# Oxygen microscopy with two-photon enhanced phosphorescent nanoprobes

Department of Biophysics and Biochemistry,
University of Pennsylvania

# Oxygen Microscopy Design Parameters

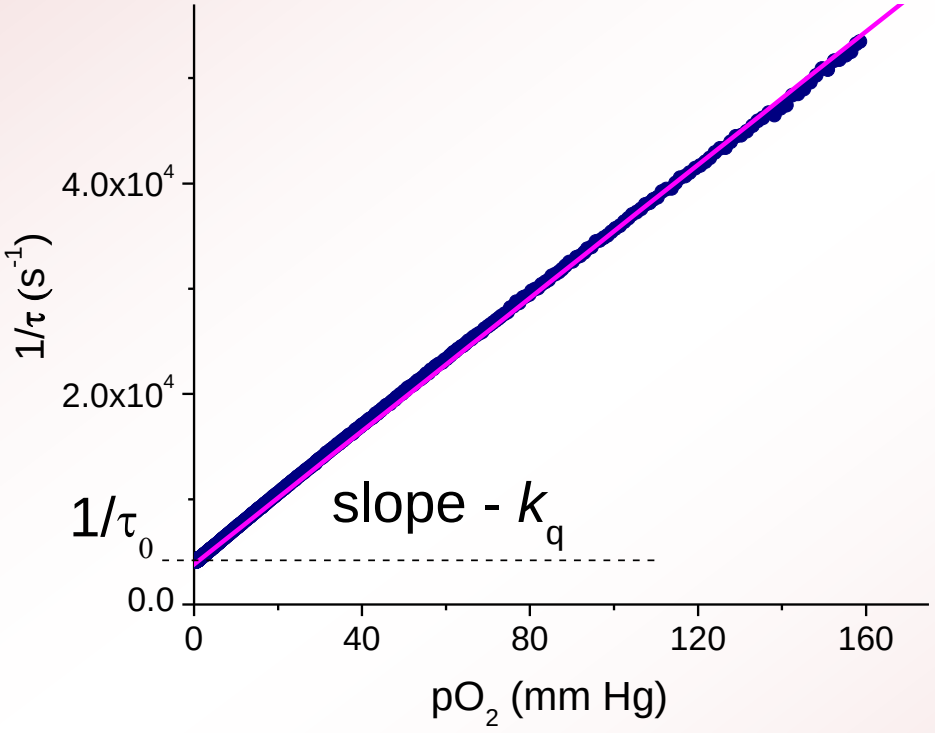
***Interested in studying distribution of oxygen at the cellular and sub-cellular level***
***Using PHOSPHORESCENCE QUENCHING***

Microscope Parameters
- Two-photon emission (optical sectioning)
- Lifetime Measurements

Probe Parameters
- Phosphorescent
- Two-photon absorbing
- Tunable Sensitivity to Oxygen
- Insensitive to other environmental effects

# Phosphorescence Quenching

- **Oxygen quenches phosphorescence of probe**
- **Concentration of oxygen can be measured by phosphorescence lifetime ($\tau$) or phosphorescence intensity (I)**

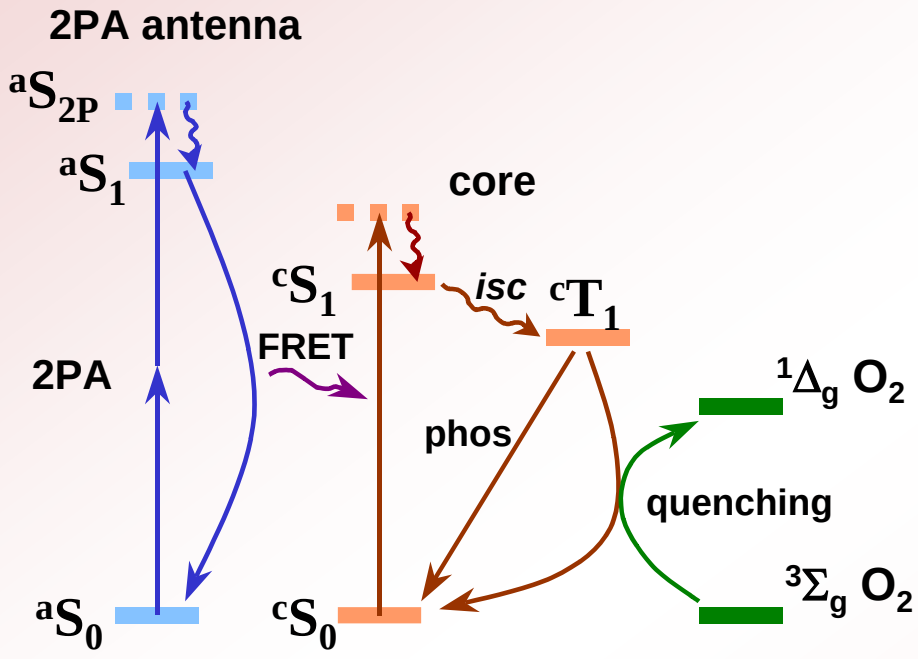


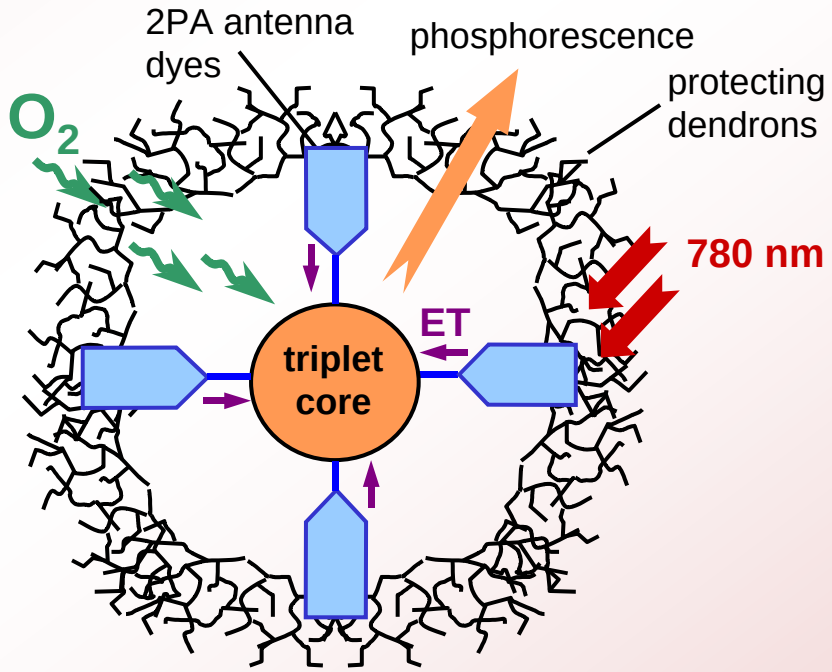

## Stern-Volmer equation

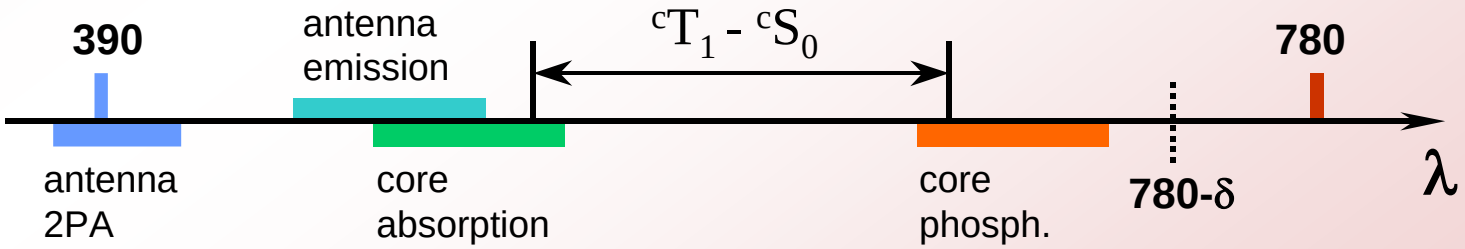
$$\frac{I_0}{I} = \frac{\tau_0}{\tau} = 1 + K_Q \tau_0 [O_2]$$

**$K_Q$, $\tau_0$ - probe-related constants.**

# Probe Design: Couple 2PA to phosphorescence *via* intramolecular FRET

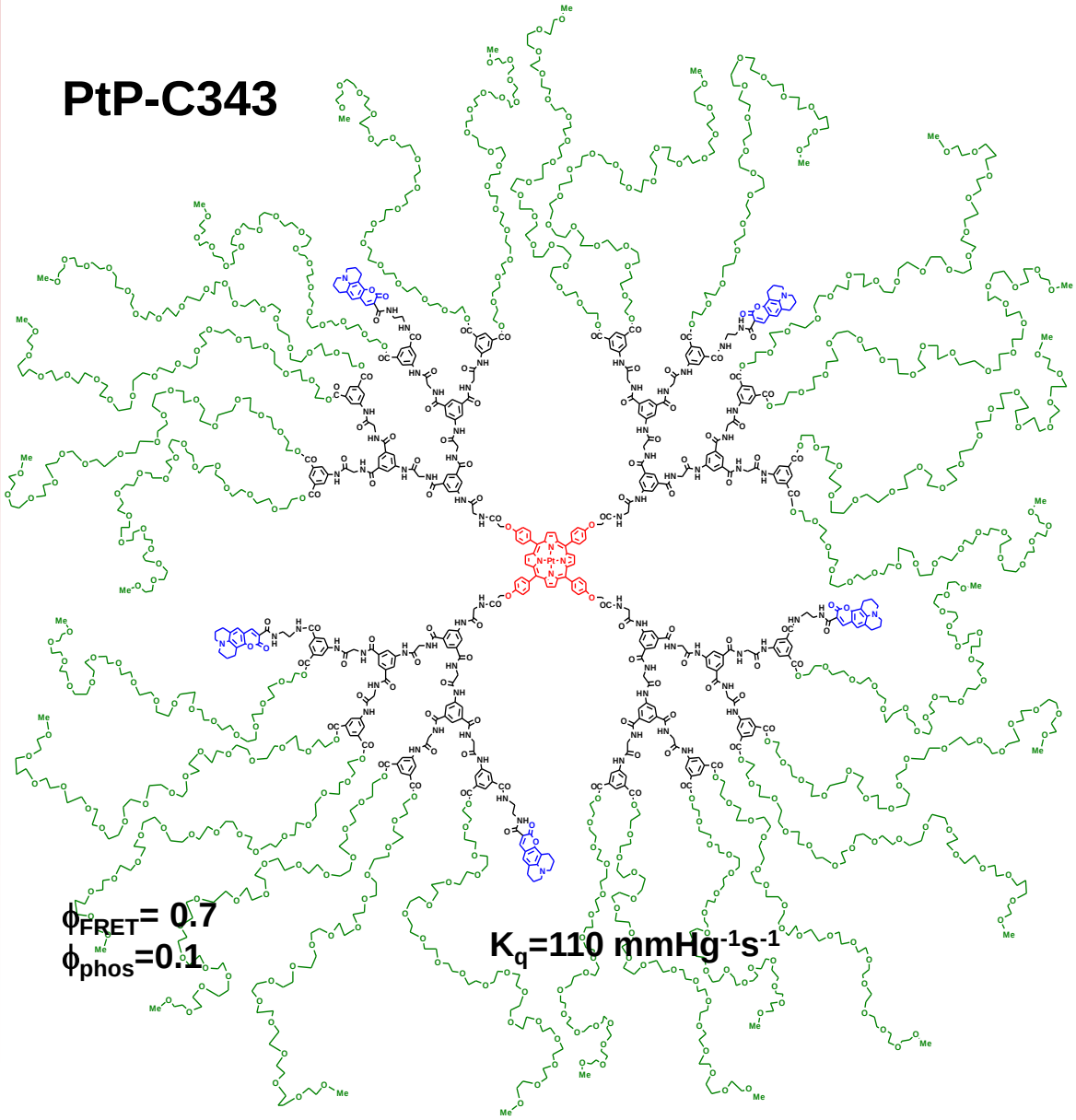


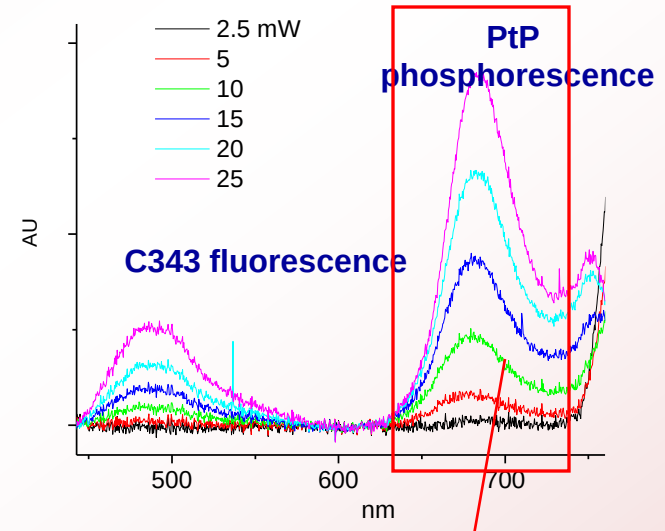



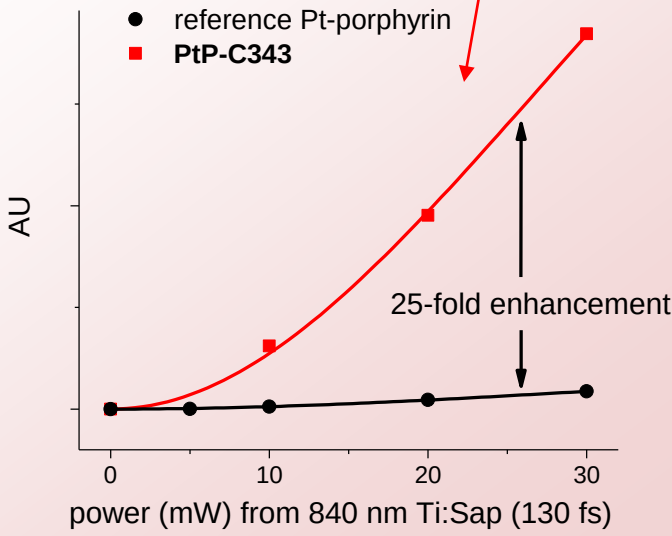



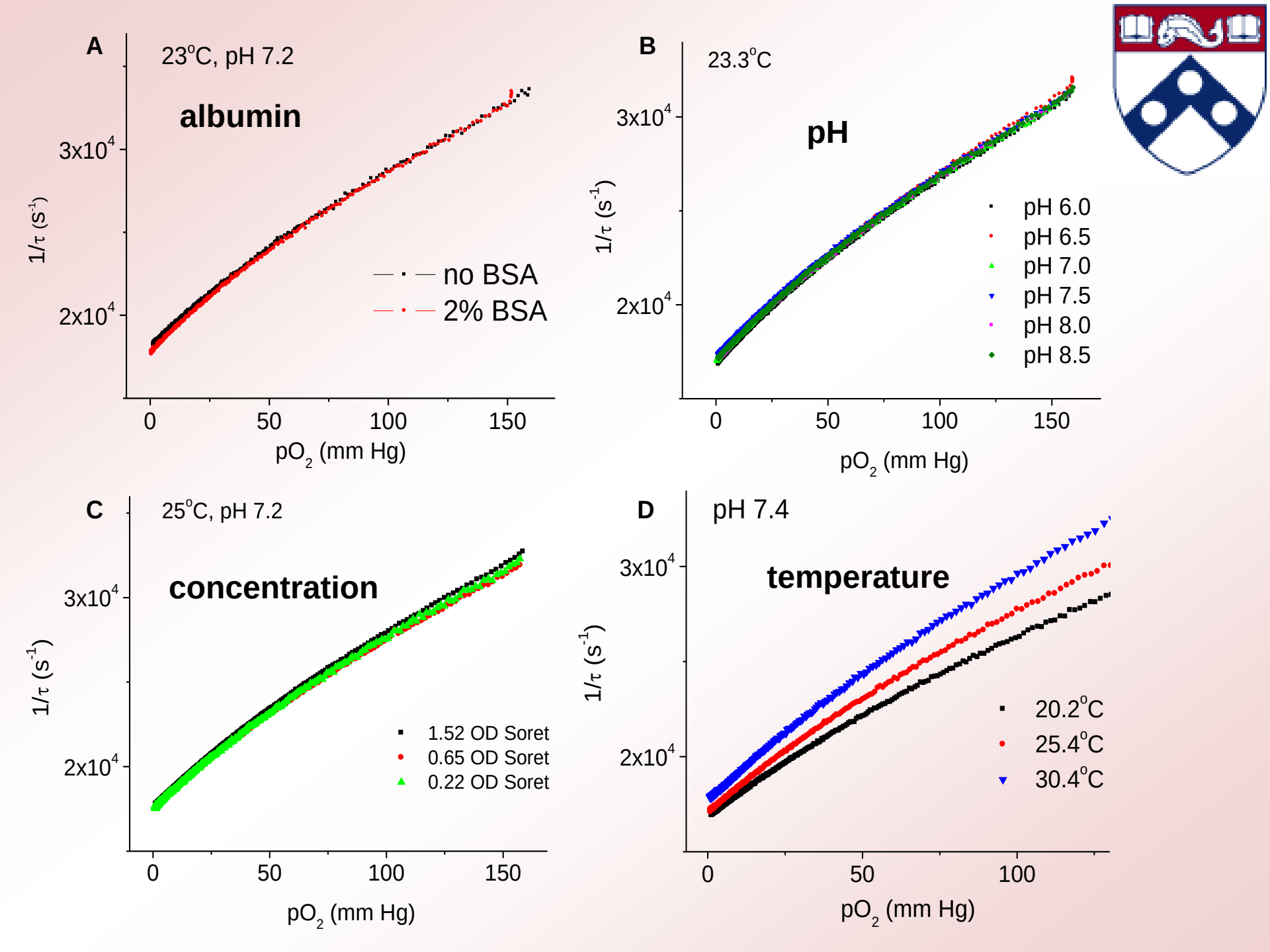

Briñas et al. *J. Am. Chem. Soc.*, 2005, **127**, 11851.

# First Generation Probe

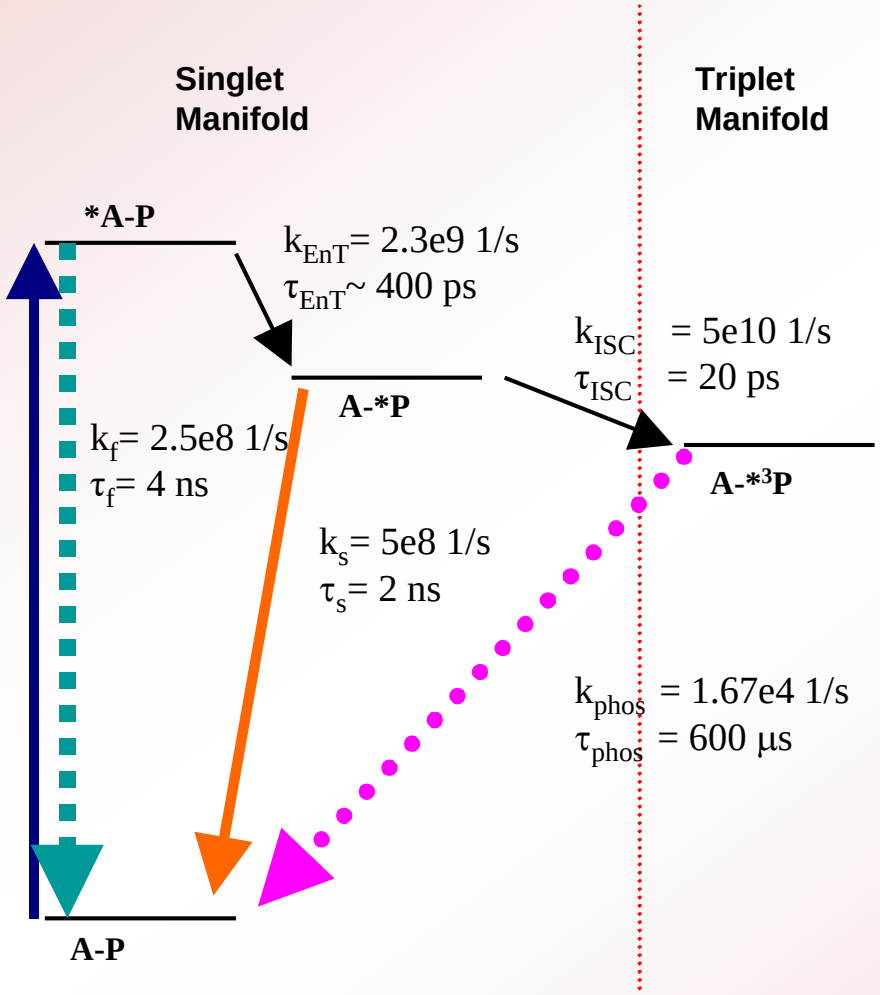


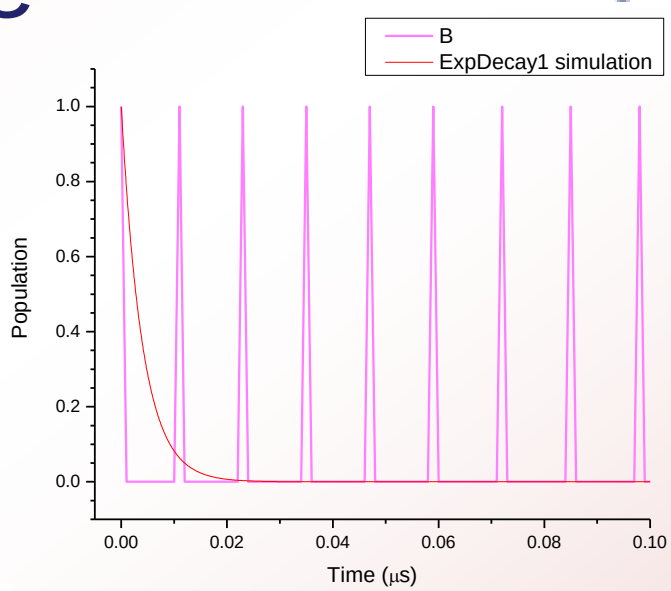



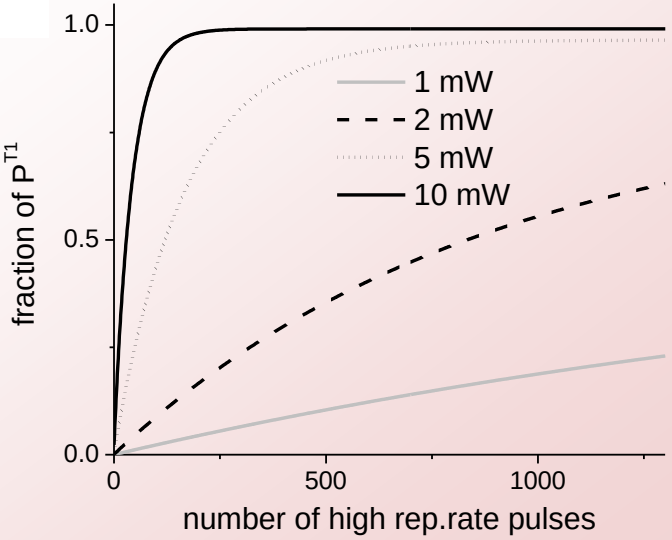




A
23°C, pH 7.2
albumin
3x10^4
2x10^4
1/τ (s^-1)
no BSA
2% BSA
0
50
100
150
pO2 (mm Hg)
B
23.3°C
pH
pH 6.0
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 8.5
C
25°C, pH 7.2
concentration
1.52 OD Soret
0.65 OD Soret
0.22 OD Soret
D
pH 7.4
temperature
20.2°C
25.4°C
30.4°C

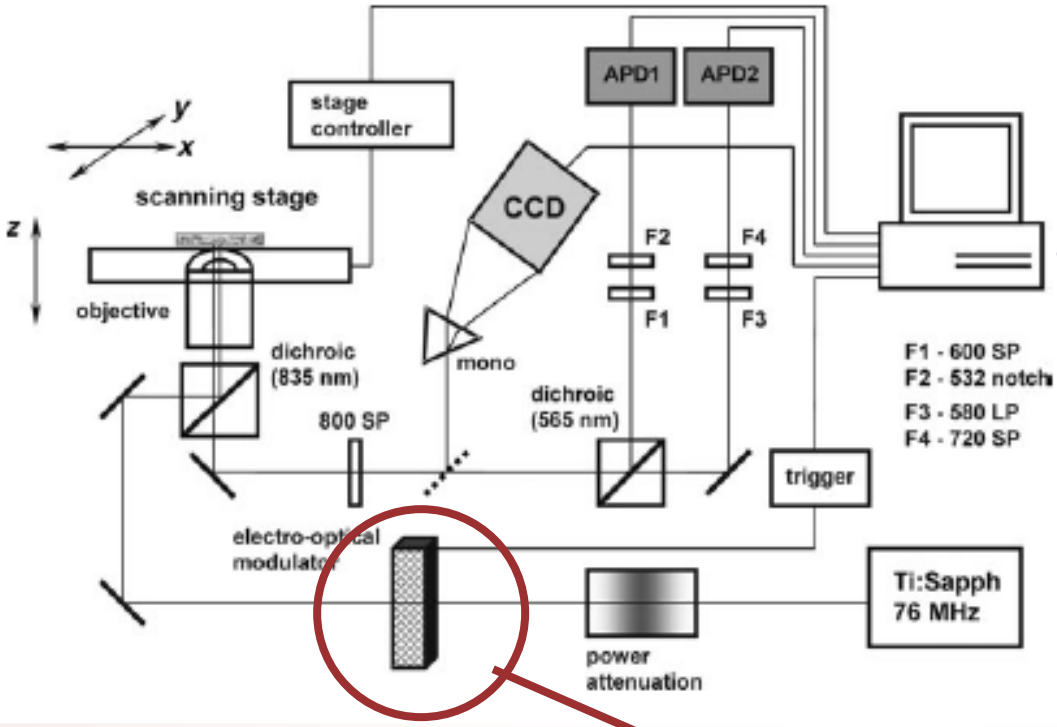
# Phosphorescence vs. Fluorescence



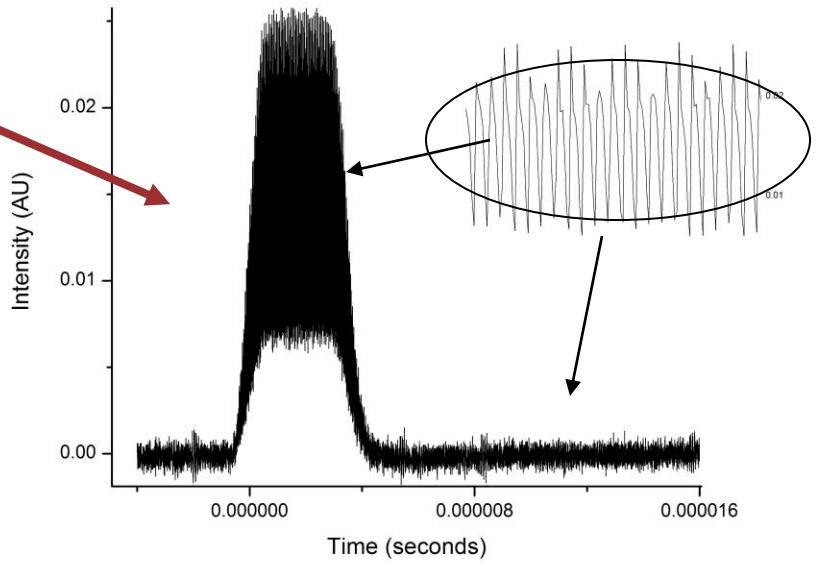



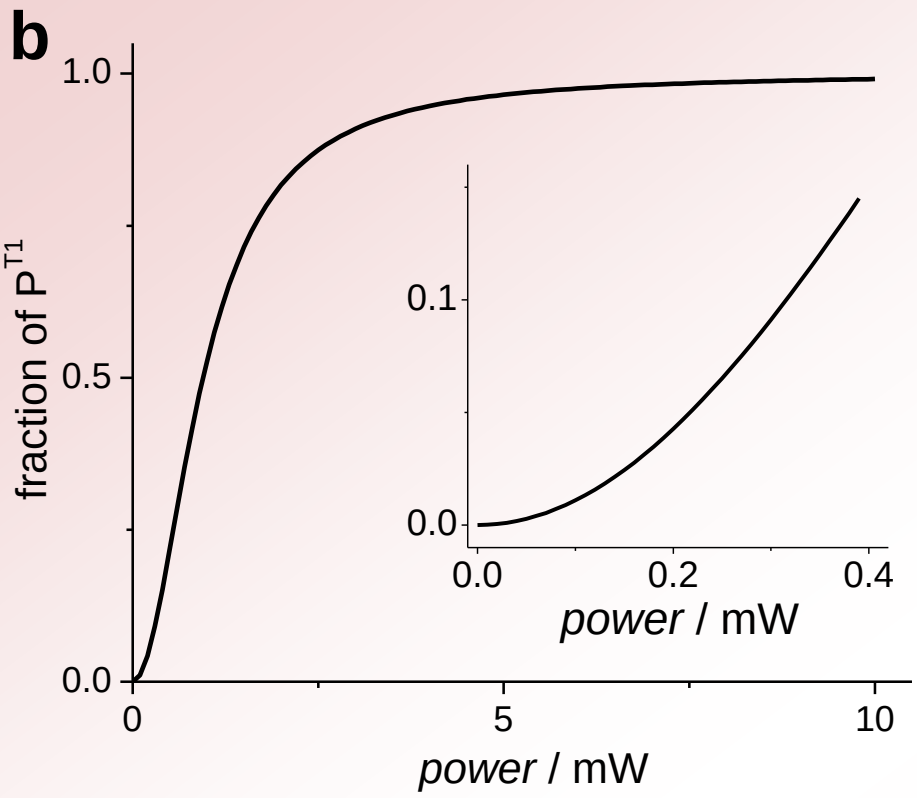

O. S. Finikova, A. Y. Lebedev, A. Aprelev, T. Troxler, F. Gao, C. Garnacho, S. Muro, R. M. Hochstrasser, and S. A. Vinogradov, "Oxygen microscopy by two-photon-excited phosphorescence," ChemPhysChem **9**(12), 1673-1679 (2008).



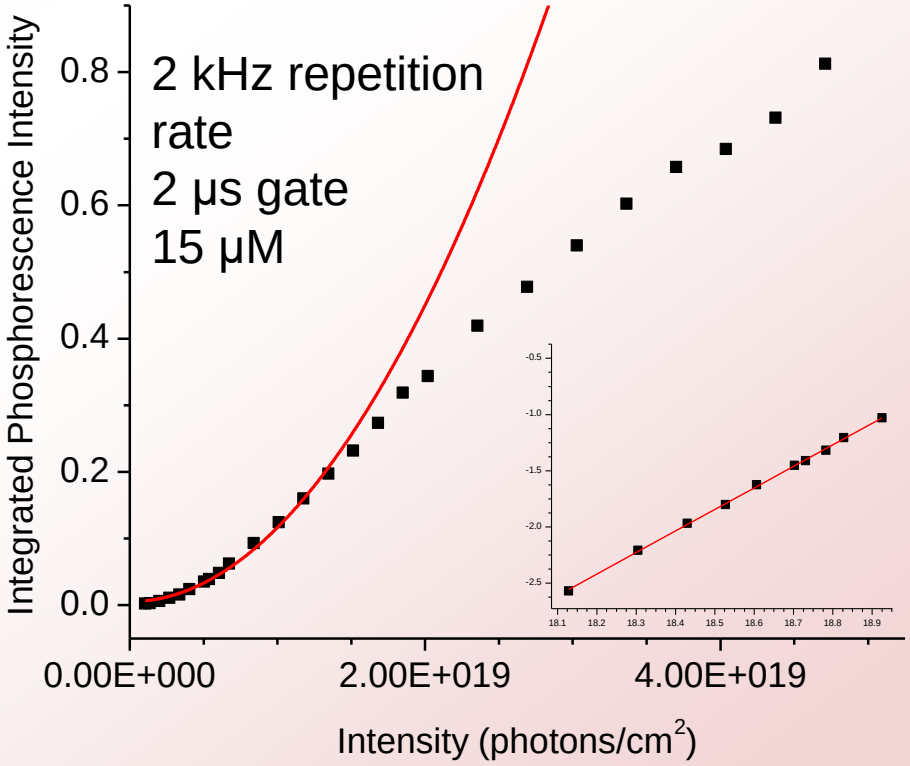

- The laser beam was not expanded before the objective, and was found to be 5.5 mm using the D86 (circular Gaussian beam profile integrated down to 1/e2 of its peak value ) metric before the microscope at 840 nm
- This means the objective is underfilled (Φ~13 mm)

- (Nikon Diaphot 300, objective (Nikon-Fluor 40/1.3 160/.170 Oil 143511, dichroic 780 SP (770DCXR, Chroma))
- . Cargille FF oil (η= 1.479, Cargille Labs, Cedar Grove, NJ USA) was used due to its extremely low auto-fluorescence.



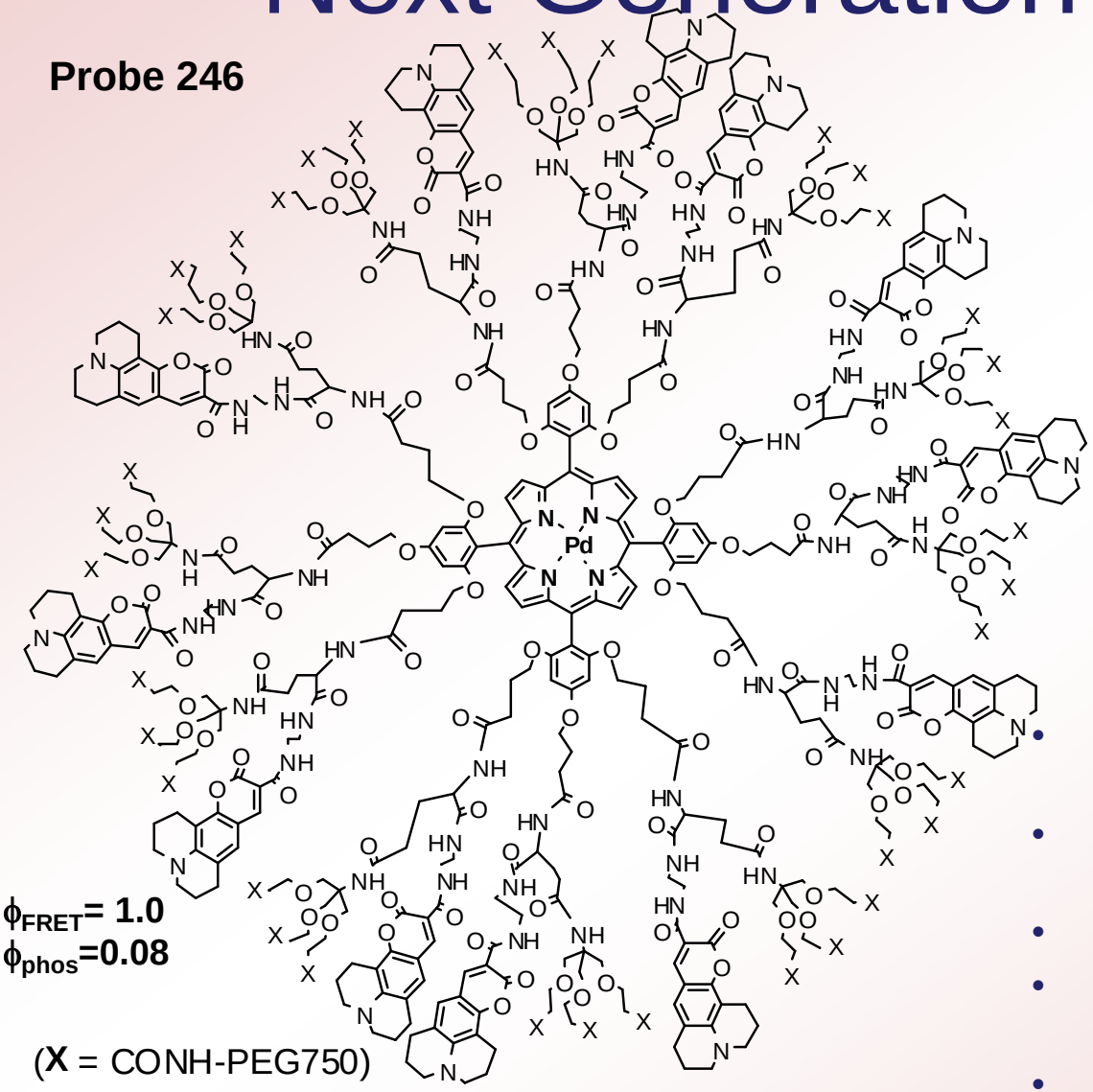



~ 60 nJ/packet (120 μW) is max power
In quadratic regime
At 10% saturation, we should collect ~20 photons ($O_2$-free, simple geometrical considerations)
Actual collection is a few photons per gate in quadratic regime (in air)



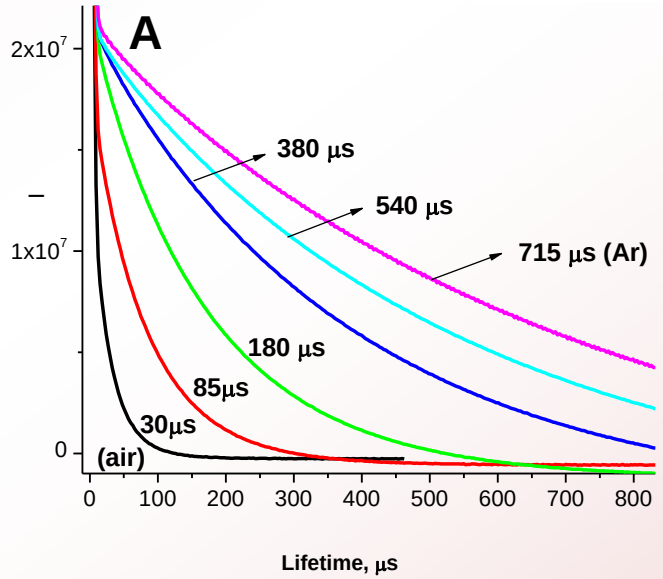
# Next Generation Probe

**Probe 246**

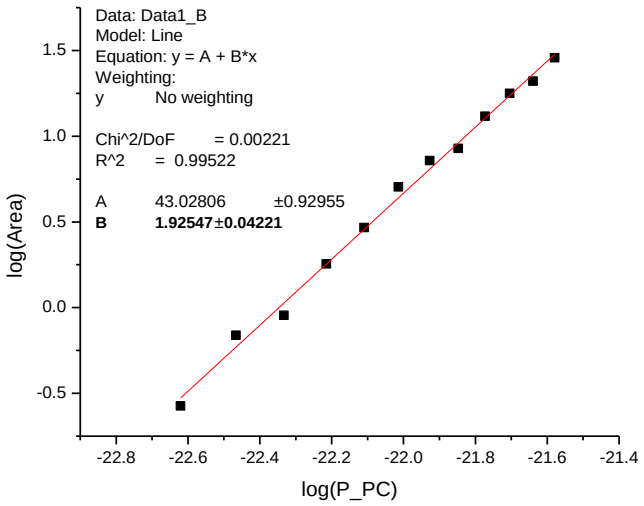



$\phi_{FRET}$= 1.0
$\phi_{phos}$=0.08

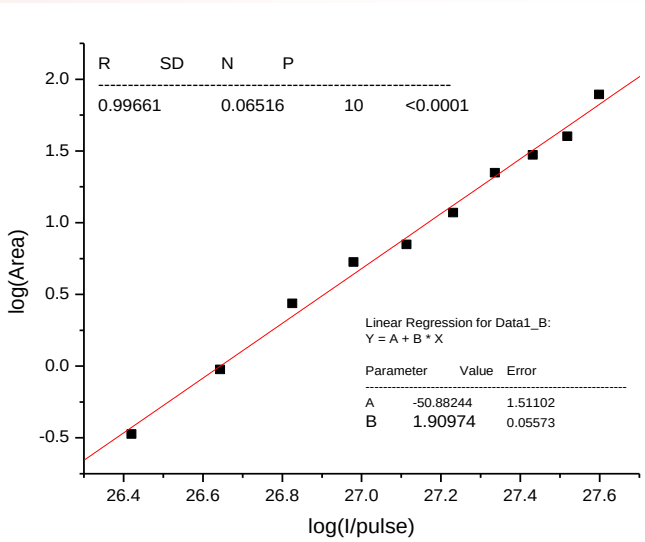
$K_q$=175 mmHg$^{-1}$s$^{-1}$

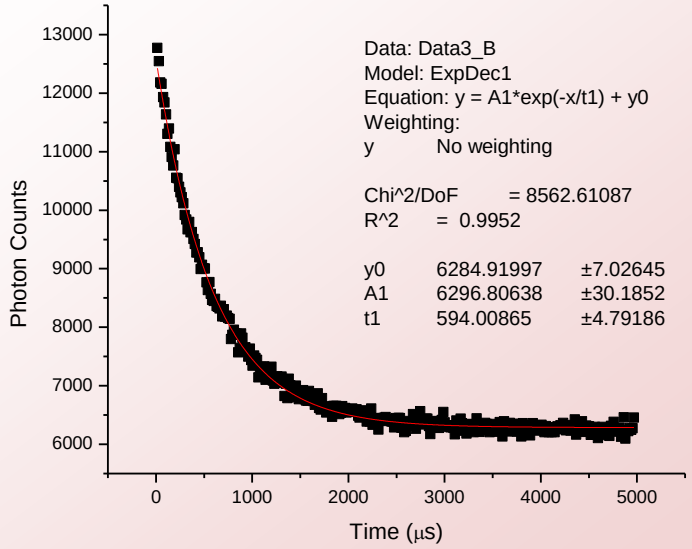



- Larger dynamic range for $O_2$ measurement
- Photo-physically identical dendrons (convergent synthesis)
- Improved Energy Transfer
- Improve TPA cross-section (more C343 antenna molecules)
- Linear Stern-Volmer Plot




Conditions:
24 μM, de-oxygenated
(top) 1.0 us gate, 200 Hz
(bottom) 1.5 us gate, 200 Hz

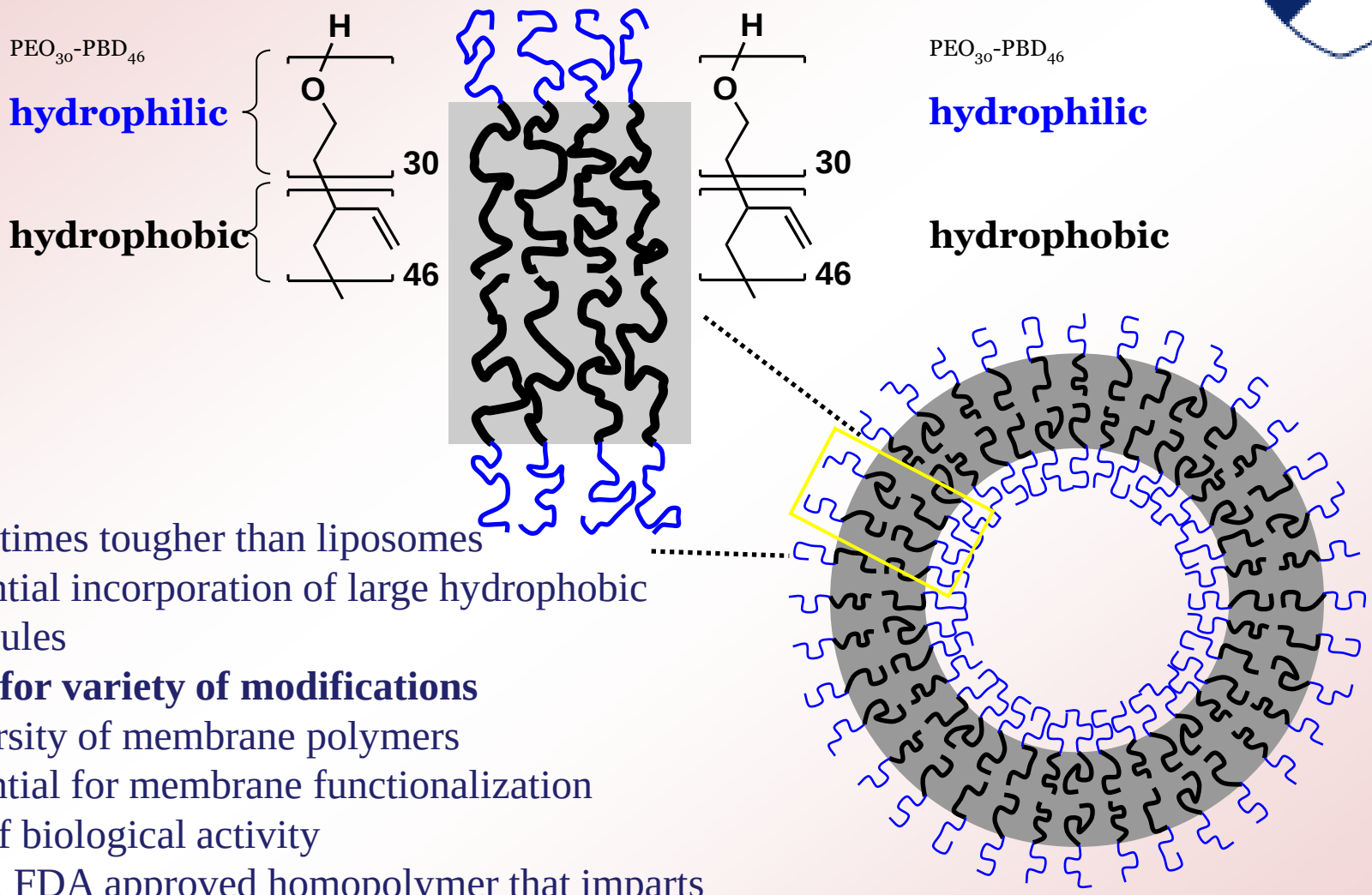


# Delivering Probe to the Cell

- Previous method delivered extremely small volumes of probe in to cell
- New methods?
  - Attachment of functional groups to the outside of the dendrimer (impedes probe function?)
  - Different types of cells (macrophages, fibroblast, HeLa)
  - Delivery methods (free dye, microinjection, **polymersome**)

# Polymersomes



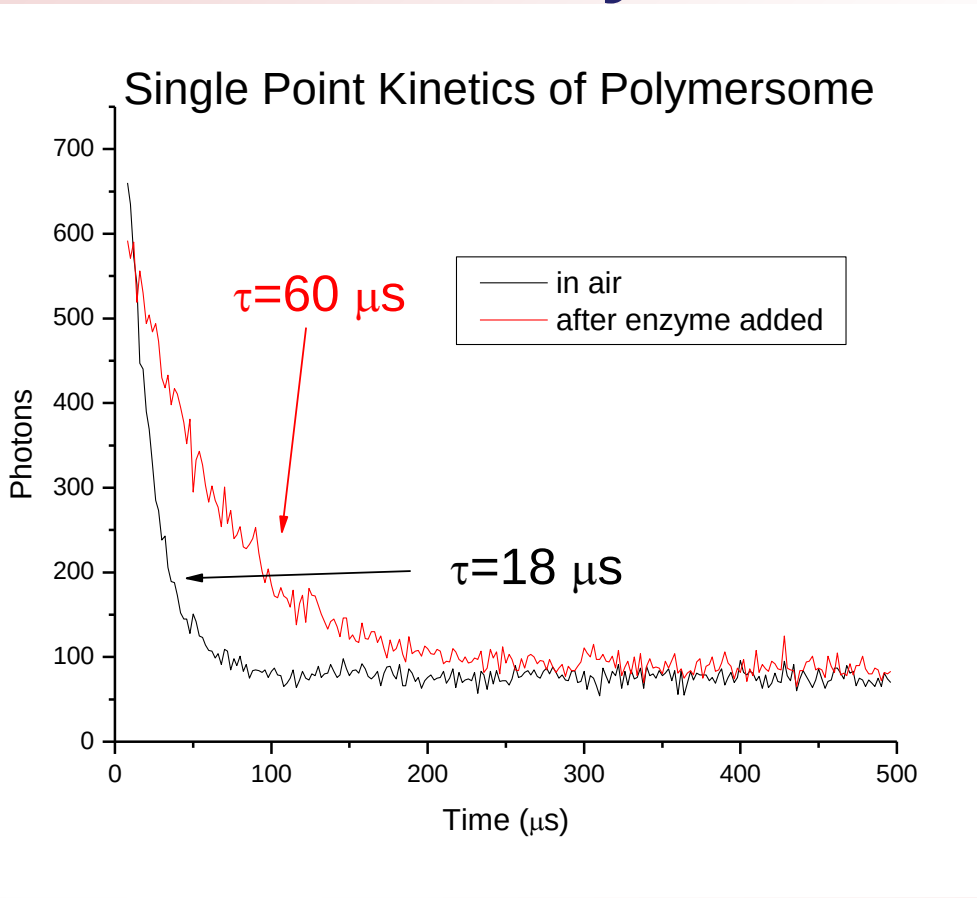

- 5-50 times tougher than liposomes
- Potential incorporation of large hydrophobic molecules

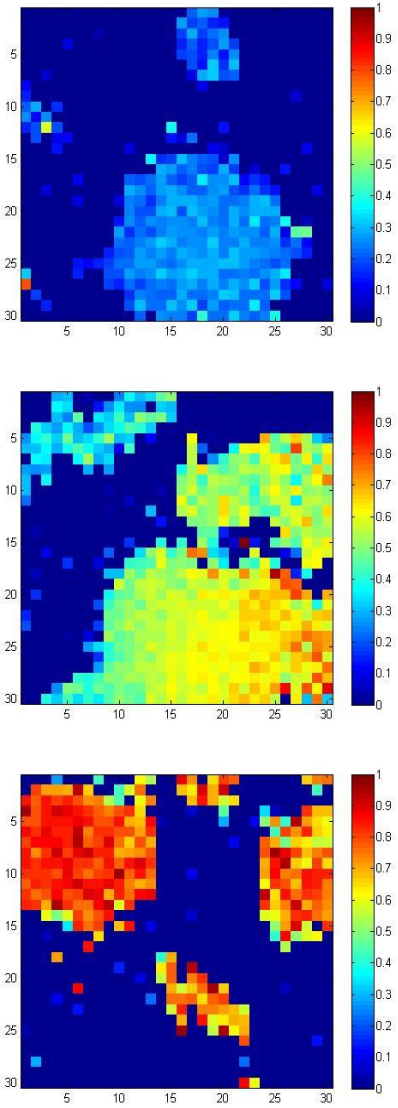
- **Potential for variety of modifications**
  - Diversity of membrane polymers
  - Potential for membrane functionalization
- Capable of biological activity
  - PEO: FDA approved homopolymer that imparts to vesicles surface biocompatability and prolonged blood circulation times

Slide Adapted from G. Robbins (Hammer Group)

Discher, B. M., Won, Y. Y., Ege, D. S., Lee, J. C. M., **Bates, F. S., Discher, D. E. & Hammer, D. A.** (1999) S*cience* 284, 1143-1146.

# Oxygen Diffuses Through Polymersome



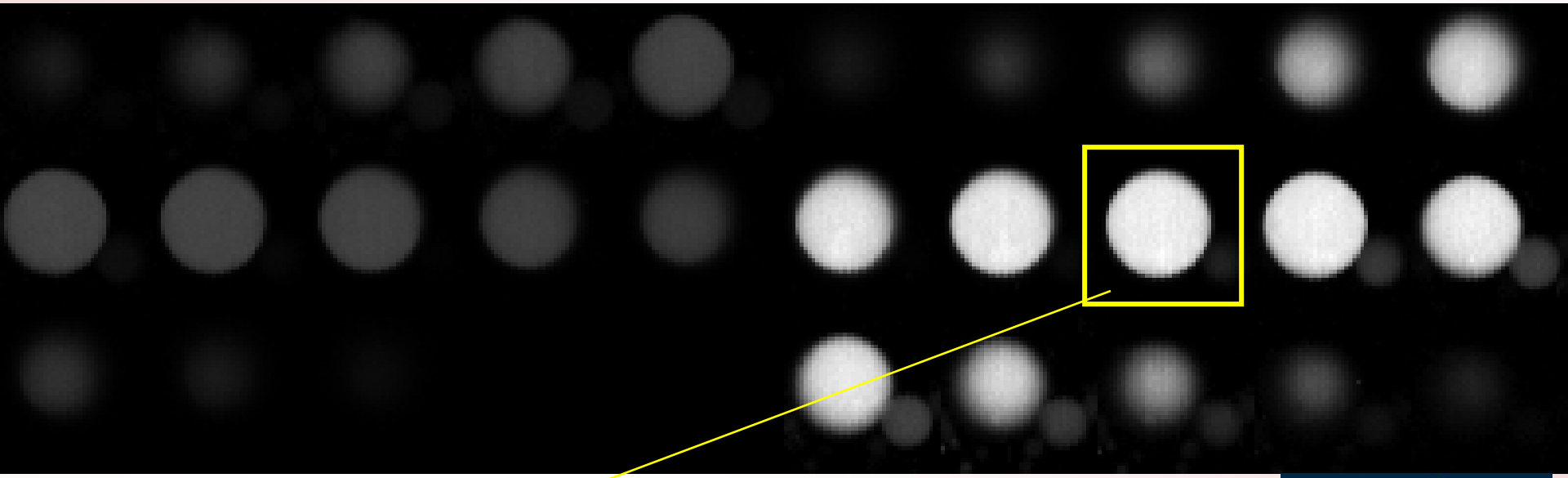



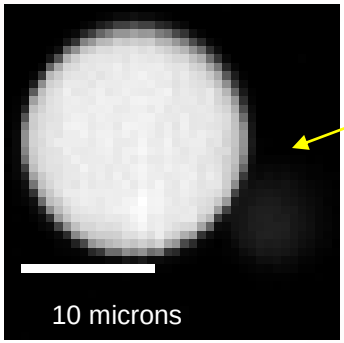
Aqueous samples can be deoxygenated by a glucose oxidase/ catalase/glucose mixture

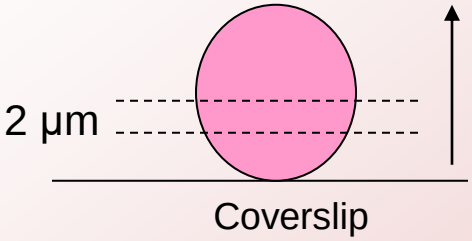
J. M. Vanderkooi, G. Maniara, T. J. Green, and D. F. Wilson, "An optical method for measurement of dioxygen concentration based on quenching of phosphorescence," Journal of Biological Chemistry **262**, 5476-5482 (1987).

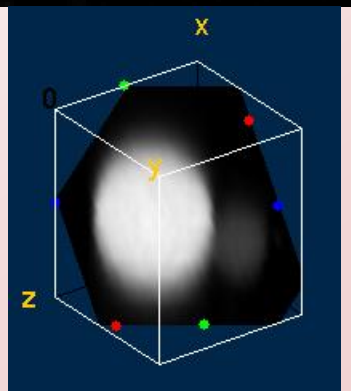
# Imaging a Polymersome Containing PtP-C343

Area from fitting to single exponential

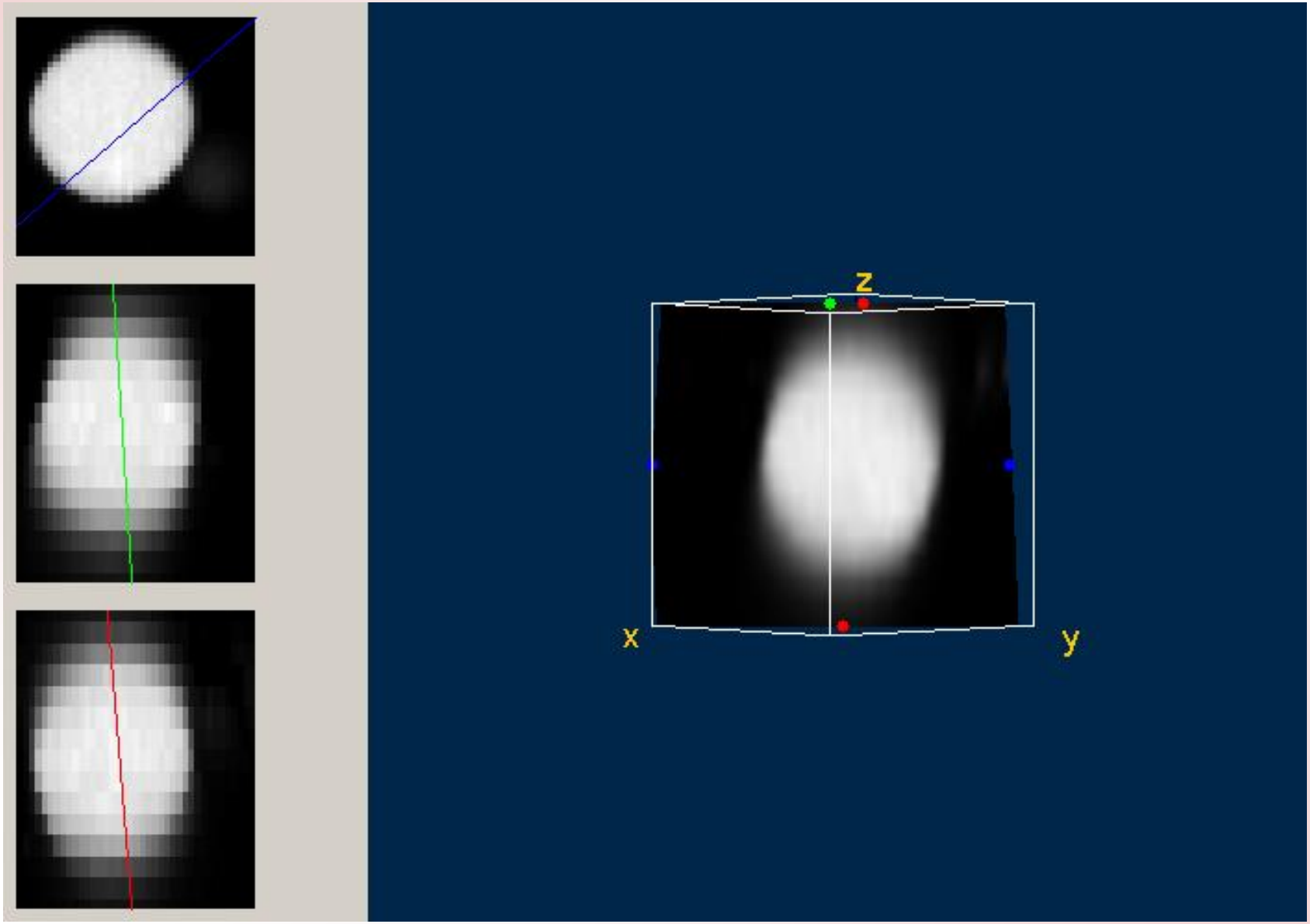





Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2008.


z
x
y

# Experimental Conditions

- A Chameleon Ultra II Ti:Sapphire oscillator provided 100 fs pulses at 80 MHz, with center wavelength of 840 nm.
- These pulses were modulated by a ConOptics Pockels cell and driver system (Pockels cell model  ; driver 302RM).  The contrast ratio between on and off was generally 100:1, as measured by a photodiode at the Pockels cell.  A Tektronix AFG3021B function generator provided both a variable voltage modulation signal to the Pockels cell driver and the trigger pulse for the photon counting unit.
- The Ti:Sapphire pulse train was modulated with a 2 us gate of ‘on pulses’ at 2000 Hz.
- Photon counts were detected by an EG&G APD (model SPCM-200) and delivered to a Hamamatsu C9744 counting unit.
- (1) Semrock RazorEdge 785SP (Semrock, Rochester, NY, USA), 2 x 800 SP (Edmund Optics), 2 x Shortpass Filter/IR 780nm  XIS0780 (Asahi Spectra)
- (2) HQ555 LP + HQ600LP (Chroma Technology)
- Additionally, a variable diameter iris was placed immediately after the exit port of the microscope and closed to <1 mm (diameter). This pinhole dramatically reduced the scattered laser light seen by the detector, but did not decrease the signal, as assessed by examination of kinetic traces at various diameters of the pinhole.

# Conclusions

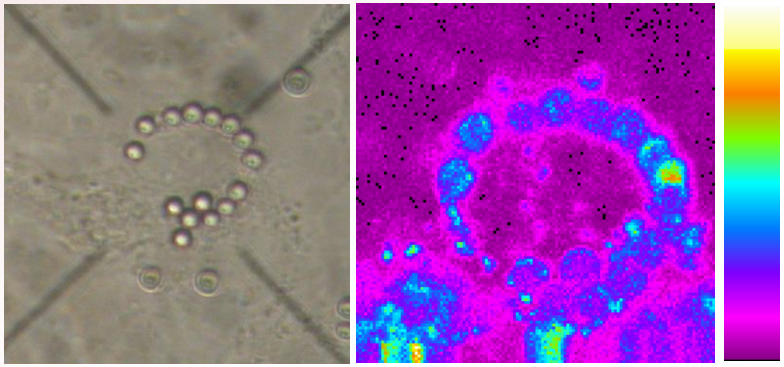
- Probes can be designed with desired functionality
- Pockels cell allows us to access the quadratic regime of the probe (improved contrast, improved resolution)
- Polymersomes can be made to contain our probe molecule, and are permeable to oxygen.  Show promise for delivery to cells and also as standards to test our system.

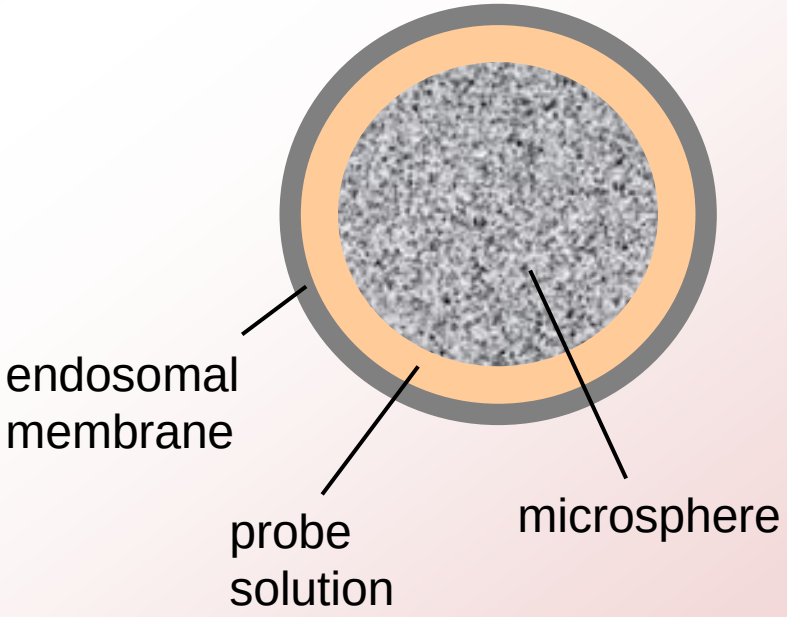
# Acknowledgements


- Vinogradov Group
  - Molly Sheehan
  - Manolis Roussakis
- RLBL (NIH NCRR)
  - Prof. Robin Hochstrasser
  - Chin-Kuei Kuo & Dr. Tom Troxler
- Greg Robbins, Hammer Lab (U. of Pa., Eng. School)
- Prof. Alexey Aprelev (Drexel, Physics)
- Funding: US NIH

# 2P oxygen imaging in cells

- 2P-probe is dissolved in the cell growth medium.
- Latex microspheres (1 or 2 μm), surface modified with ICAM, are internalized *via* endocytosis together with the probe.
- **Co-internalized probe serves as an intracellular pO2 indicator**



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# Why Two Photon?