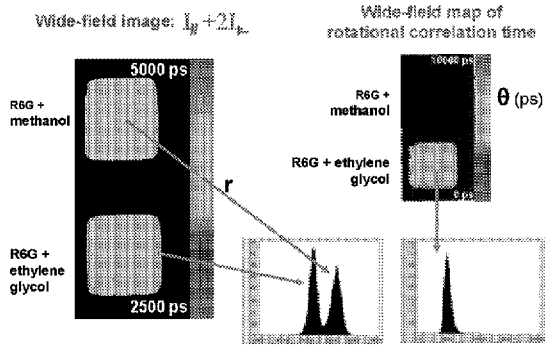


image polarization anisotropy
⇒ map rotational correlation time

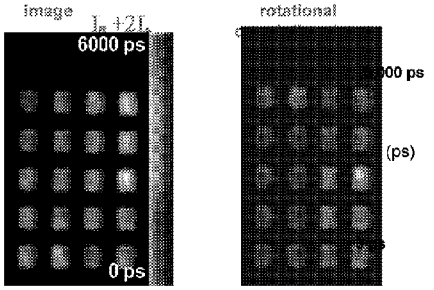
Wide-field images of "total fluorescence decay" and rotational correlation time of Rhodamine 6G



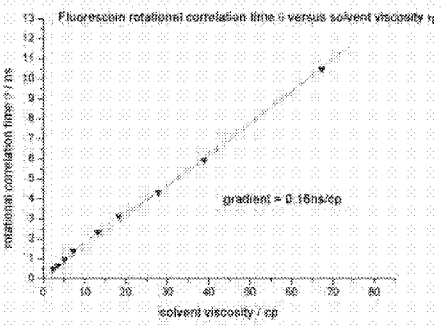
Fluorescence Lifetime and Anisotropy Imaging (FLAIm): Fluorescein in NaOH/glycerol

% glycerol weight in wells

0	0	63.0	63.0
29.9	29.9	67.4	67.4
38.4	38.4	72.4	72.4
46.9	46.9	75.9	75.9
53.3	53.3	81.0	81.0



Fluorescence Lifetime and Anisotropy Imaging (FLAIm): Fluorescein in NaOH/glycerol

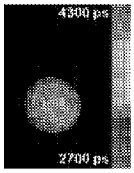


Fluorescence Lifetime and Anisotropy Imaging (FLAIm): application to cells?

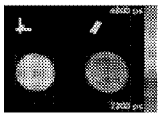
CFSE stained B cells

FLIM map
Vertical excitation

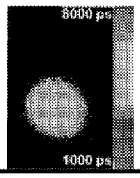
Fluorescence (total) lifetime map



FLIM map
Vertical excitation



Rotational correlation time map



Outlook for microscopy and biomedical imaging

- More functional imaging
  - *FLIM and FRET, multi-spectral imaging*
  - *Super-resolution*
  - *PSF engineering, e.g. 4 Pi microscope, STED*
- Multi-modal microscopes
  - *Combining confocal microscopy with OCT, THG, multi-photon fluorescence microscopy*
- High-speed imaging
  - *Wide-field optical sectioning*
  - *Multi-foci microscopes*
  - *High speed cameras and multi-channel imaging*
- Compact user-friendly low-cost laser technology

For more information...

For a review of the development of ultrafast laser technology:
French, P.M.W., *The Generation of Ultrashort Laser Pulses.* Reports on Progress in Physics, 1995. 58(2): p. 169-262.

For a review of biomedical optics:
French, P. M. W., *Biomedical Optics in the 21st Century.* Physics World, (June 1999) 41-46
http://physicsweb.org/article/world/12/6/8

For a review of fluorescence lifetime imaging:
Cole, M.J., J. Siegel, S.E.D. Webb, R. Jones, K. Dowling, M.J. Dayel, D. Parsons-Karavassilis, P.M.W. French, M.J. Lever, L.O.D. Sucharov, M.A.A. Neil, R. Juskaitis, and T. Wilson, *Time-domain whole-field fluorescence lifetime imaging with optical sectioning.* Journal of Microscopy, 203 (2001) 246-257
See also OPN November 2002 at www.osa.org

http://photonics.ic.ac.uk — *Look for biomedical optics pages and recent conference presentations available online*

Further reading

http://micro.magnet.fsu.edu/primer/index.html

Denk, W., Strickler, J. H. & Webb, W. W. 2-Photon Laser Scanning Fluorescence Microscopy. *Science* **248**, 73-76 (1990).

Muller, M., Squier, J., Wilson, K. R. & Brakenhoff, G. J. 3D microscopy of transparent objects using third-harmonic generation. *Journal of Microscopy-Oxford* **191**, 266-274 (1998).

Webb, R. H. Confocal optical microscopy. *Reports on Progress in Physics* **59**, 427-471 (1996).

Andersson Engels, S., Klinteberg, C., Svanberg, K. & Svanberg, S. In vivo fluorescence imaging for tissue diagnostics. *Physics in Medicine and Biology* **42**, 815-824 (1997)

Cole, M. J. et al. Time-domain whole-field fluorescence lifetime imaging with optical sectioning. *Journal of Microscopy-Oxford* **203**, 246-257 (2001)

Schrader, M. & Hell, S. W. 4Pi-confocal images with axial superresolution. *Journal of Microscopy-Oxford* **183**, 189-193 (1996)

Straub, M. & Hell, S. W. Multifocal multiphoton microscopy: a fast and efficient tool for 3-D fluorescence imaging. *Biotmaging* **6**, 177-185 (1998)

Klar, T. A., Jakobs, S., Dyba, M., Egner, A. & Hell, S. W. Fluorescence microscopy with diffraction resolution barrier broken by stimulated emission. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 8206-8210 (2000)