Fluorescence Nanoscopy

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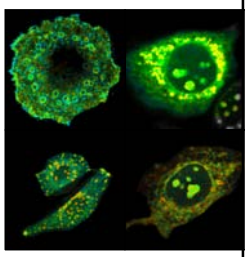


Outline

- The Abbe's (diffraction) limit and nanoscopy
- Fundamentals and opportunities of FLIM/FRET
- Visualizing cell metabolism with autofluorescence imaging
- Coming development

One of the Microscopy Challenges: Elucidating Molecular Dynamics in Biology without Destroying the Molecules

Diffract Limit: **A barrier originated from the Uncertainty Principle**

Ernst Abbe memorial at Jena, Germany:

$$\Delta x \Delta p > \frac{\hbar}{2}$$
$$\Delta E \Delta t > \frac{\hbar}{2}$$

Diffraction Limit: $\Delta R \sim \lambda/NA$

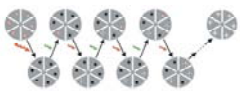
Difficult for life science to be alive at $\lambda < 300$ nm

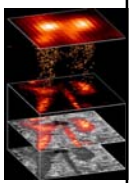
Nanoscopy: Seeing Beyond the Diffraction Limit

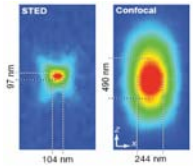
From micro to nano

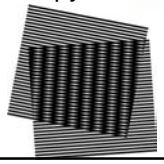
200 nm →100 nm → 30 nm

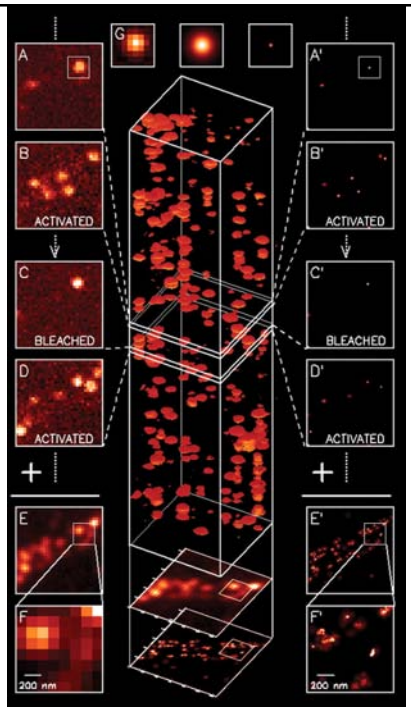
1. Stochastic optical reconstruction microscopy (STORM), 2006, Xiaowei Zhuang.
2. Photoactivated localization microscopy (PALM), 2006, by Eric Betzig, Harald Hess, and Samuel Hess.
3. Stimulated emission depletion (STED) by Stefan Hell.
4. Saturated structured illumination microscopy (SSIM) by Mats Gustafsson.







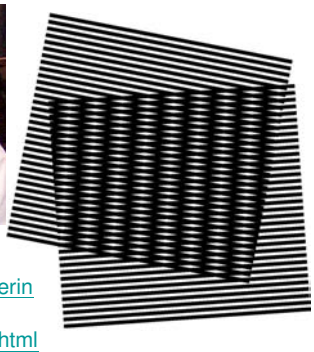


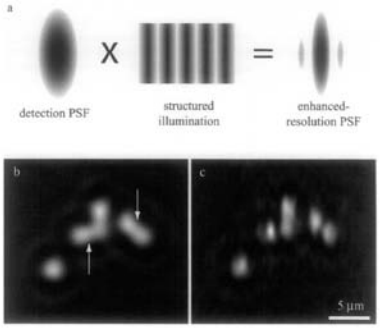


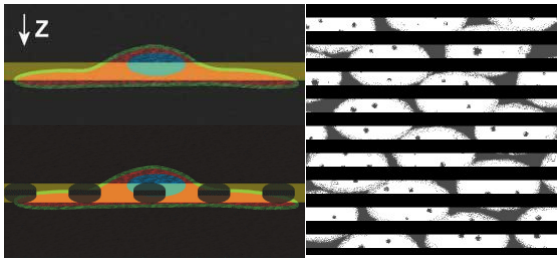
The principle behind PALM. A sparse subset of PA-FP molecules that are attached to proteins of interest and then fixed within a cell are activated (**A** and **B**) with a brief laser pulse at $_{act}$ = 405 mm and then imaged at $_{exc}$ = 561 mm until most are bleached (C). This process is repeated many times (**C** and **D**) until the population of inactivated, unbleached molecules is depleted. Summing the molecular images across all frames results in a diffraction-limited image (**E** and **F**). However, if the location of each molecule is first determined by fitting the expected molecular image given by the PSF of the microscope [(**G**), center] to the actual molecular image [(G), left], the molecule can be plotted [(G), right] as a Gaussian that has a standard deviation equal to the uncertainty $_{x,y}$ in the fitted position. Repeating with all molecules across all frames (**A**' through **D**') and summing the results yields a superresolution image (**E**' and **F**') in which resolution is dictated by the uncertainties $_{x,y}$ as well as by the density of localized molecules. Scale: 1 x 1 µm in (F) and (F'), 4 x 4 µm elsewhere.

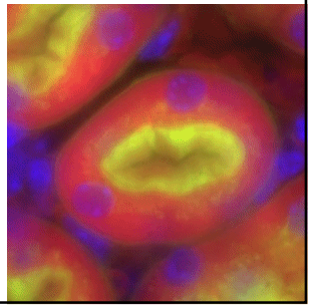
A moiré pattern and structured illumination

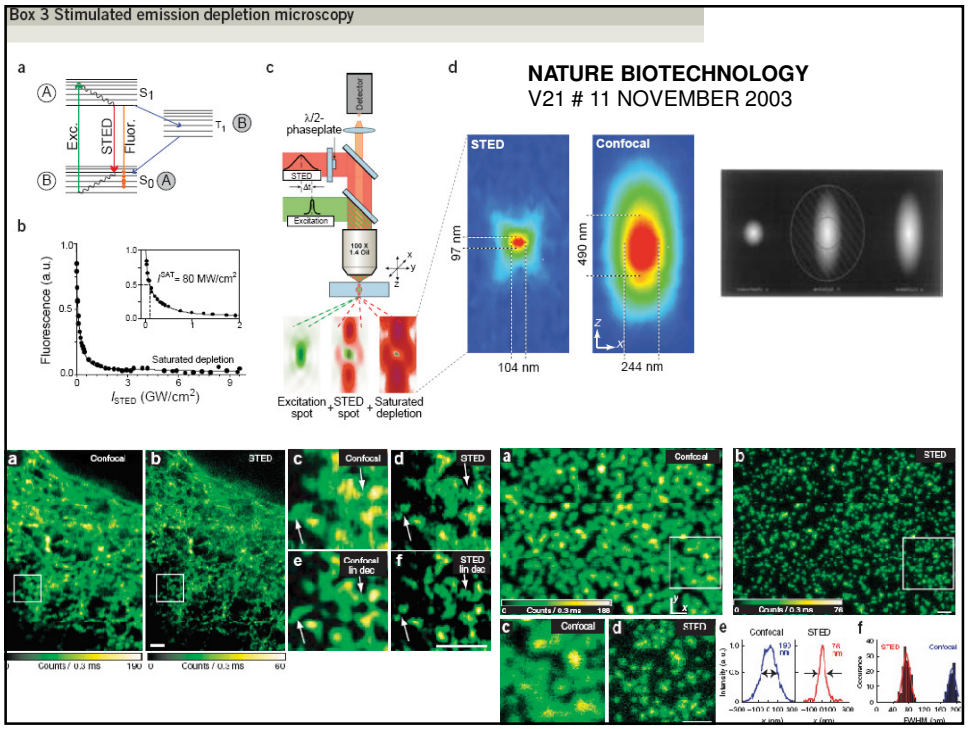
Mats Gustaffson
http://www.bioengineering.ucsf.edu/faculty-mats_gustafsson.vp.html

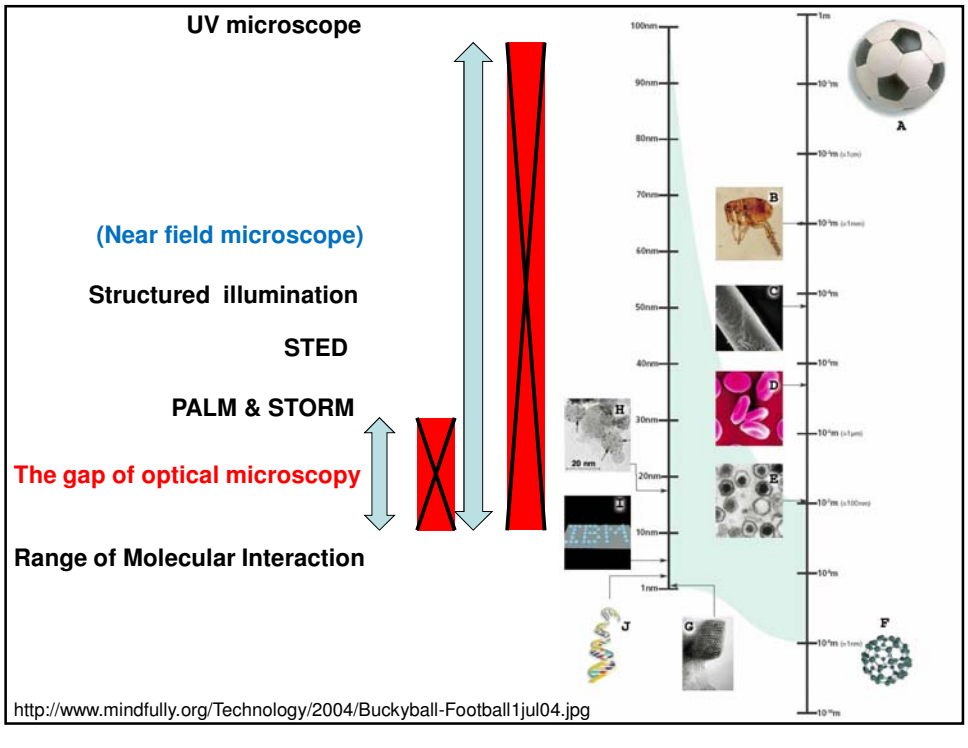









Box 3 Stimulated emission depletion microscopy
NATURE BIOTECHNOLOGY
V21 # 11 NOVEMBER 2003
STED
Confocal
97 nm
490 nm
104 nm
244 nm
Excitation spot
STED spot
Saturated depletion


UV microscope
(Near field microscope)
Structured illumination
STED
PALM & STORM
The gap of optical microscopy
Range of Molecular Interaction
http://www.mindfully.org/Technology/2004/Buckyball-Football1jul04.jpg

Nanoscopy:
Seeing Beyond the Diffraction Limit

From micro to nano

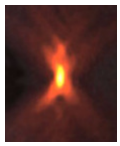
200 nm →100 nm → 30 nm

1. Note that the critical distance for bio-interaction is ~10 nm.
2. The available photons and the signal-to-noise ratio.
3. $\Delta x \Delta p > \frac{\hbar}{2}$ ⟸ **The "brute force" dimension**

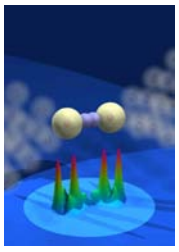
   $\Delta E \Delta t > \frac{\hbar}{2}$ ⟸ **the "free" dimension**

PSF Volume $\propto \frac{1}{(NA)^4}$

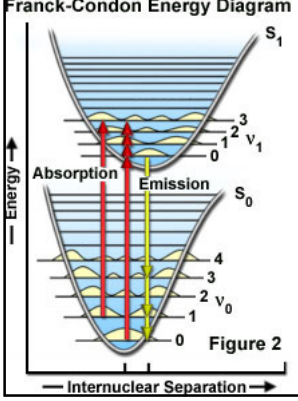
With the time domain techniques, the "ensemble average" of molecular dynamics is recorded within the point spread function volume.

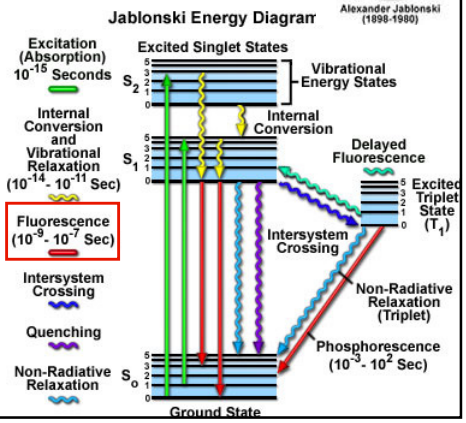


Starting with Molecular States __
Franck-Condon and Jablonski Energy Diagram



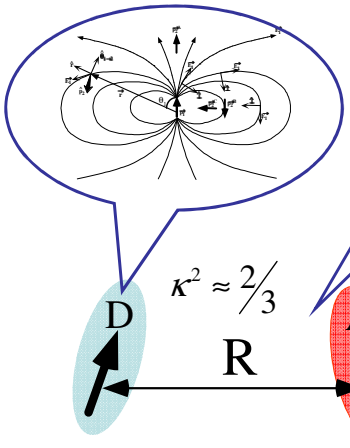
Alexander Jablonski (1898-1980)



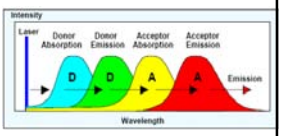


Dipole-dipole interaction

1. Van der Waal force
2. FRET (Förster/Fluorescence Resonant Energy Transfer)



$$k_T = \frac{1}{\tau_{r_D}} \left(\frac{R_0}{R} \right)^6$$

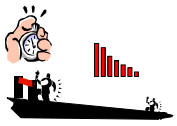


Criteria of FRET

- Overlapping of the emission spectrum of D and the absorption spectrum of A. (> 30%).
- Close proximity of two fluorophores (within 1 to 10 nm).
- Favorable mutual orientation.
- High quantum yield of the donor.

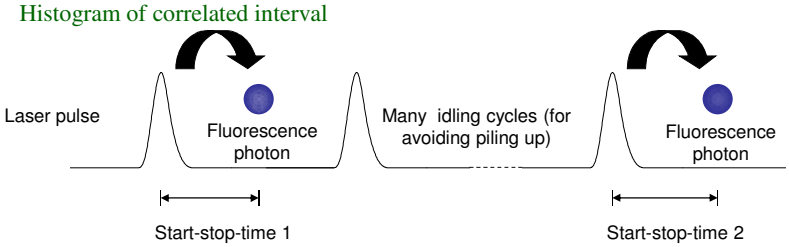
Fluorescence Lifetime Measurement

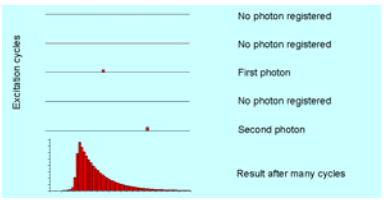
A Time-domain Technique: Time Correlated Single Photon Counting (TCSPC)

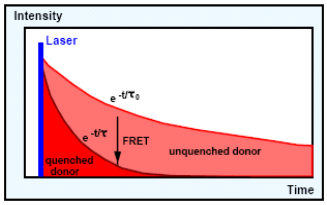


1. **The concept of a very fast stop watch**
2. **Naturally compatible with confocal microscope using pulsed lasers.**

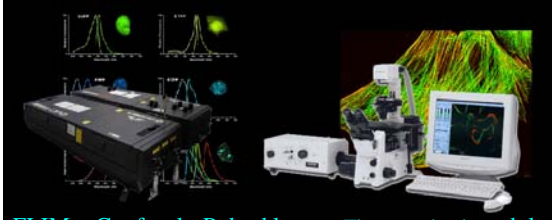
Histogram of correlated interval

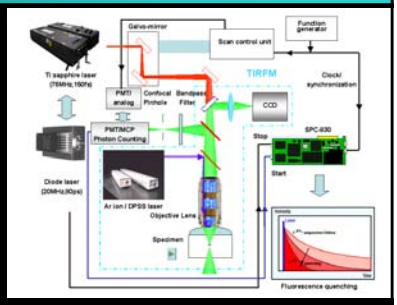




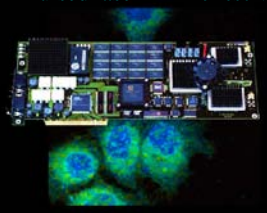


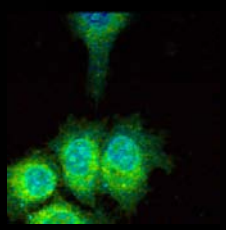
2-p Time-correlated single photon counting microscopy

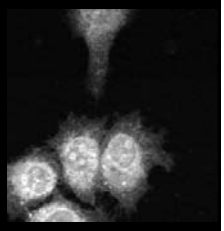




FLIM = Confocal+ Pulsed laser + Time-resolved module







The Advantages of Photon Counting

- High temporal resolution (~0.1 ns)
- Great signal-to-noise ratio (the SMD capacity)
- Intensity fluctuations in light source is not critical
- Intuitive data interpretation

The Significance of FLIM/FRET

- **To distinguish "co-localization" and "binding" with nanometer (1~10 nm) sensitivity**
- **In vivo and in-situ observation**

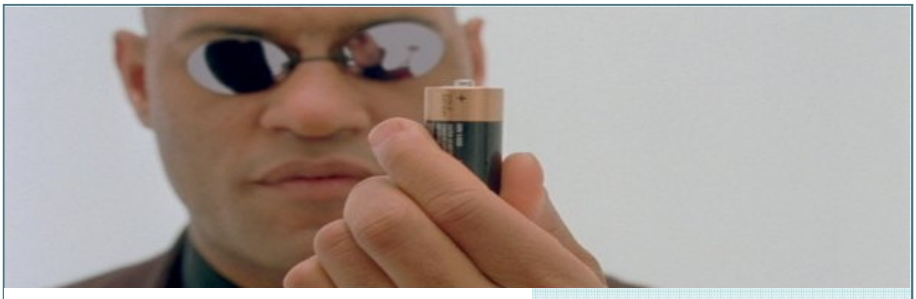
FLIM is far more quantitative than ratio imaging (intensity FRET), not affected by photobleaching, dilution, ..etc.

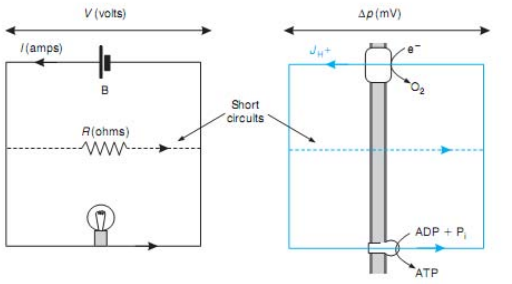
The FLIM Platforms

- 2-p
- Portable
- Multi-channel
- Miniaturized
- STED-FLIM
- Others (Micro-laser engraving, induction powering)

Cell Vitality and Metabolism through Auto-fluorescence

We are batteries





ATP (Adenosine triphosphate)
The main energy source and transporter in the cells.

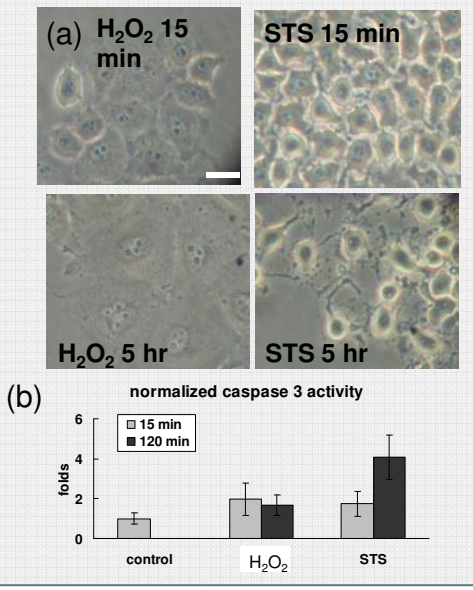
A Ratio Imaging

$$I_c(t) = \int_{-\infty}^{\infty} I_{instr}(t)\{I_0 + F(t)\}dt$$

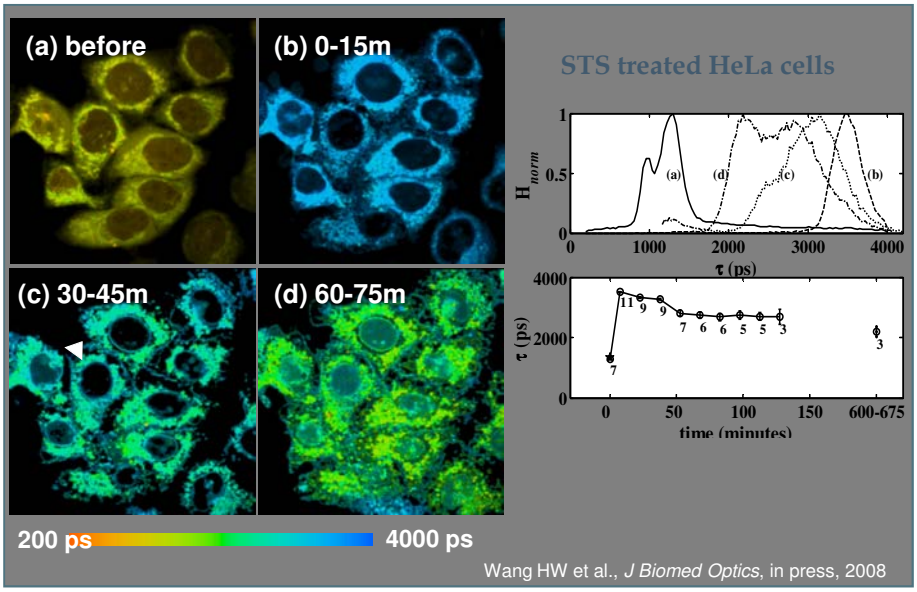
$$F(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}$$

$$\frac{a_1}{a_2}$$

Brightfield imaging

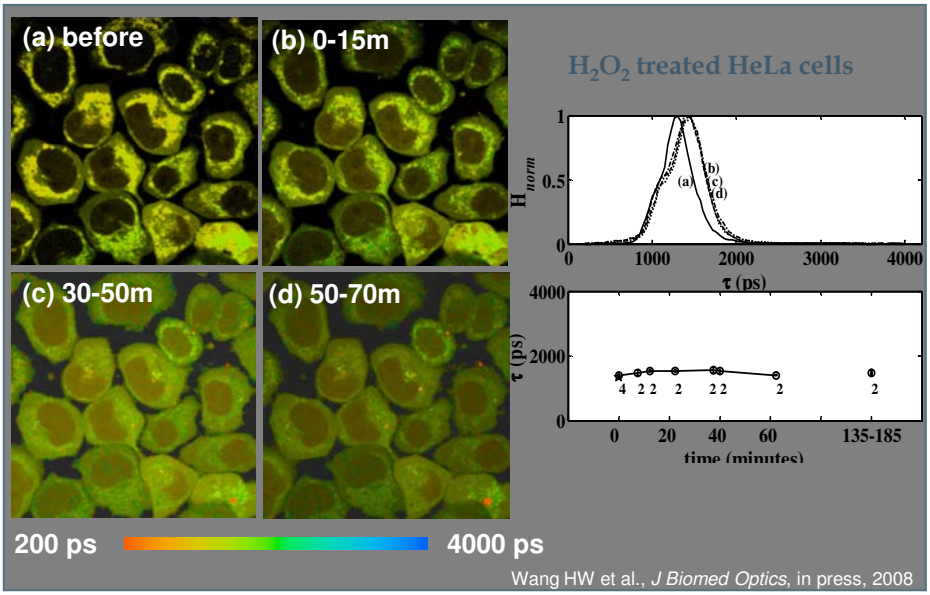


Lifetime imaging: the “death raise”

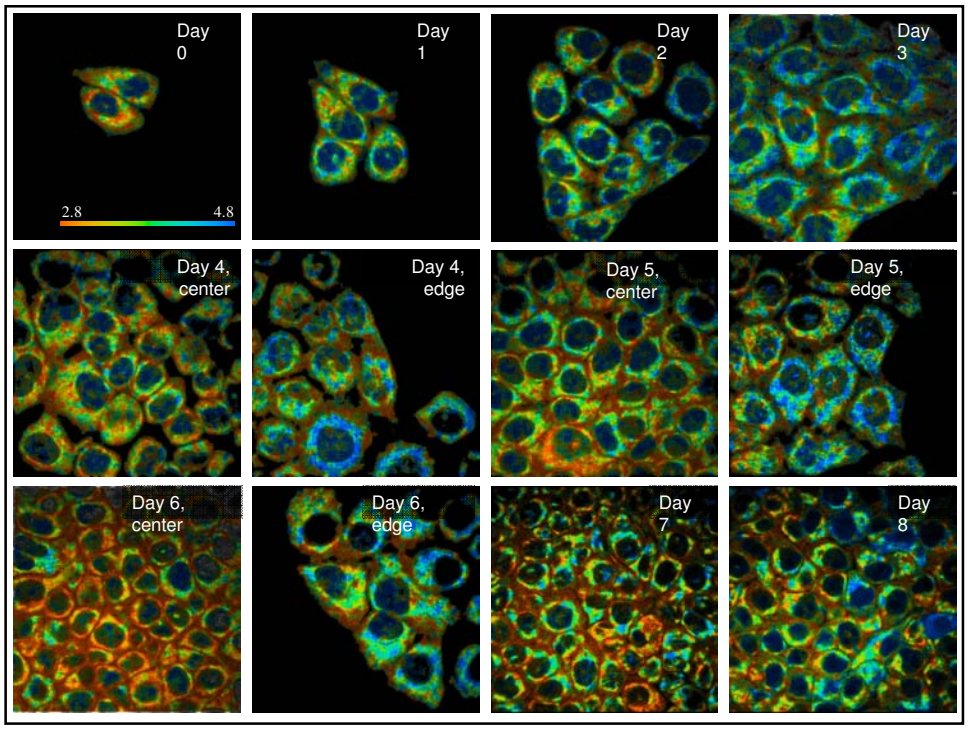


Wang HW et al., *J Biomed Optics*, in press, 2008

Lifetime of necrosis: still

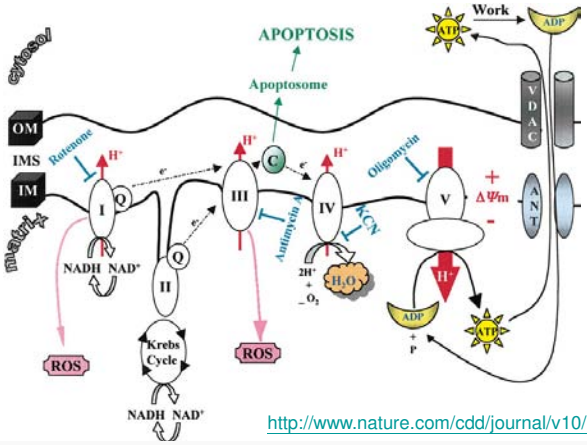


Wang HW et al., *J Biomed Optics*, in press, 2008



Mitochondrial functions during cell death, a complex (I–V) dilemma

J-E Ricci[1], N Waterhouse[2] and D R Green[1]
Correspondence: Green, E-mail: DGreen5240@aol.com



http://www.nature.com/cdd/journal/v10/n5/full/4401225a.html

NADH → *Complex I* → Q → *Complex III* → cytochrome *c* → *Complex IV* → O_2

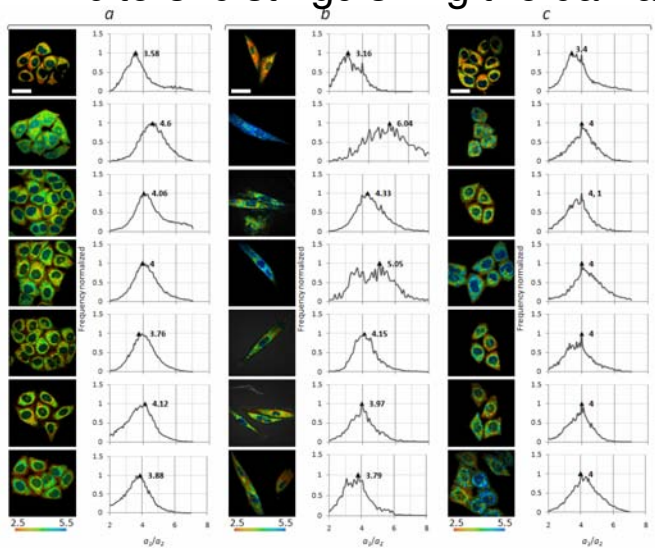
(rotenone)

Complex II

(Antimycin A, Myxothiazol, Stigmatellin)

(Cyanide, sulfide, azide, and carbon monoxide)

Inhibitors: distinguishing the damage

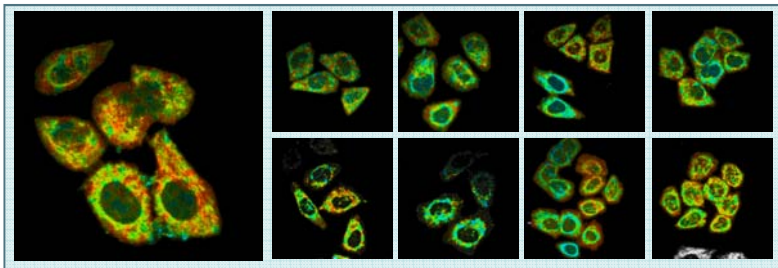


Rotenone, 1.5uM, HeLa (Parkinson's disease, pesticide, insecticide, etc)

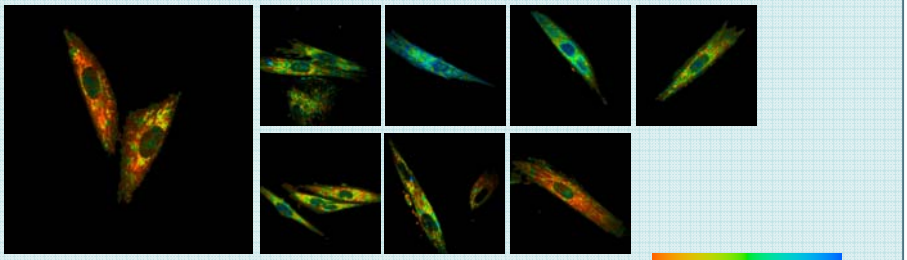
KCN, 1.5uM, WI38

KCN, 1.5uM, HeLa

Inhibitors: distinguishing the damage



Rotenone, 1.5uM, HeLa (Parkinson's disease, pesticide, insecticide, etc)

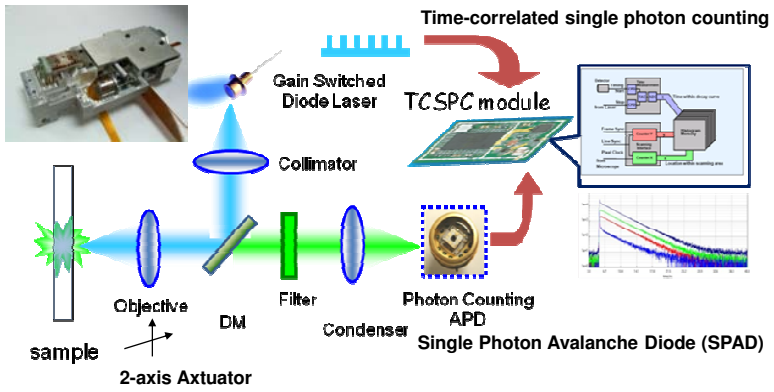


KCN, 1.5uM, WI38

75%

90%

Pick-up Head for Bio-sensing



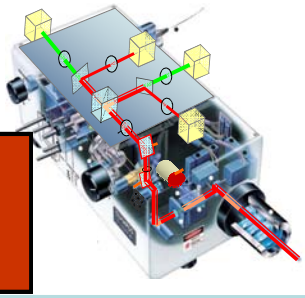
Phase1 : **FLIM-based Pick-up Head**
Phase2 : **FLIM-CARS based Pick-up Head**

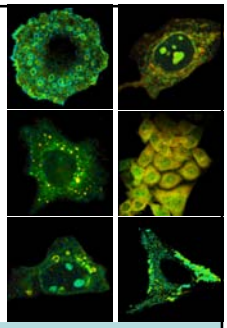
Schematic design of FLIM-based Blue pick-up head (PUH) for rapid, inexpensive read o
Such a PUH will permit High Throughput Screening of novel HCV anti-viral drugs.

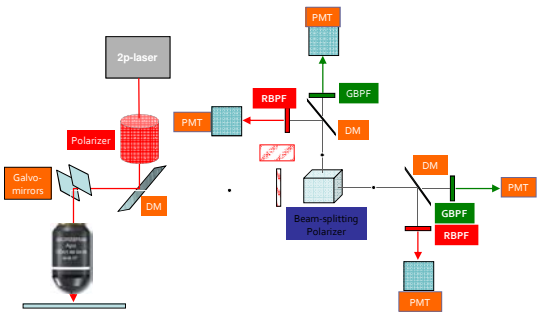
MODERN OPTICS LABORATORY, NYMU

Argus

- User configurable and friendly
- Compact and portable
- Cost-effective
- Rapid know-how transfer







Improved Functions
- 4 simultaneous detections
-Increased temporal resolution
-Polarization-resolved detection
- Anisotropy measurements

Portability
Designed as an add-on can be easily moved between systems with slight additional calibration

Thank You for Your Attention !