

Coherent anti-Stokes Raman Scattering (CARS) Microscopy for Biological Imaging and Spectroscopy

Selected Topics in Biophotonics (EAD289)

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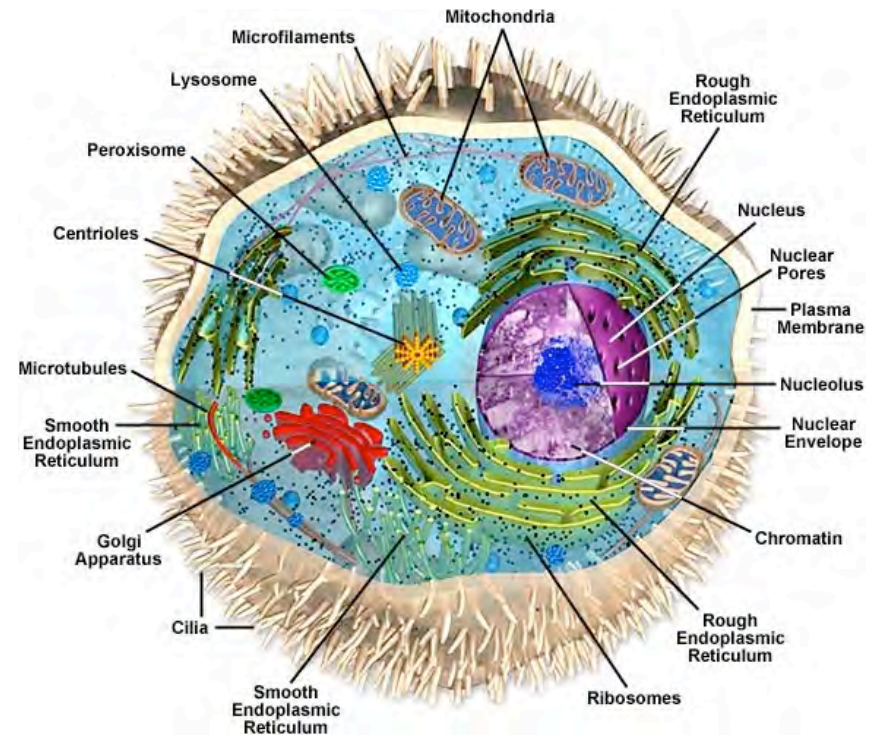
Outline



- Motivation
- Background theory on Raman spectroscopy
- Spontaneous Raman imaging
- Background theory on Coherent Anti-Stokes Raman Scattering (CARS)
- CARS Instrumentation
- Brief Introductions to F-CARS, E-CARS, M-CARS
- Application of CARS to cell imaging
- Future directions
- Summary

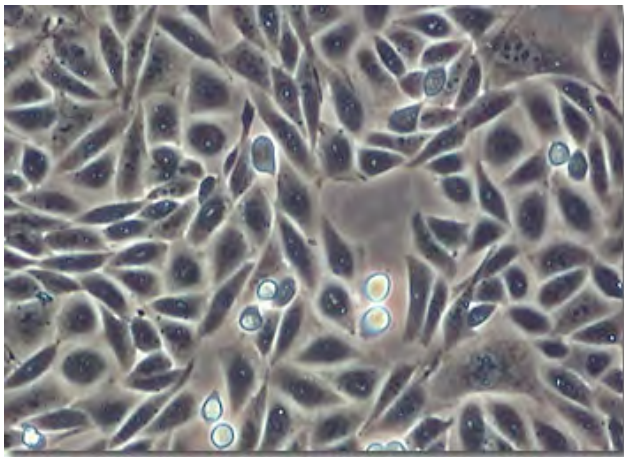
Live cell imaging requires the development of new optical microscopy methods

- Specificity
- Sensitivity
- Dynamic live cell imaging
- Long term live cell imaging

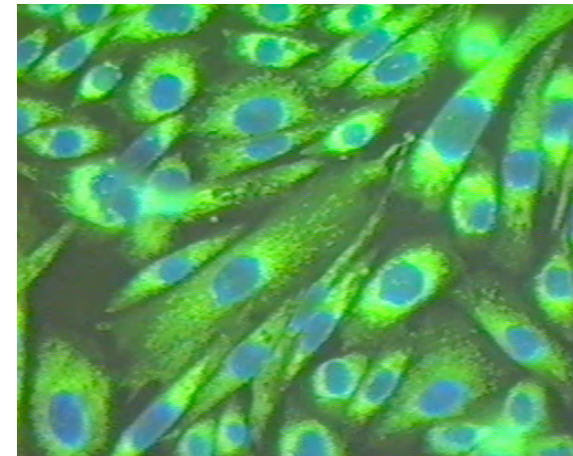


Current state of conventional optical microscopy

Phase contrast



Fluorescence



- (+) Low cost
- (+) Easy to use

- (-) No chemical information
- (-) Low 3D-resolution

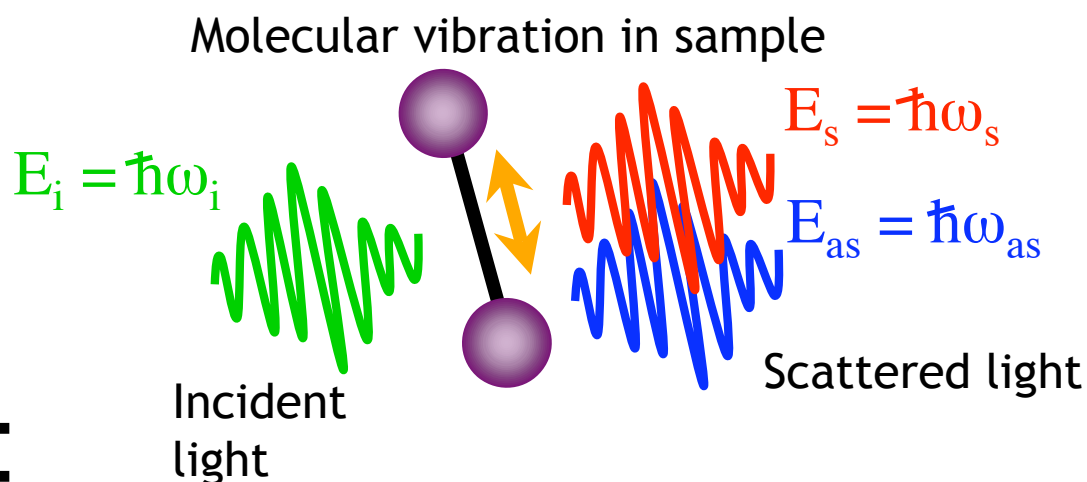
- (+) Specific labeling
- (+) 3D information with confocal and multiphoton microscopy

- (-) Photobleaching - no long term studies
- (-) Toxicity, cell fixation - perturbs cell function

Raman scattering is the interaction of photons and intrinsic molecular bonds



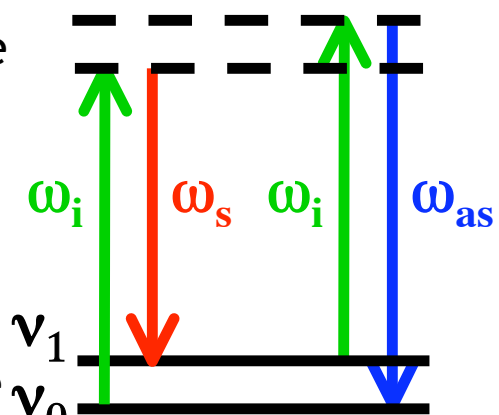
C.V. Raman
1930 Nobel Prize



Excited state

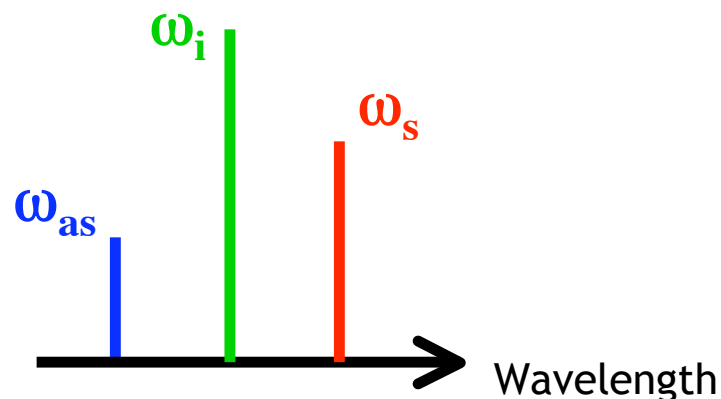


Virtual state



Ground state

Stokes anti-Stokes



Boltzmann distribution



Polarizability induced dipole equation

Classical picture of Raman and Rayleigh scattering with a diatomic molecule

$E = E_o \cos(\omega_i t)$ Electric field of incident light oscillating at frequency

$\mu_{ind} = \alpha E = \alpha E_o \cos(\omega_i t)$ Induced dipole from this E-field

$\alpha = \alpha_o + (r - r_{eq}) (d\alpha / dr)$ Molecular polarizability changes with bond length

$r - r_{eq} = r_{max} \cos(\omega_{vib} t)$ The bond length oscillates at vibrational frequency

$\alpha = \alpha_o + (d\alpha / dr)r_{max} \cos(\omega_{vib} t)$ Polarizability oscillates at vibrational frequency

$$\mu_{ind} = \alpha_o E_o \cos(\omega_i t) + (1/2) E_o r_{max} (d\alpha / dr) [\cos((\omega_i + \omega_{vib}) t) + \cos((\omega_i - \omega_{vib}) t)]$$

Rayleigh

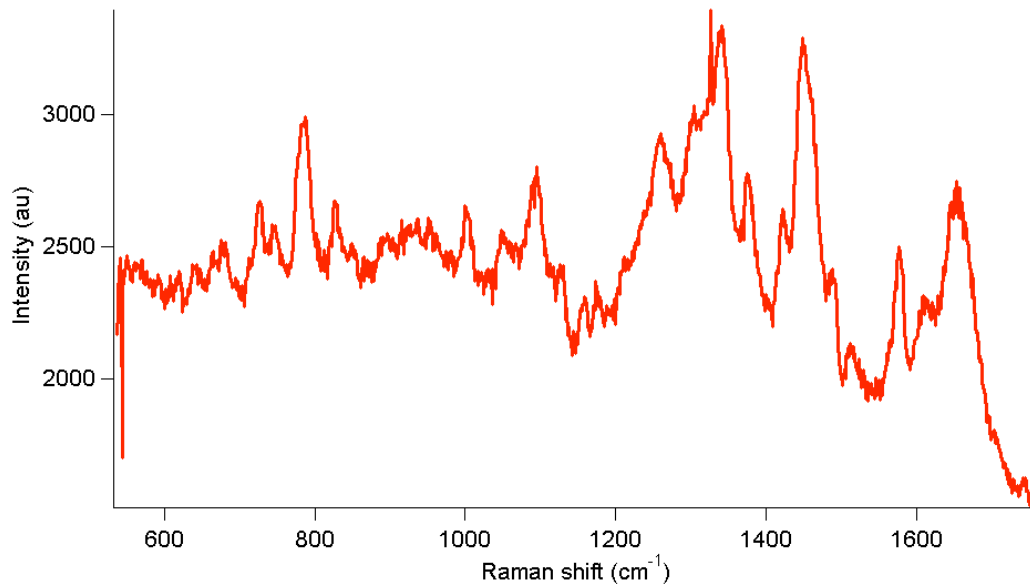
Anti-Stokes

Stokes

Raman spectra of cells provide a wealth of biological information



Single live human T cell



$k = 1/\lambda$ (cm^{-1}) Wavenumber units

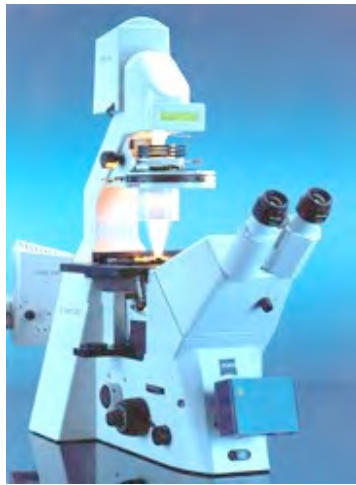
Raman shift = $(1/\lambda_{incident}) - (1/\lambda_{scattered})$

Raman frequency in wavenumber units (cm^{-1})

Assignment*

618	p: C-C twist
640	p: C-S str., C-C twist Tyr
666	G, T, Tyr, G bk in RNA
678	G (C-2'-endo-anti)
725	A
746	T
785	U, T, C, bk: O-P-O
831	O-P-O asym. str., Tyr
852	Tyr. ring breath.
893	bk, p: C-C skeletal
1003	Phe, C-C skeletal
1031	Phe, p: C-N str.
1053	C-O str., p: C-N str.
1093	O-P-O sym. str., p: C-N
1126	p: C-N str.
1156	p: C-C, C-N str.
1175	Tyr, Phe, p: C-H bend
1208	A, T, p: amide III
1257	A, T, p: amide III
1263	T,A, p: C-H bend
1302	P: amide III
1318	G, p: C-H def.
1337	A, G, p: C-H def.
1373	T, A, G
1421	A, G
1447	p: C-H ₂ def.
1485	G, A
1510	A
1575	G, A
1605	Phe, Tyr, p: C=C
1615	Tyr, Trp, p: C=C
1655–1680	p: amide I, T, G, C

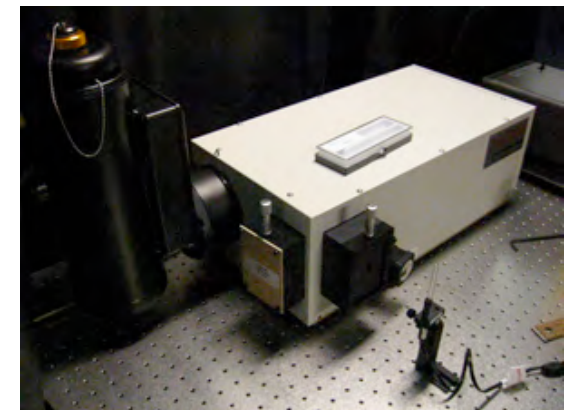
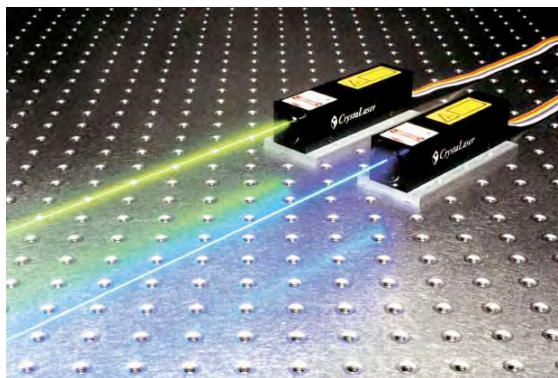
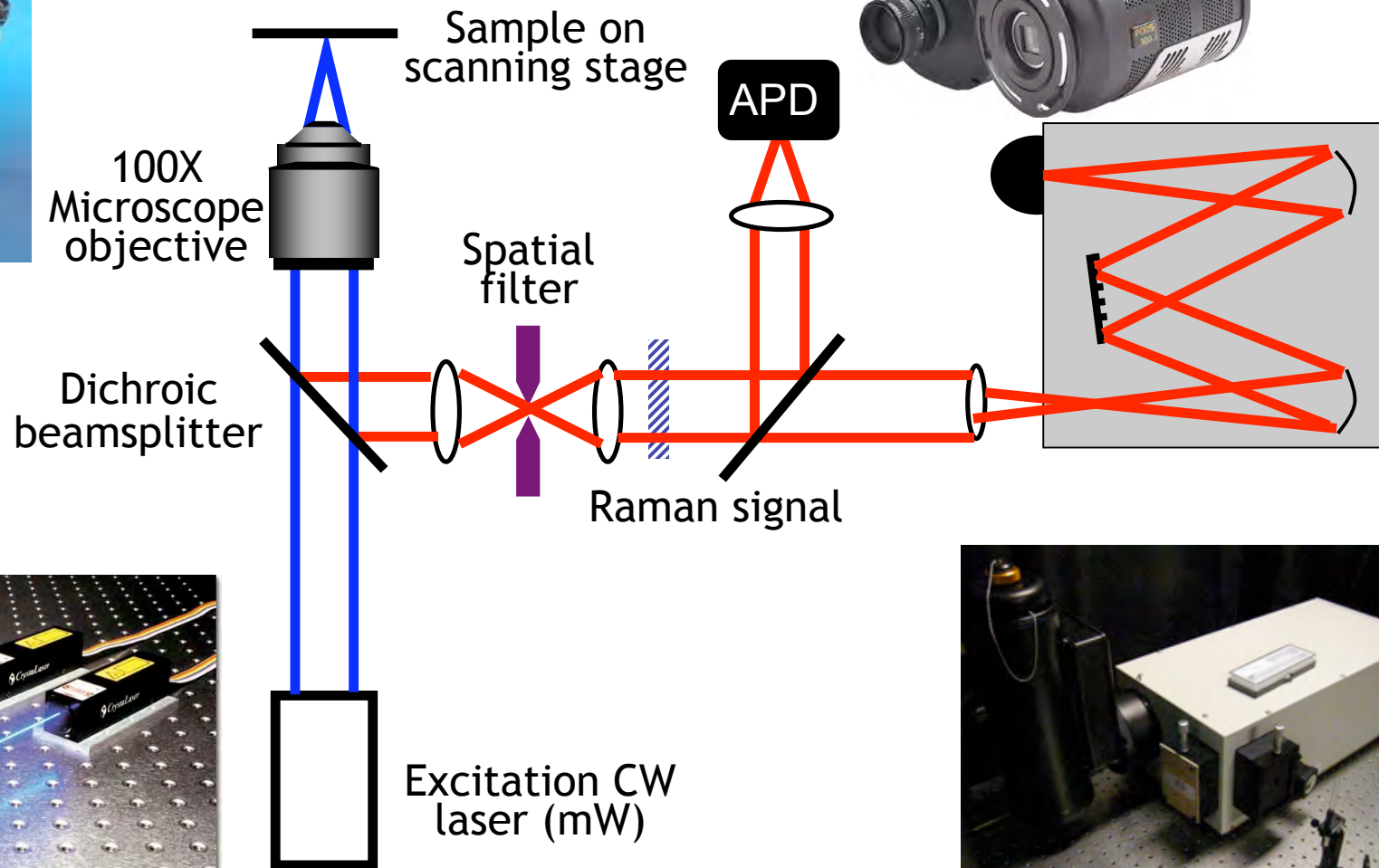
Confocal Raman microscope for microspectroscopy and imaging



CCD chip



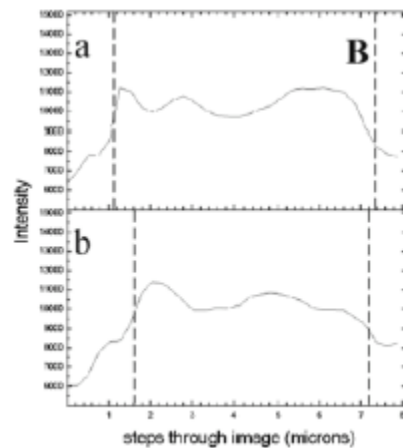
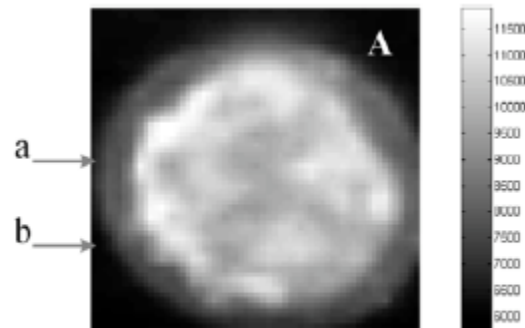
CCD camera



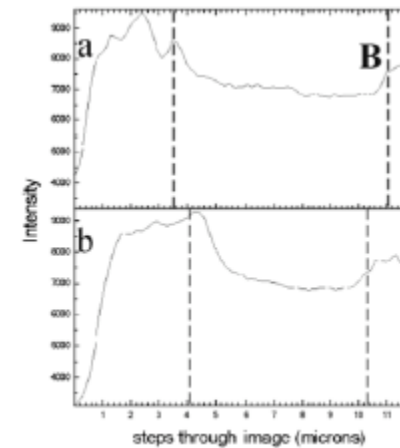
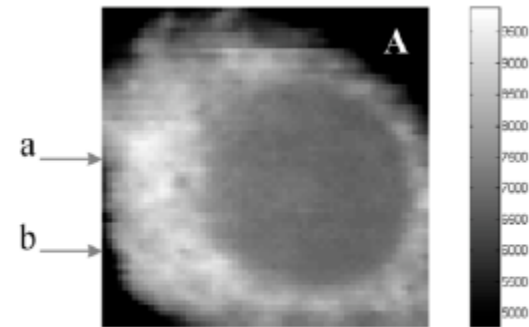
Raman images of formaldehyde fixed human cells



Single, fixed peripheral blood lymphocyte in buffer



Single, fixed lens epithelial cell in buffer

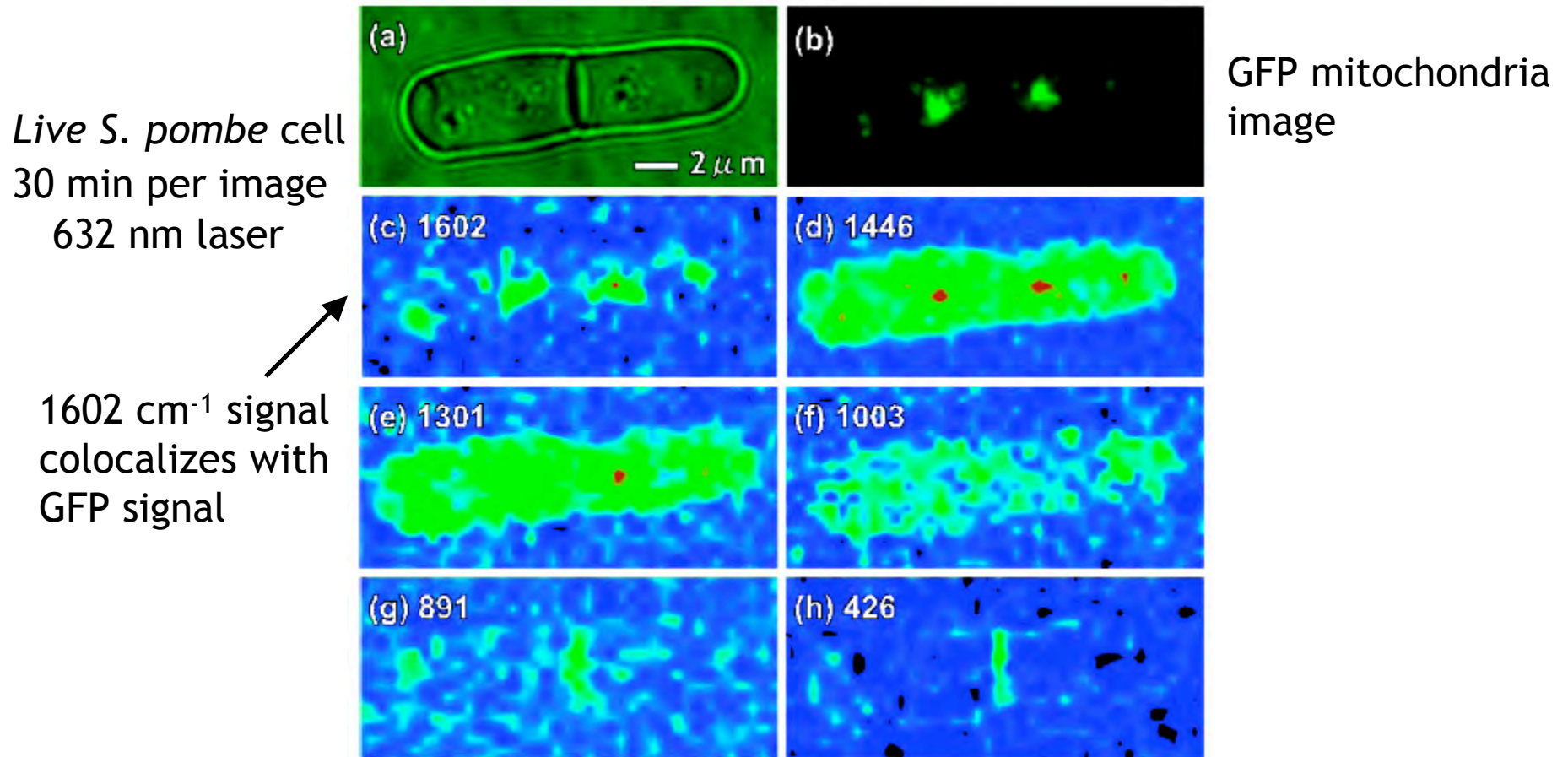


2850 cm^{-1} symmetric CH_2 protein vibration, 120 mW 657 nm laser
~ 400 nm resolution, 1 hour acquisition time

Raman mapping combined with fluorescence microscopy for multi-modal analysis



Raman mapping of chemical components in *S. pombe* cells



Advantages and limitations of spontaneous Raman imaging

Advantages

- Minimally invasive technique
- Non-photobleaching signal for live cell studies
- Works under different conditions (temperatures and pressures)
- Chemical imaging without exogenous tags
- Works with different wavelengths

Limitations

- Fluorescence interference
- Limited spatial resolution
- Weak signal - long integration times

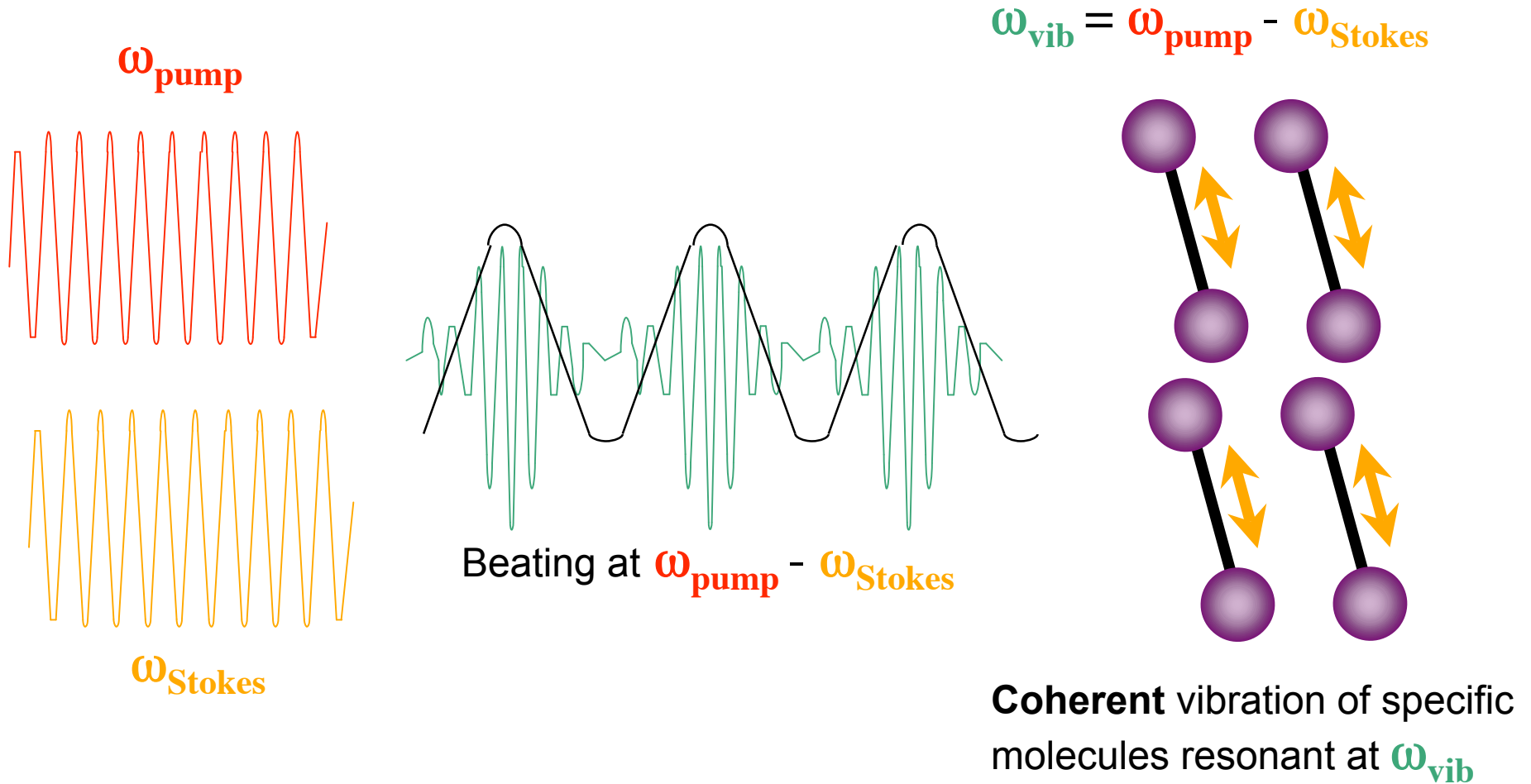
Raman scattering is extremely inefficient (10^{-30} cm² cross sections)
1 in 10^8 incident photons are Raman scattered

Why develop CARS?

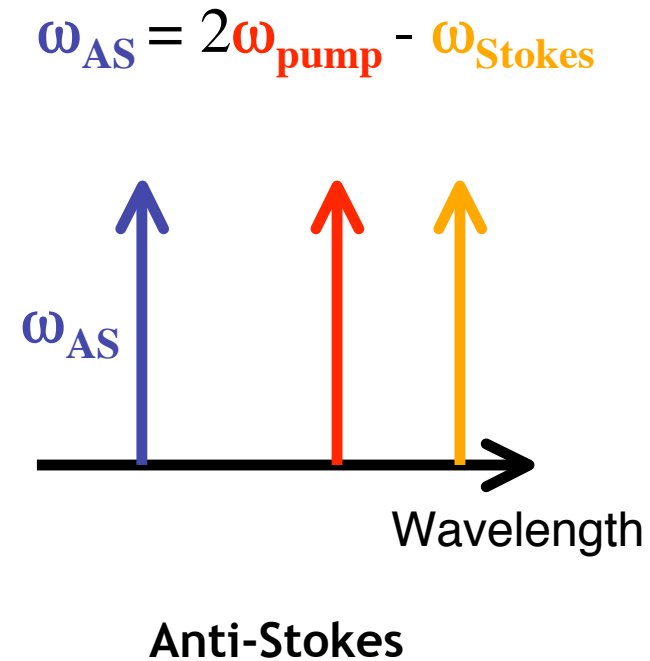
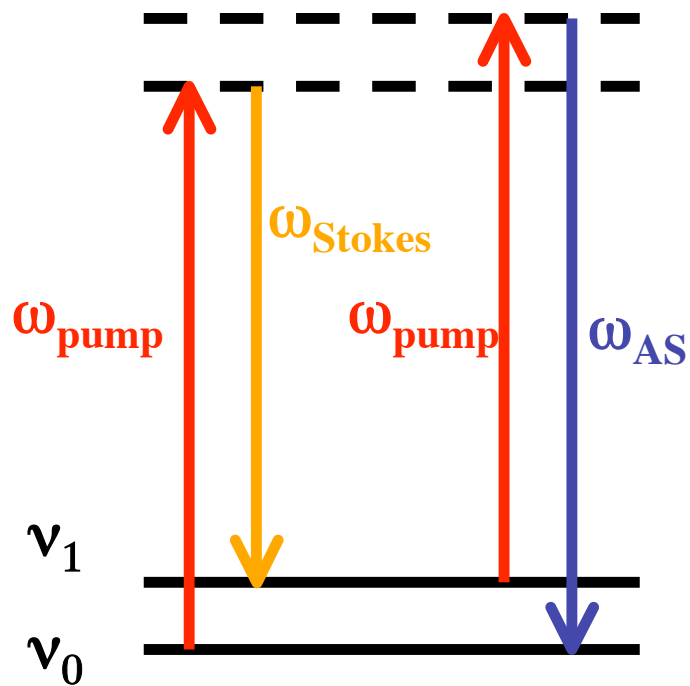


- More sensitive (stronger signals) than spontaneous Raman microscopy - faster, more efficient imaging for real-time analysis
- Contrast signal based on vibrational characteristics, no need for fluorescent tagging.
- CARS signal is at high frequency (lower wavelength) - minimal fluorescence interference
- Higher resolution

CARS uses two laser frequencies to interact resonantly with a specific molecular vibration



CARS signals are generated at wavelengths shorter than the excitation wavelengths (anti-Stokes)



CARS is a third order nonlinear optical process, requiring high intensity laser pulses

Polarization

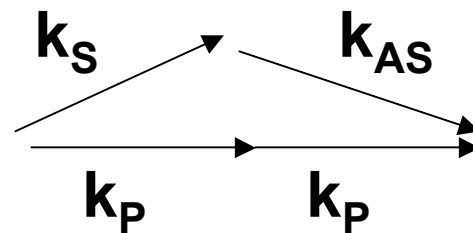
$$P(t) = \chi^{(1)} E(t) + \chi^{(2)} E(t)^2 + \chi^{(3)} E(t)^3 + \dots$$

Higher order terms becomes important when peak powers are high

For CARS,
$$P_{AS} = \chi^{(3)} E_p^2 E_s$$

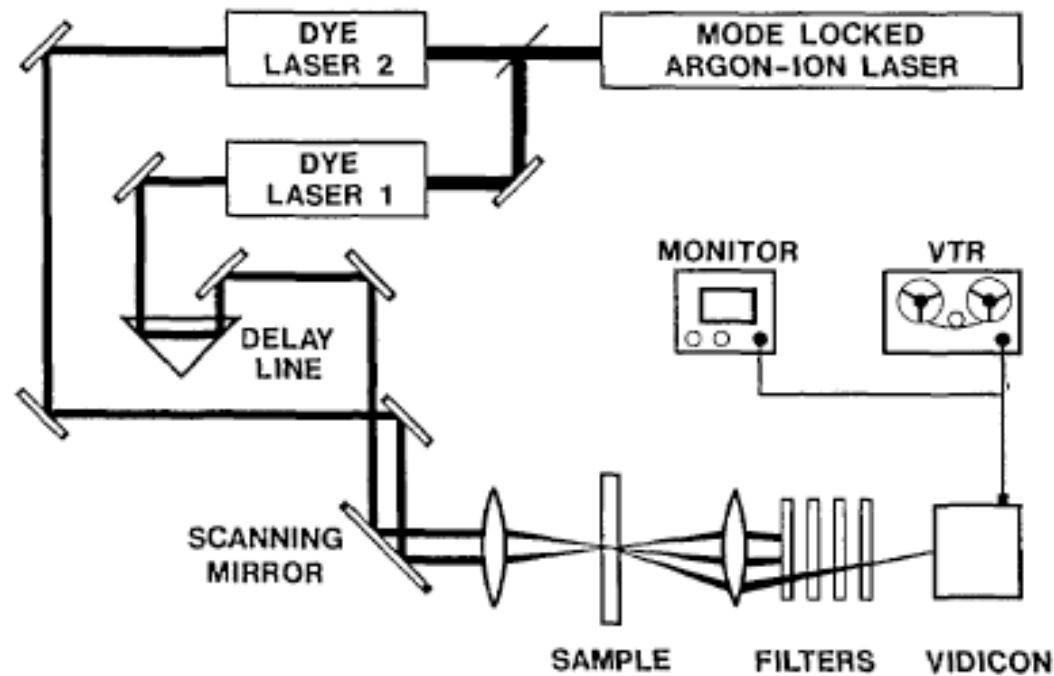
Requires high intensity, pulsed laser sources (ps, fs)

$$I_{AS} = I_p^2 I_s [\sin (\Delta kz/2) / (\Delta kz/2)]^2$$



Phase matching conditions

First CARS microscope demonstrated in 1982



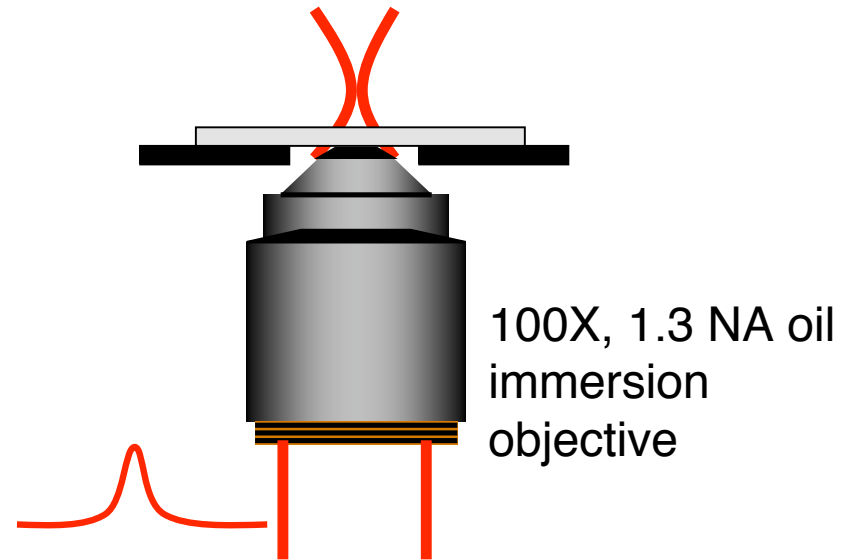
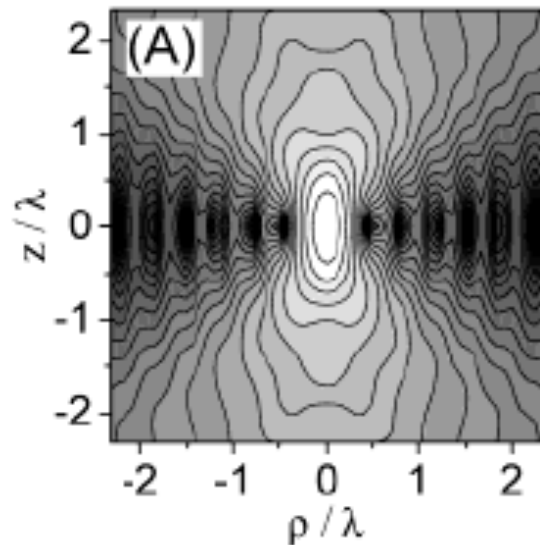
- Drawbacks of this configuration for biological imaging
 - Laser wavelengths at 565-700 nm
 - Phase matching configuration difficult to implement practically

Major improvements developed in 1999 for biological imaging



- Tight focusing conditions relax phase matching conditions
- Advancement in laser technology
- Near IR light reduces potential laser damage to cells, tissue
- Collinear geometry makes it much easier to implement
- 3-D sectioning, through cells, tissue

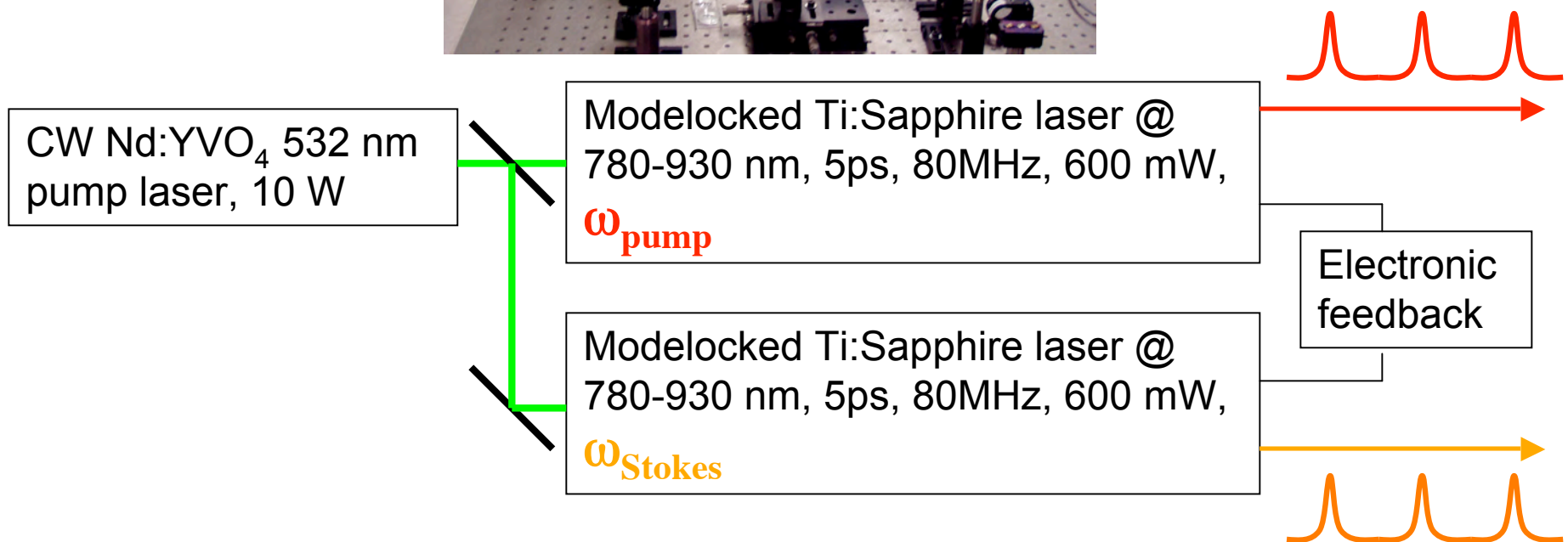
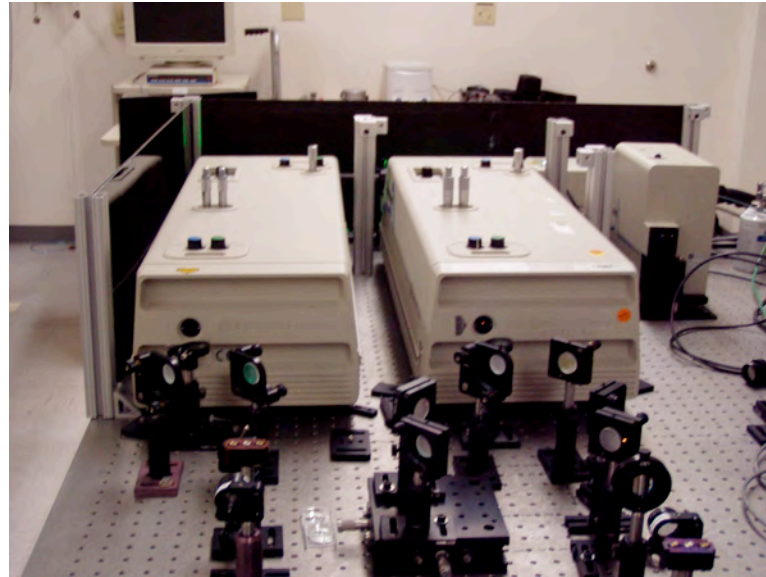
Tight focusing using a high NA objective is key for CARS microscopic imaging



Intensity distribution of an optical field focused by a 1.4 NA objective

- Phase matching condition relaxed
- Tight focus generates highest intensity at laser focus
- CARS signal generated within focal volume
- 3-D sectioning capability

Two synchronized Ti:Sapphire lasers provide two frequencies for CARS excitation



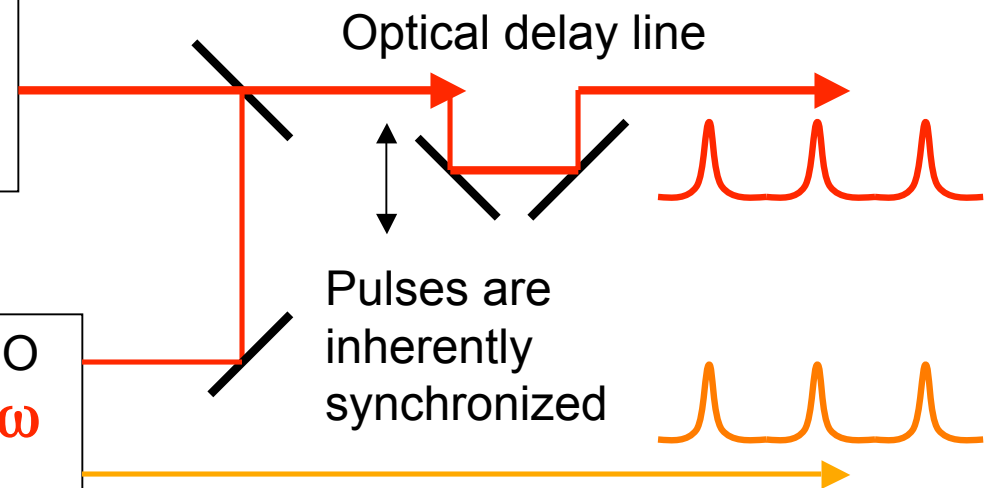
Optical parametric oscillators are another type of system used for CARS microscopy



Modelocked Nd:Vanadate Pump Laser @ 1064 nm, 7ps, 76MHz, 10W, ω_{Stokes}

Intracavity doubled sync-pumped OPO
780 nm – 920 nm, 5ps, 76MHz, 2W, ω

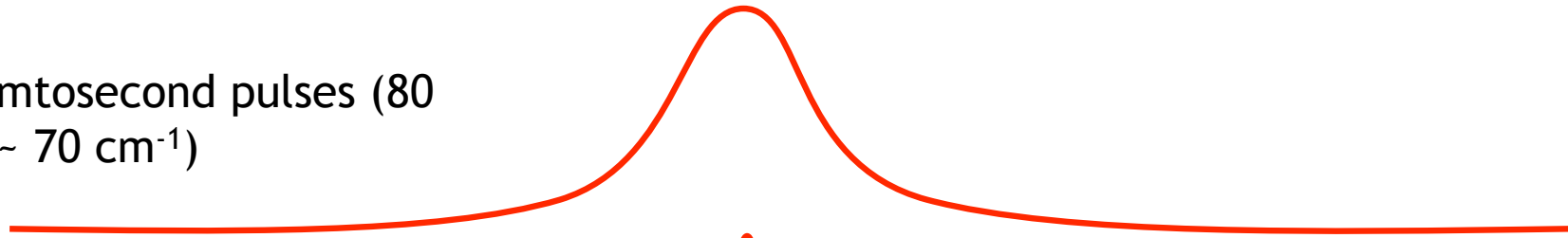
pump



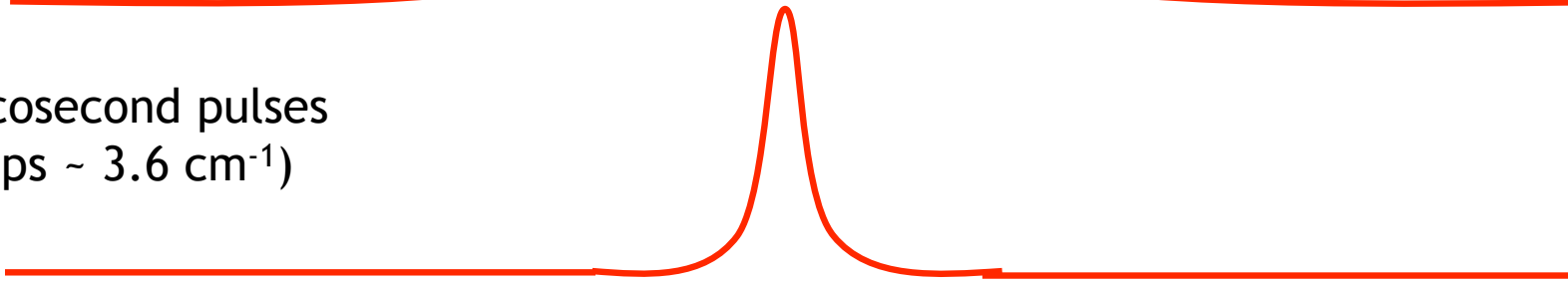
Picosecond or femtosecond pulses, which is better? There are several tradeoffs



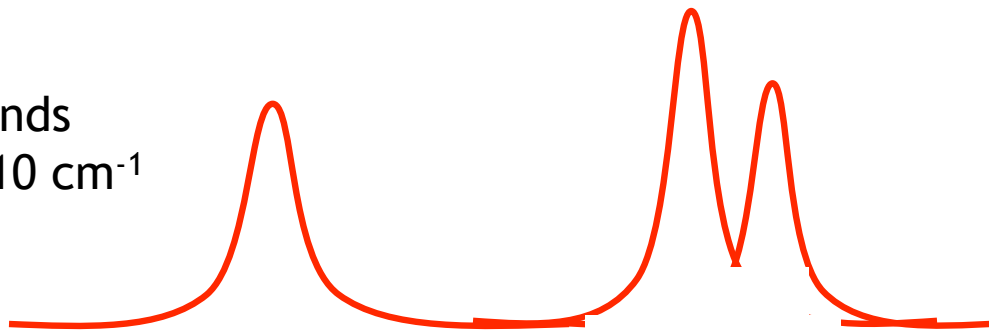
Femtosecond pulses (80 fs ~ 70 cm^{-1})



Picosecond pulses (5 ps ~ 3.6 cm^{-1})



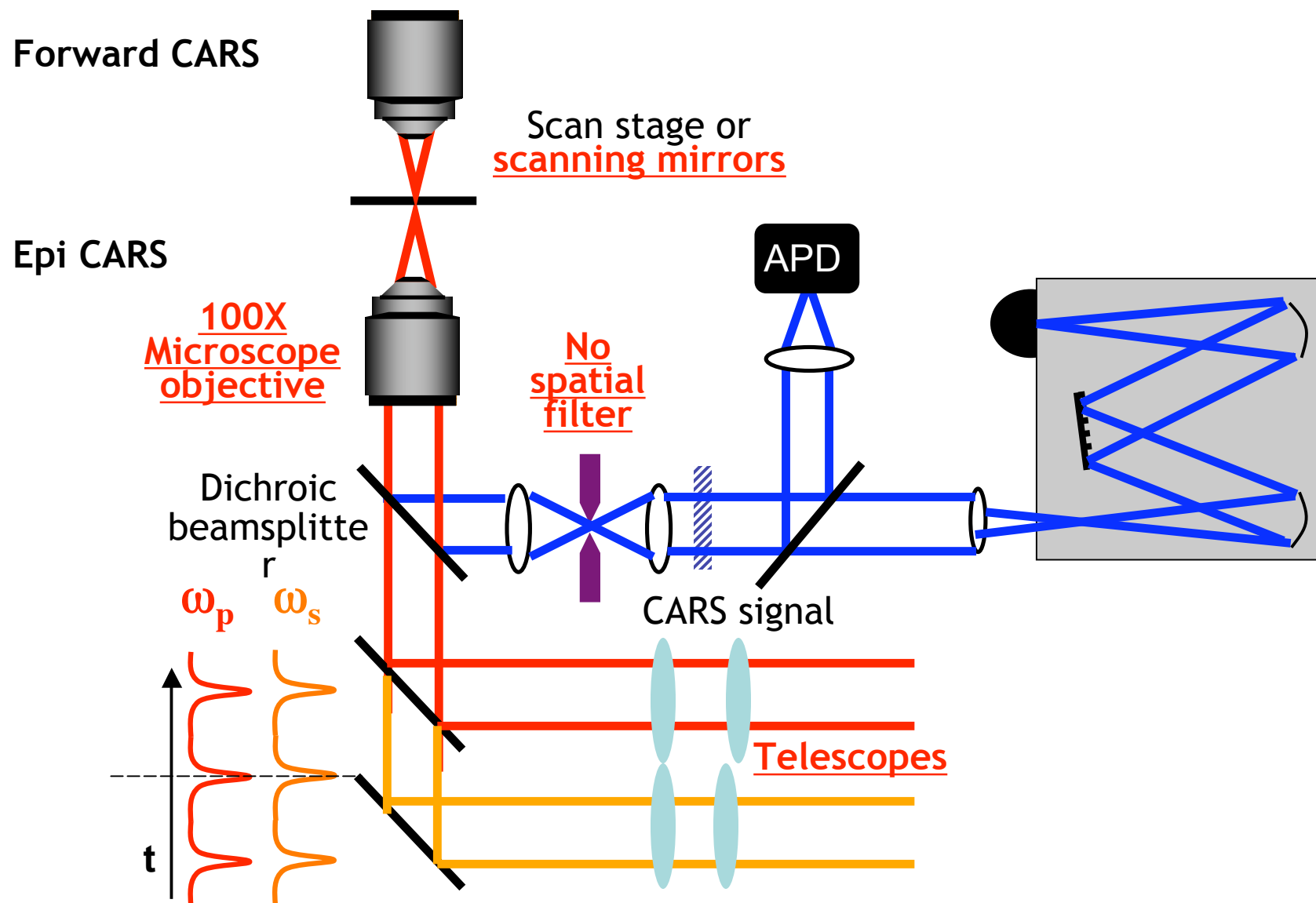
Raman bands typically 10 cm^{-1}



Wavenumber

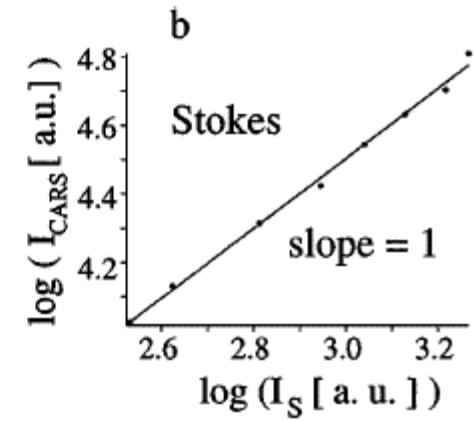
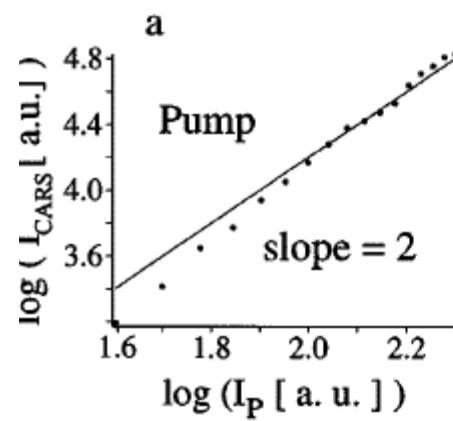
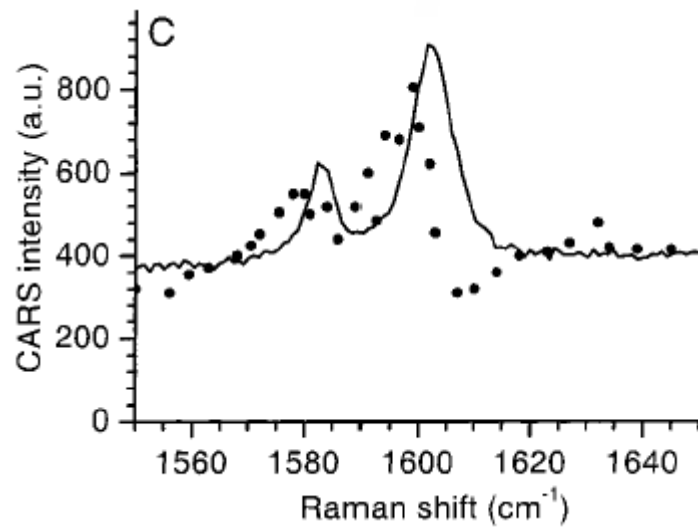
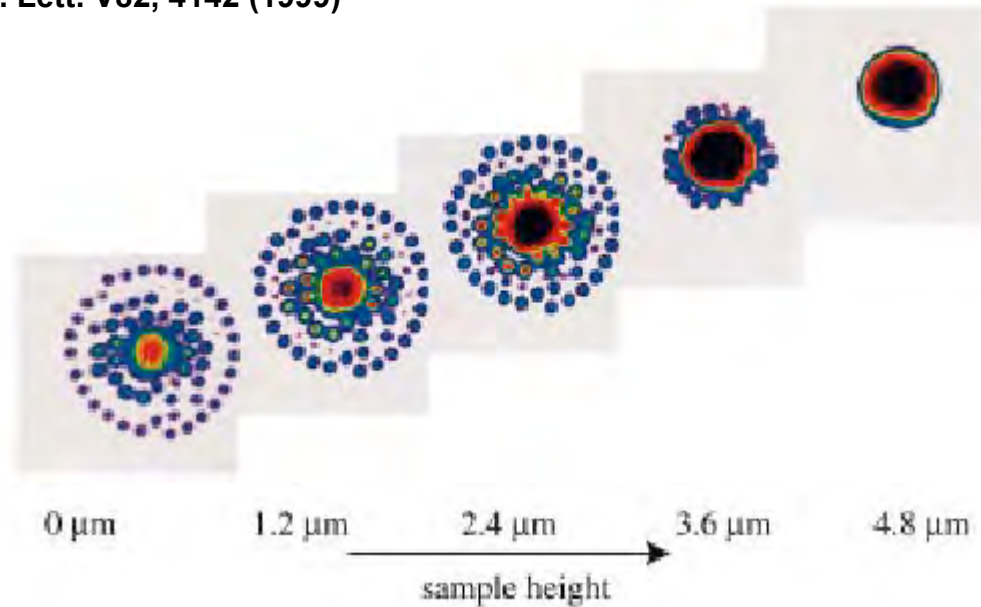
Ps pulses focus all energy to a single Raman band to maximize coherent vibration, at expense of losing peak intensity and multiplex advantage with fs pulses

Key components in a CARS microscope setup

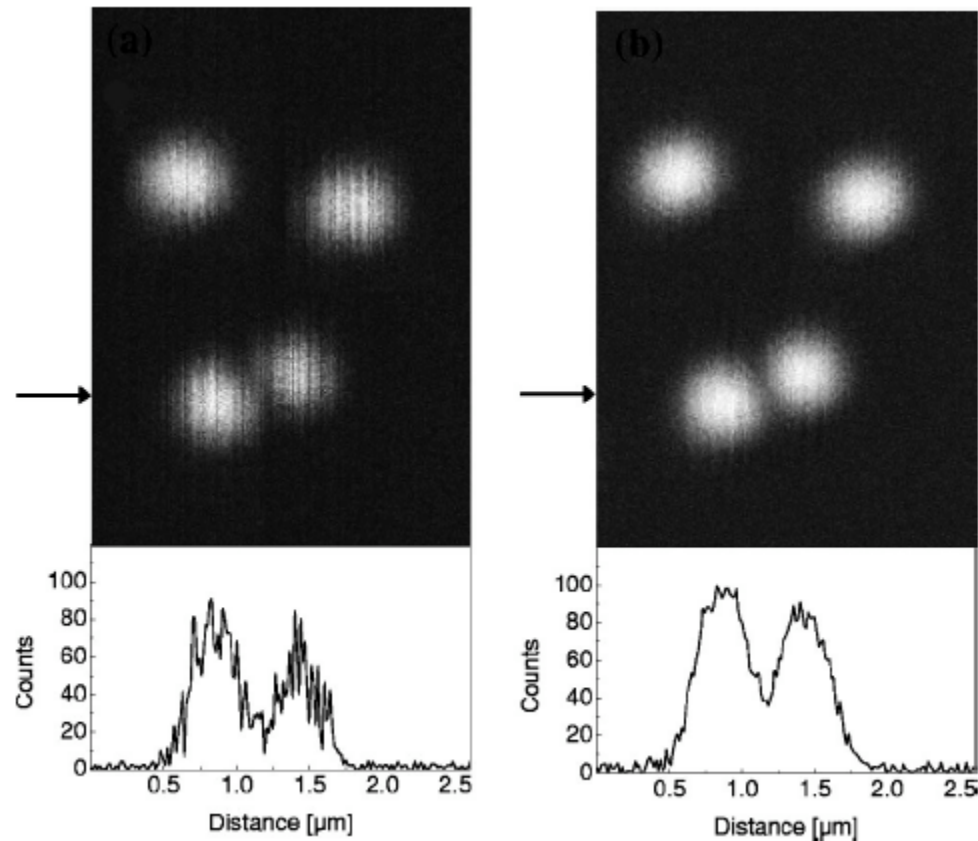


First demonstration on 910 nm polystyrene beads

Zumbusch et. al., Phys. Rev. Lett. V82, 4142 (1999)



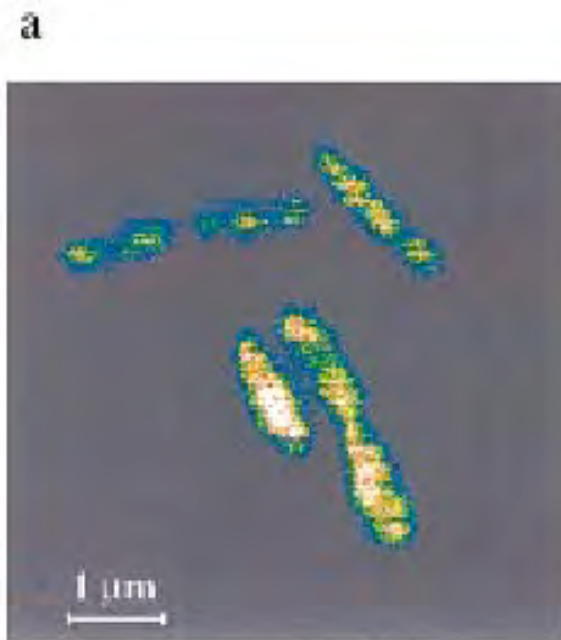
Jitter between two laser trains affects the quality of the CARS image



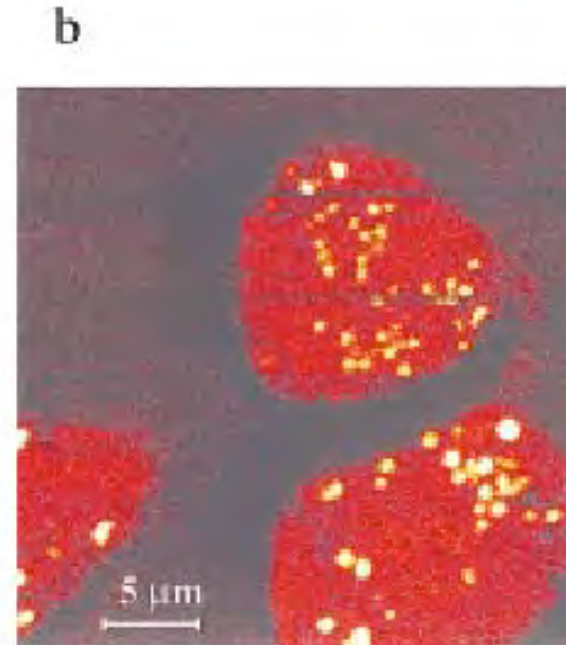
0.5 μm polystyrene beads
0.3 mW, 0.1 mW pump, stokes
22 seconds to acquire image

Examples of live cell imaging

853 nm (100 μ W) and 1135 nm (100 μ W) tuned to
Raman shift of 2913 cm^{-1} C-H vibration



Unstained live bacterial cells. Signal due to cell membranes.

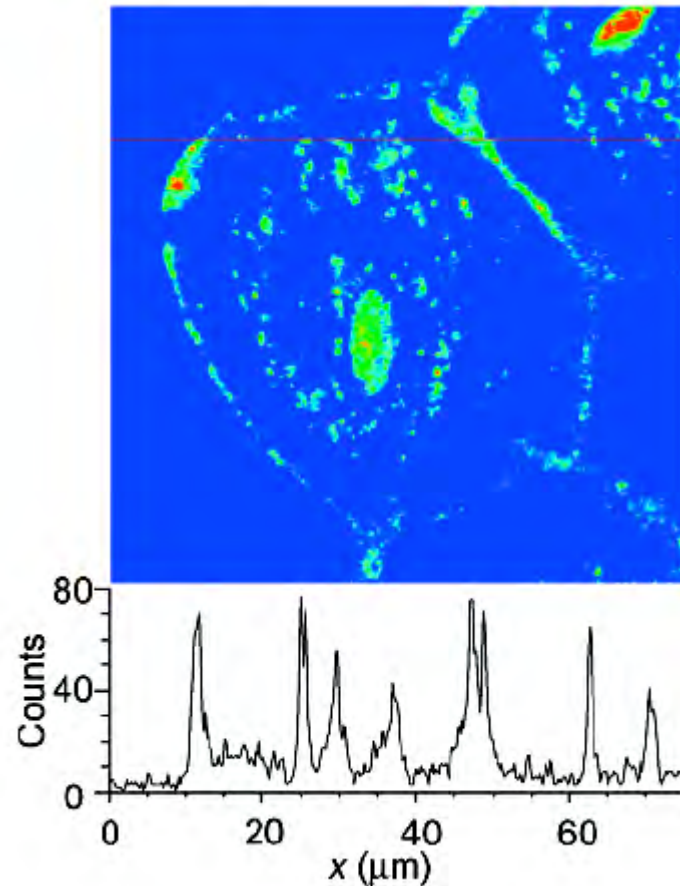


Unstained live HeLa cells. Bright spots due to mitochondria.

Example : CARS image of protein, nucleic acid in a single cell



Unstained live human epithelial cell

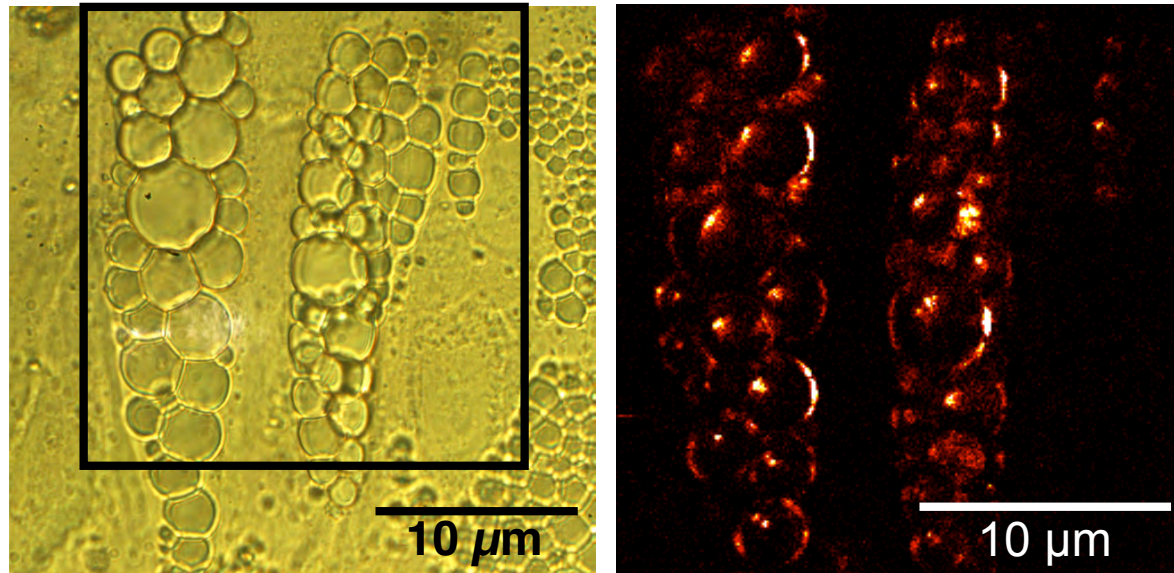


Laser powers - 2 and 1 mW, tuned to 1570 cm^{-1} (protein, nucleic acid)
image acquired in 8 min, smallest feature $<300\text{ nm}$

Example : CARS image of MSC-derived adipocytes rich in lipid structures



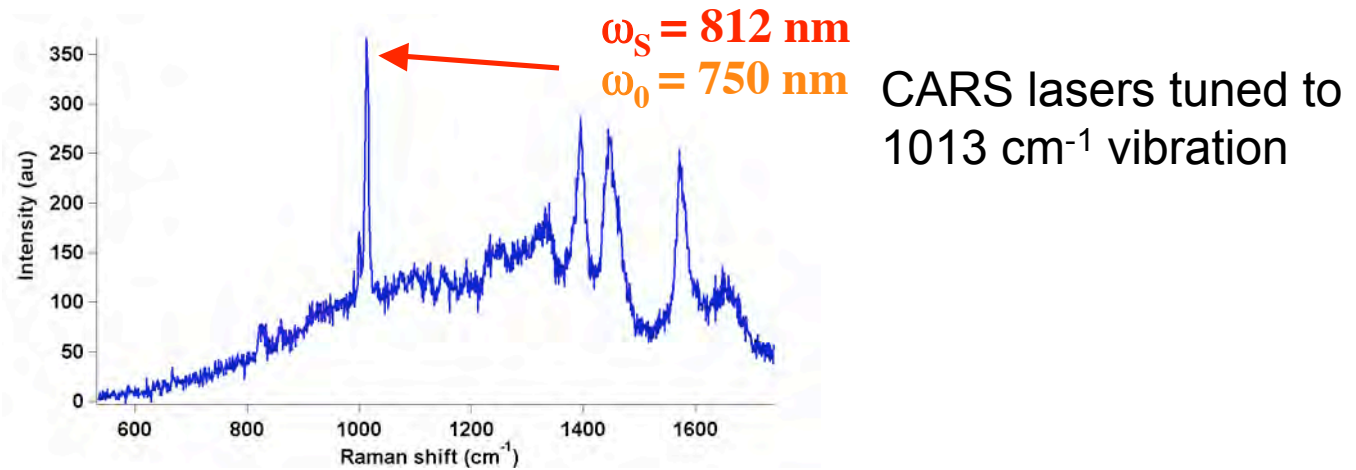
**CARS tuned to
2845 cm^{-1}
lipid mode**



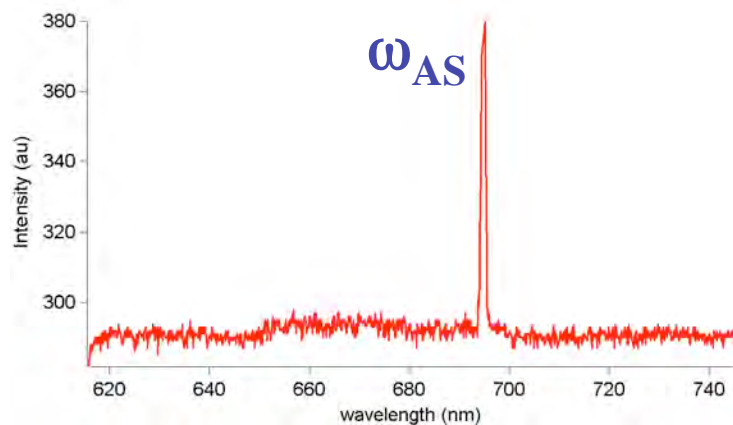
**MSC-derived
adipocytes
(fat cells)**

Courtesy: Iwan Schie, Tyler Weeks, Gregory McNerney

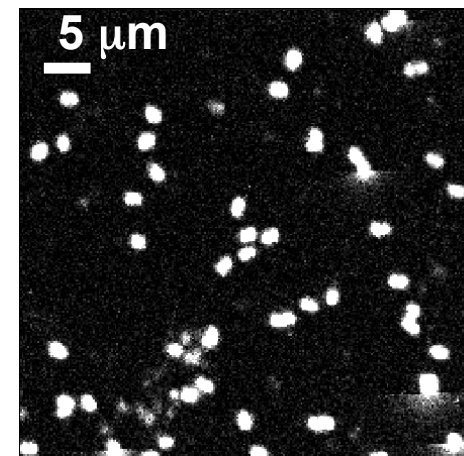
Example : CARS imaging of bacterial spores



Raman spectrum of bacterial spore



CARS signal at 697 nm

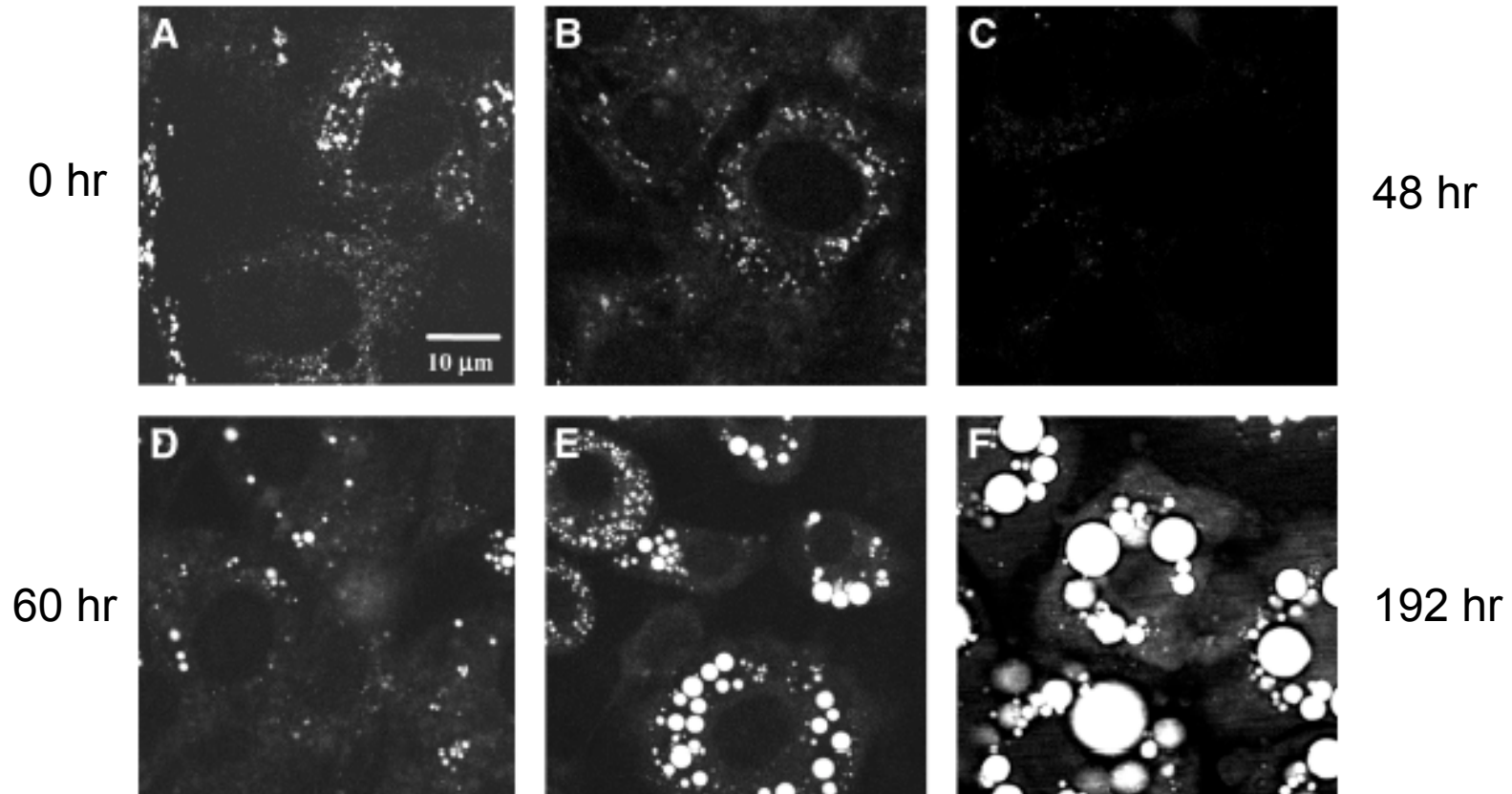


CARS image of spores on glass substrate

Long-term dynamic cell processes can be monitored with CARS microscopy

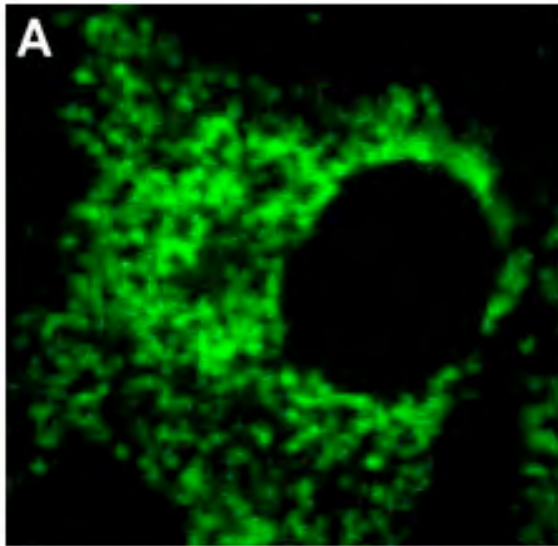


Conversion of 3T3-L1 fibroblast cells to adipocyte (fat) cells

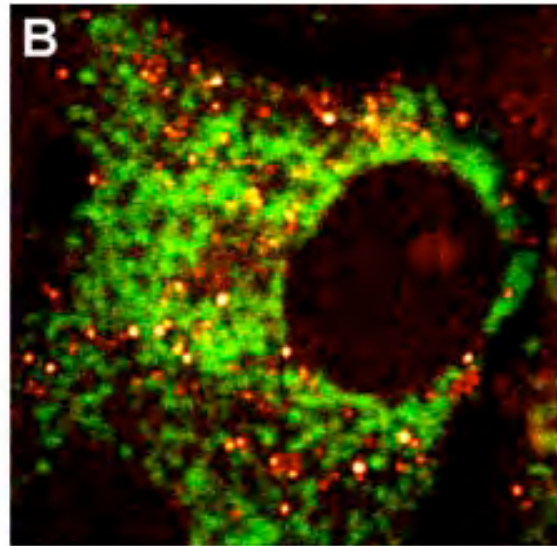


Imaging of triglyceride droplets at 2845 cm^{-1} (lipid vibration)

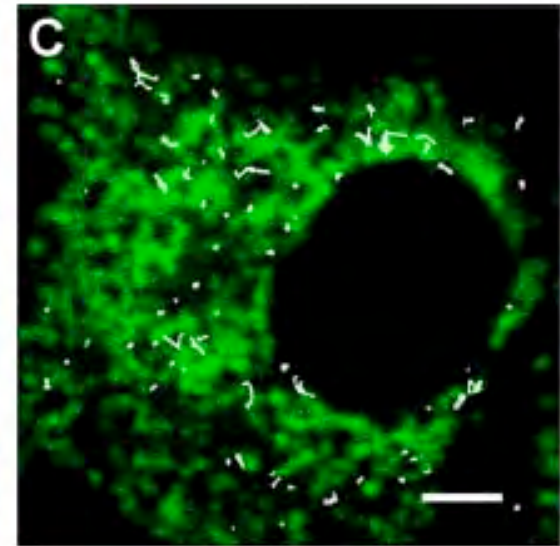
Tracking trajectories of organelles inside single living cells



A Two photon fluorescence - mitochondria



B CARS image of lipid droplets overlaid on TPF image



C Trajectory of droplets by repeated CARS imaging

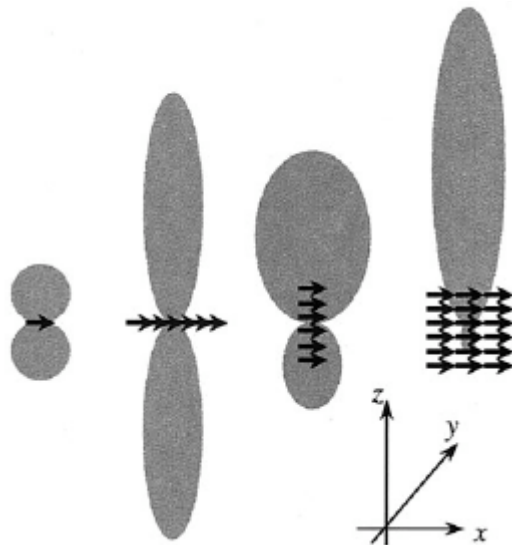
Radiation pattern in the forward and backward directions may not be symmetrical



Incoherent microscopy : Radiation is symmetrical in both forward and backward direction (Fluorescence, 2 photon fluorescence, Spontaneous Raman)

Coherent microscopy : Radiation pattern is not symmetrical (CARS, SHG, THG)

Small scatter radiates as a single dipole



Bulk scatterers add constructively in the forward direction

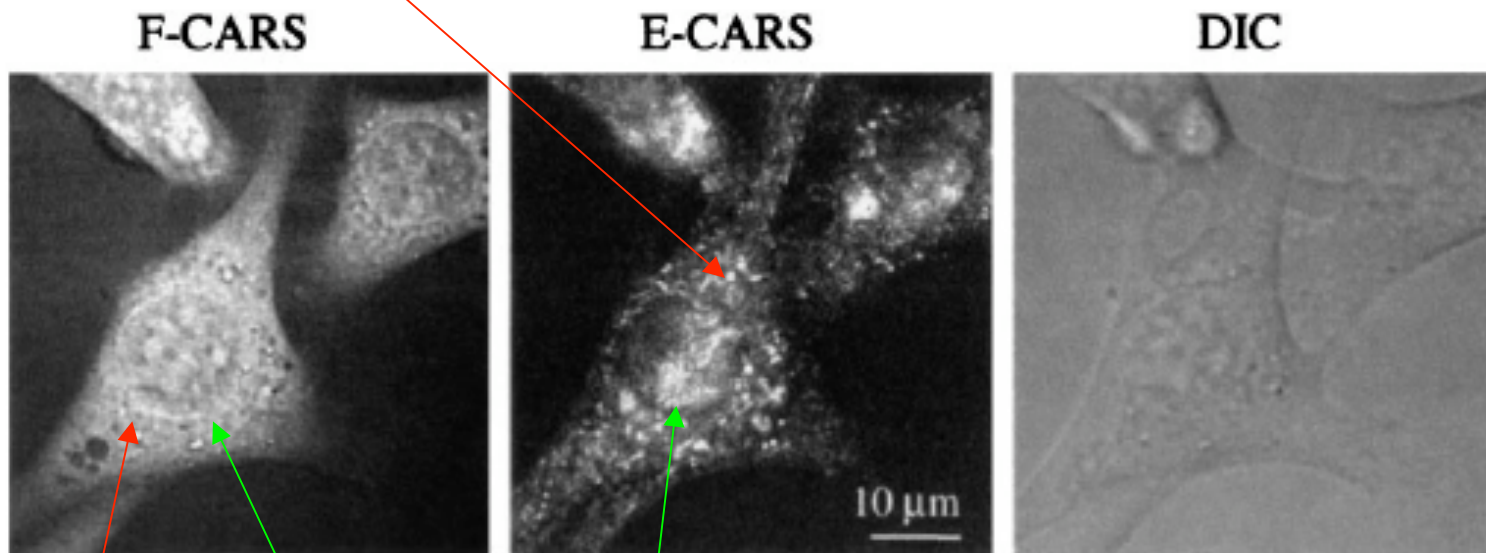
F-CARS detects large scatters, E-CARS detects small scatterers

Comparison of F-CARS and E-CARS image



Small scatterers in cytoplasm visible in E-CARS

NIH 3T3 cells
C-H 2870 cm^{-1} lipid membrane

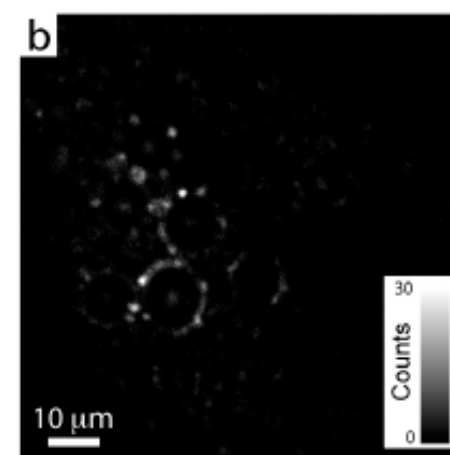
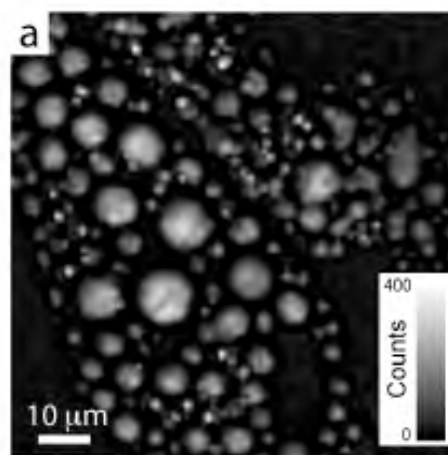
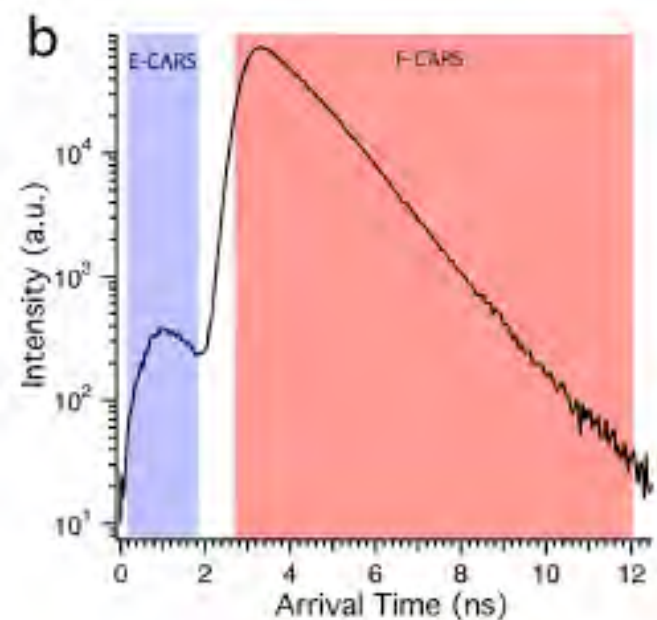
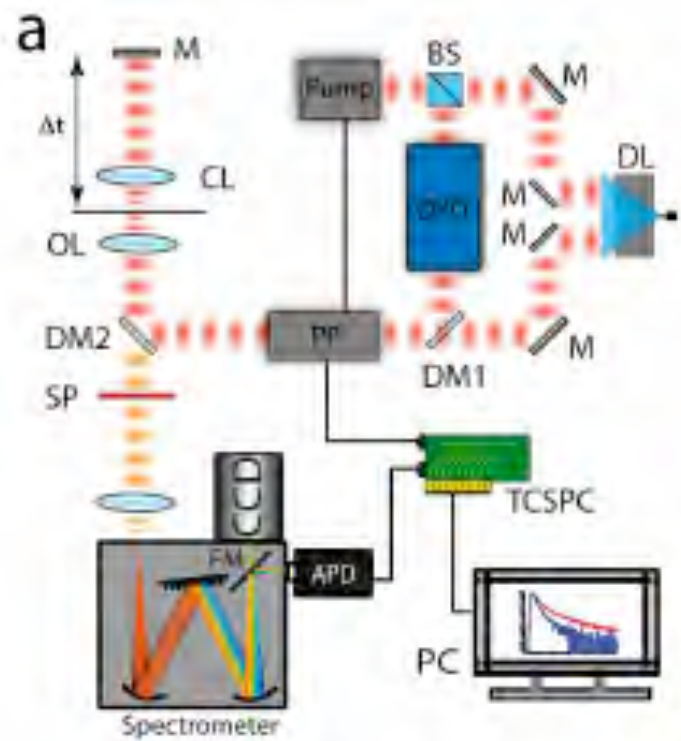


Dark image due to destructive interference in E-CARS

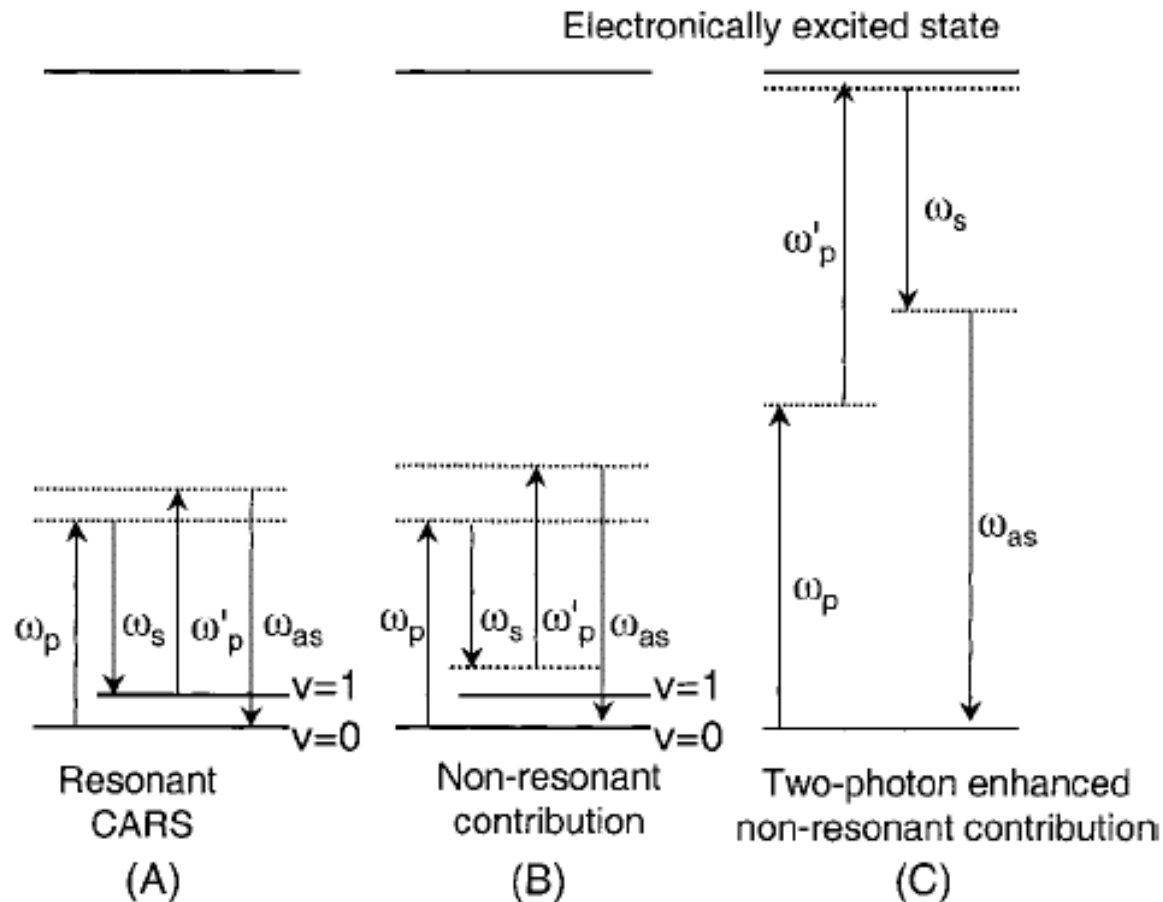
Nuclear membrane edge visible in F-CARS, large axial length

Cytoplasm overwhelmed by solvent signal

Detection of F-CARS and E-CARS using one detector is possible by temporal separation of the two signals



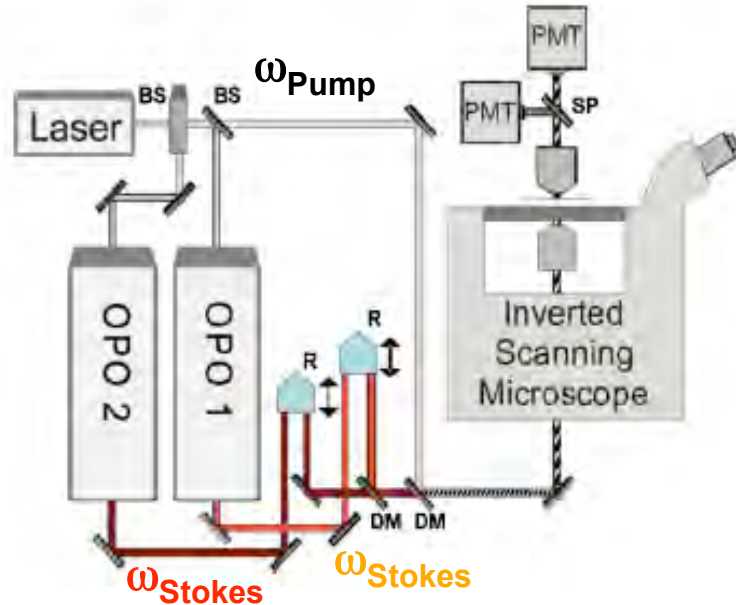
Nonresonant background is a major issue in CARS microscopy



Polarization CARS (P-CARS) – Cheng et. al, Optics Letters, V26 1341 (2001)

Epi-CARS (E-CARS) – suppression of bulk background solvent

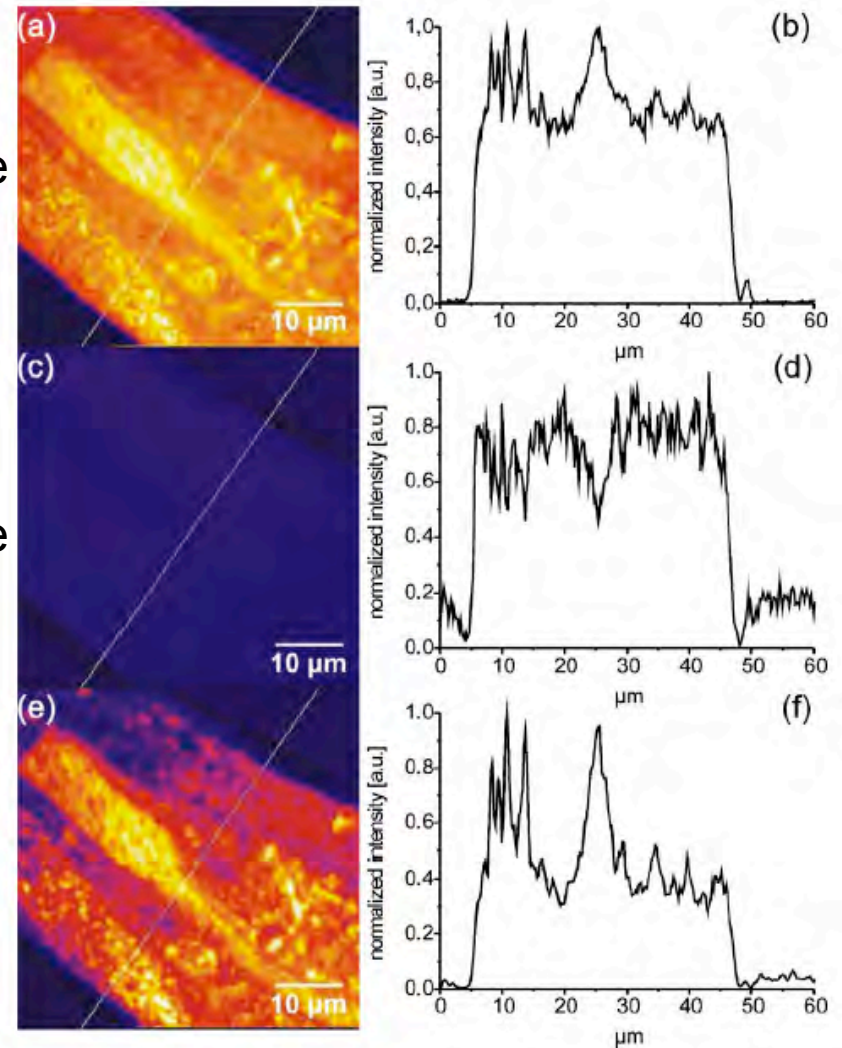
Dual pump CARS microscopy can be used to subtract nonresonant background



On resonance

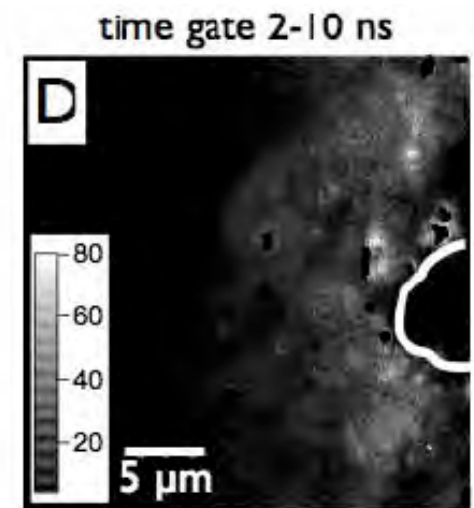
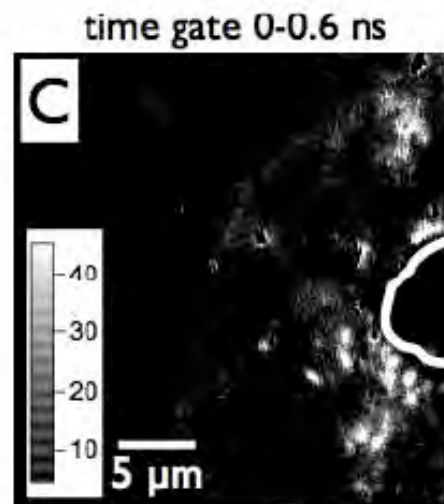
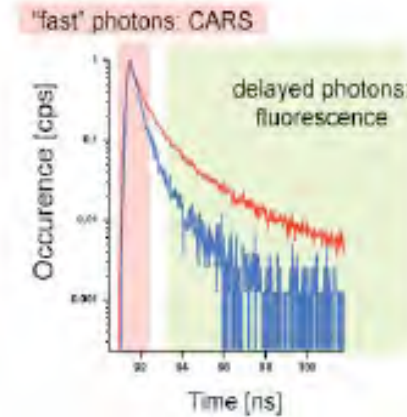
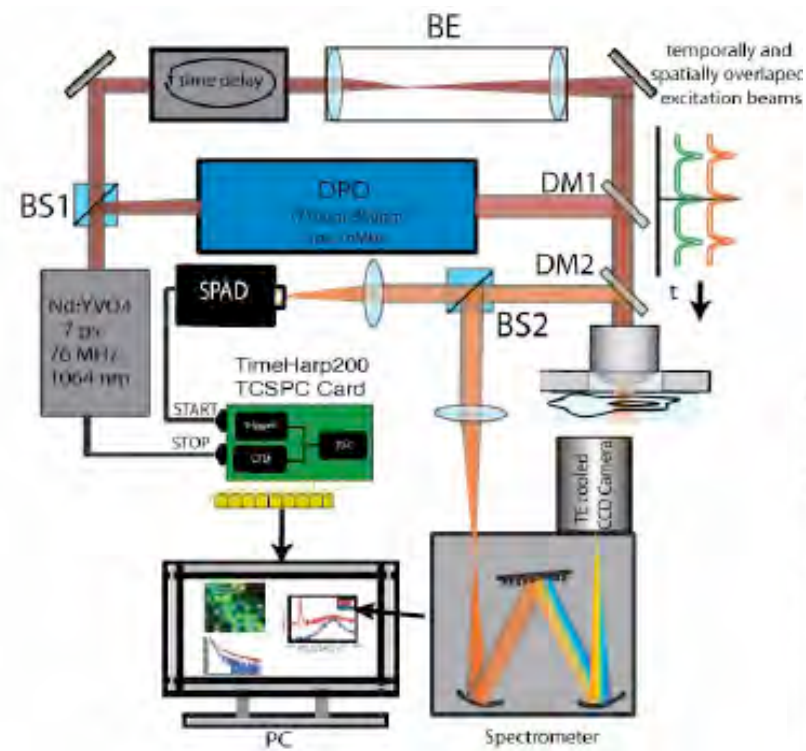
Off resonance

Difference

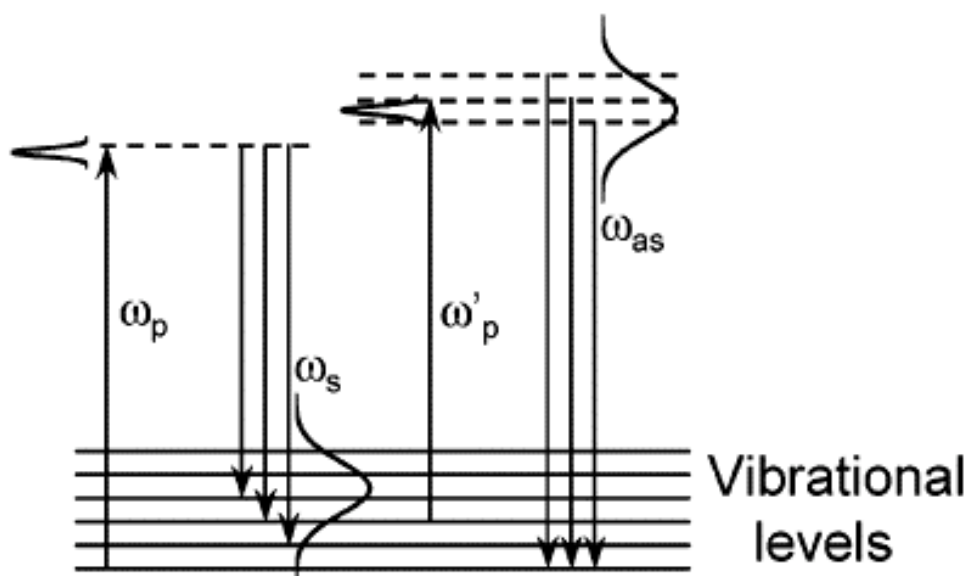


Autofluorescence (2-photon) from the sample may overwhelm the CARS signal

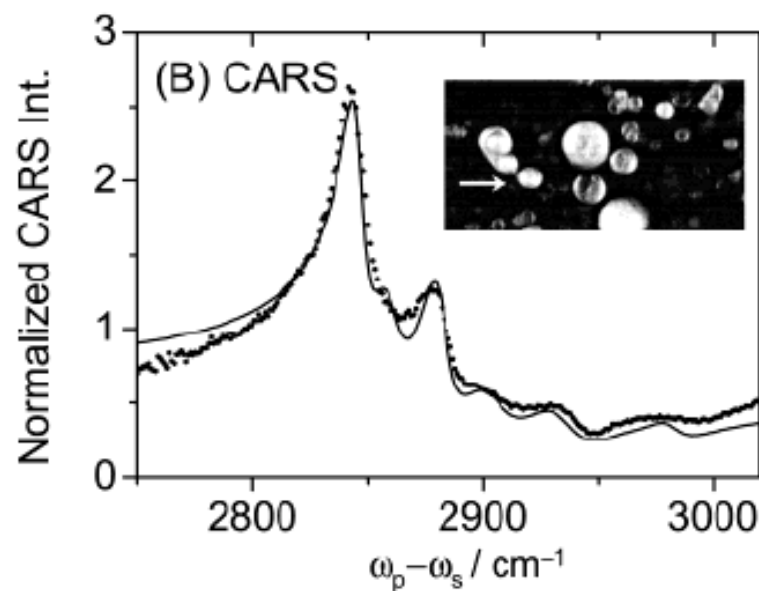
- Raman lifetimes \sim ps
- Fluorescence lifetimes \sim ns



Multiplexed CARS (M-CARS) has been developed for CARS spectroscopy



Population of multiple levels simultaneously (ps-fs combination)



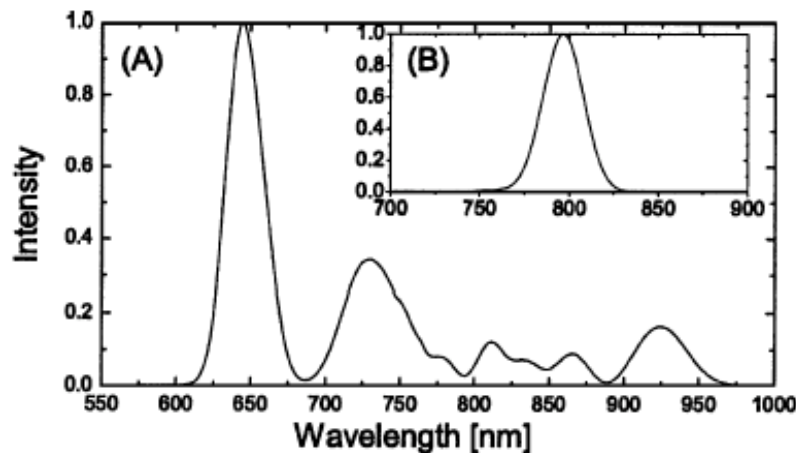
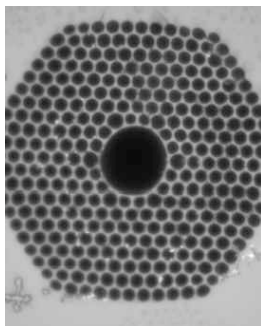
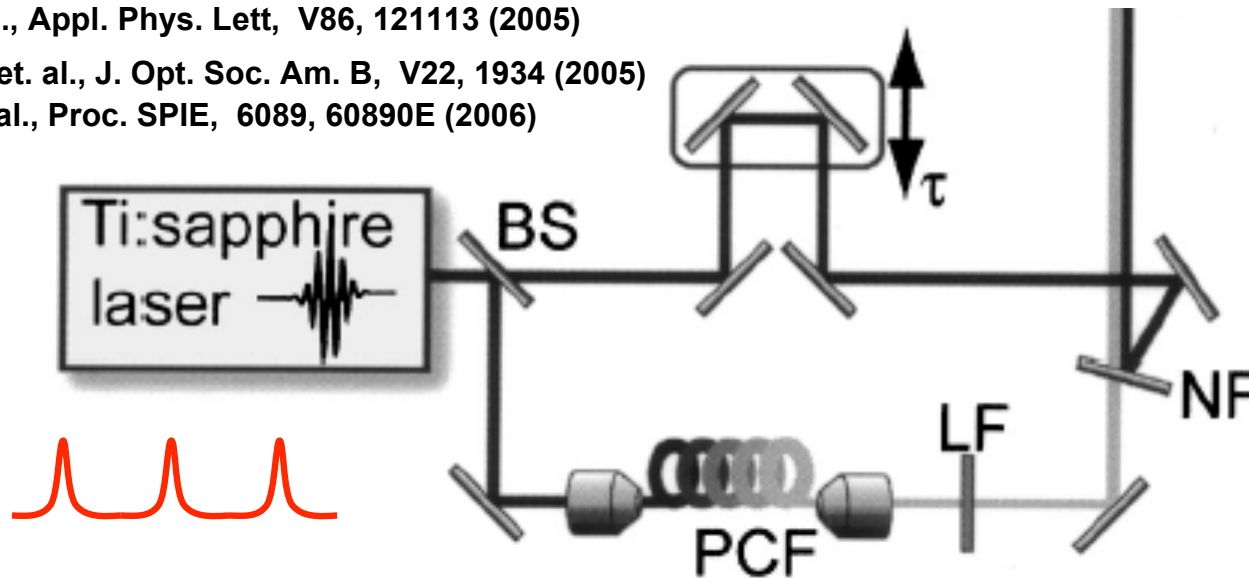
CARS spectrum of DOPC vesicle in the C-H stretching region ($\sim 150 \text{ cm}^{-1}$)

Supercontinuum generation in a photonic crystal fiber can function as a broad source for M-CARS

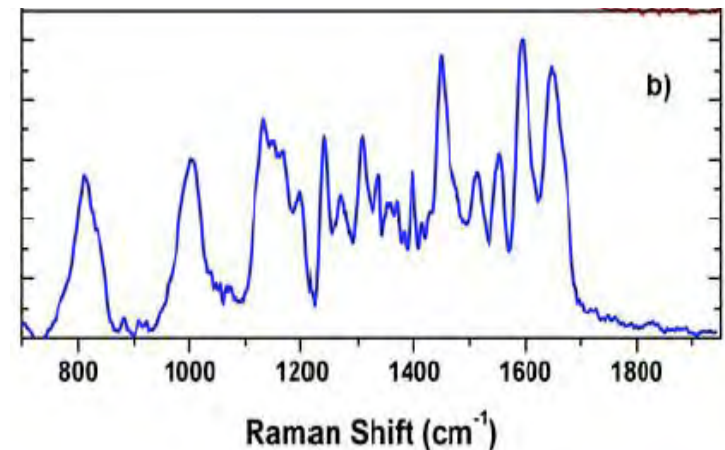
Kano et. al., Appl. Phys. Lett, V86, 121113 (2005)

Andresen et. al., J. Opt. Soc. Am. B, V22, 1934 (2005)

Petrov et. al., Proc. SPIE, 6089, 60890E (2006)



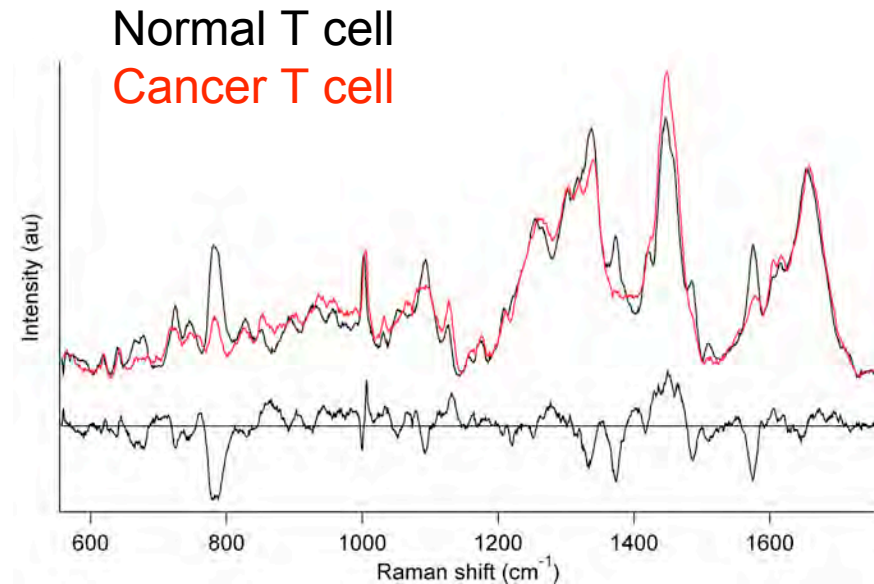
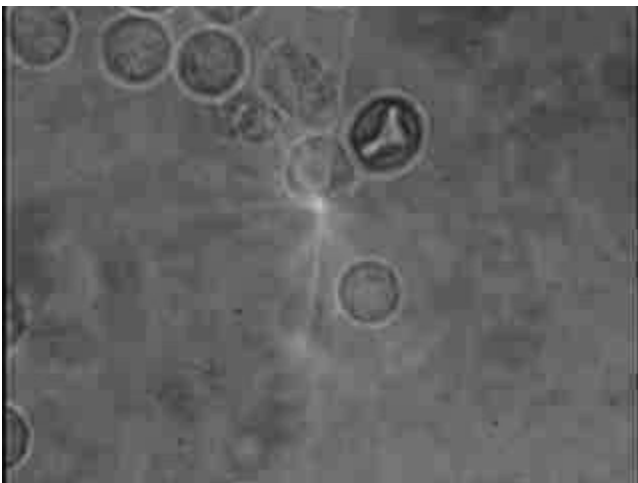
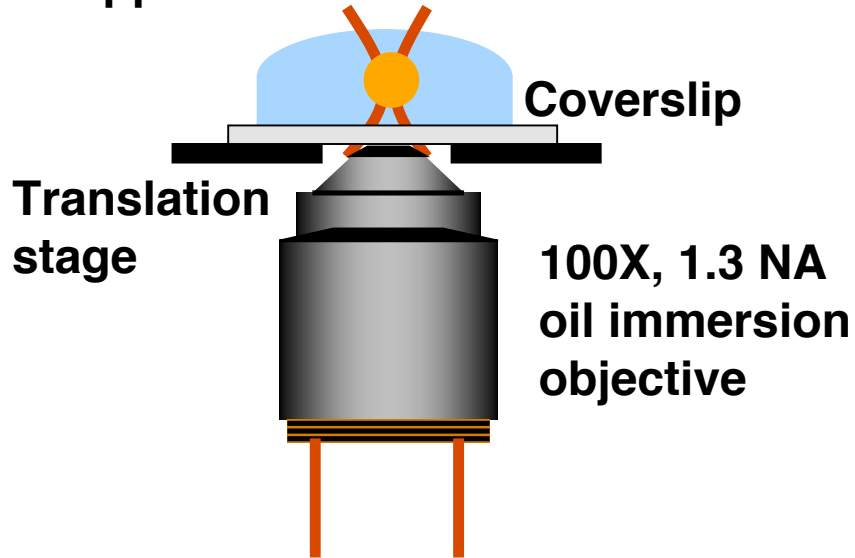
Supercontinuum generation



CARS spectrum of bacterial spore –
1 second acq. time

We have been applying Raman spectroscopy for single cell cancer detection

Trapped cell



Spontaneous Raman spectra takes
2 minutes per cell

Future applications : CARS cytometry for rapid, label-less cancer cell detection and sorting

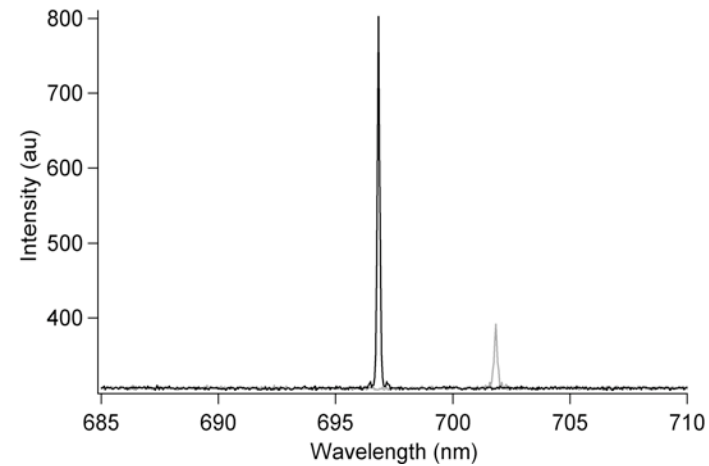


We have demonstrated optical trapping combined with CARS for faster spectral analysis

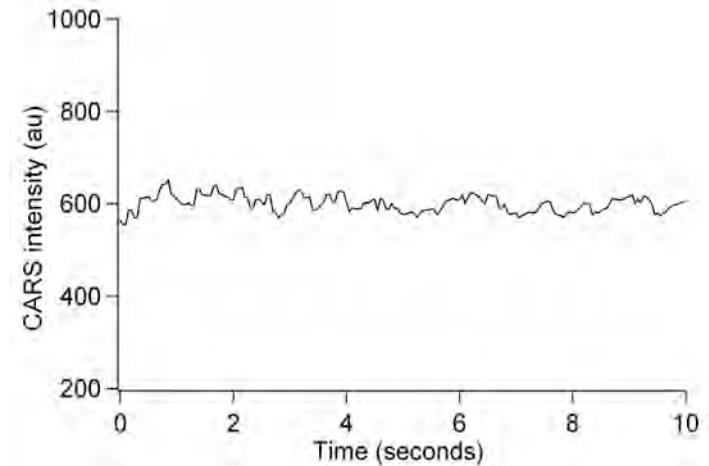


Trapped polystyrene bead using two CARS beams

Potential solution for faster chemical analysis of cells



CARS signal from a C=C bond



Microsecond temporal resolution

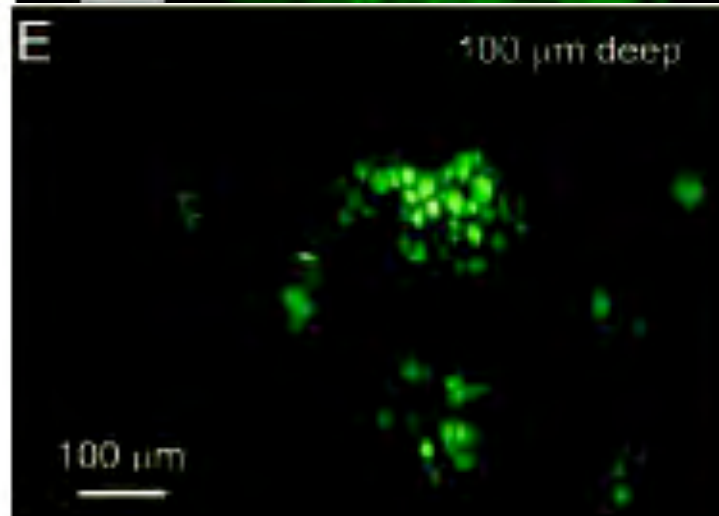
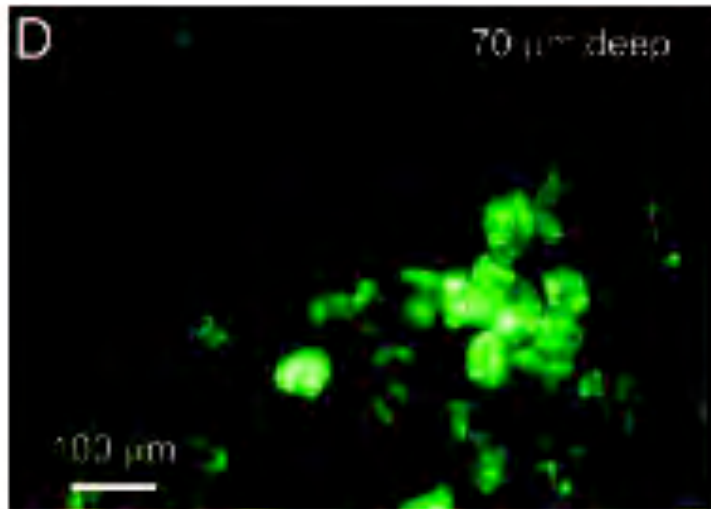
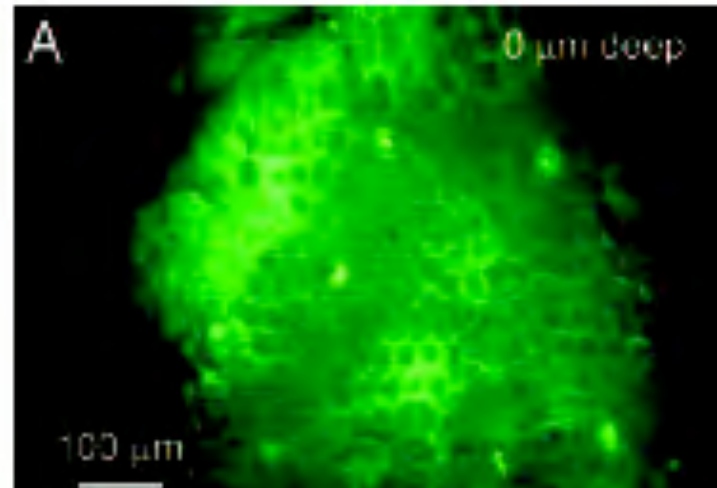
Future applications : CARS *in-vivo* imaging



2845 cm^{-1} vibration C-H lipid



Stratum
corneum

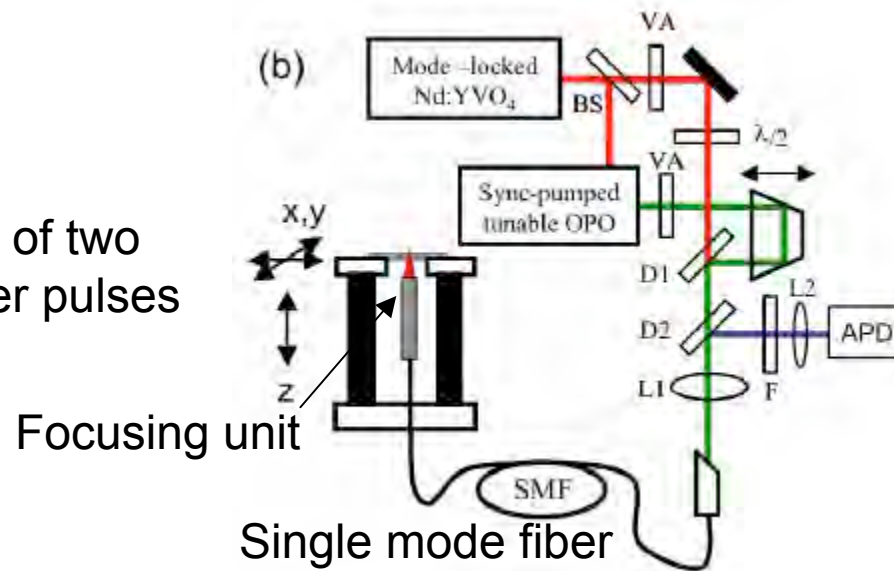


Adipocytes of the dermis

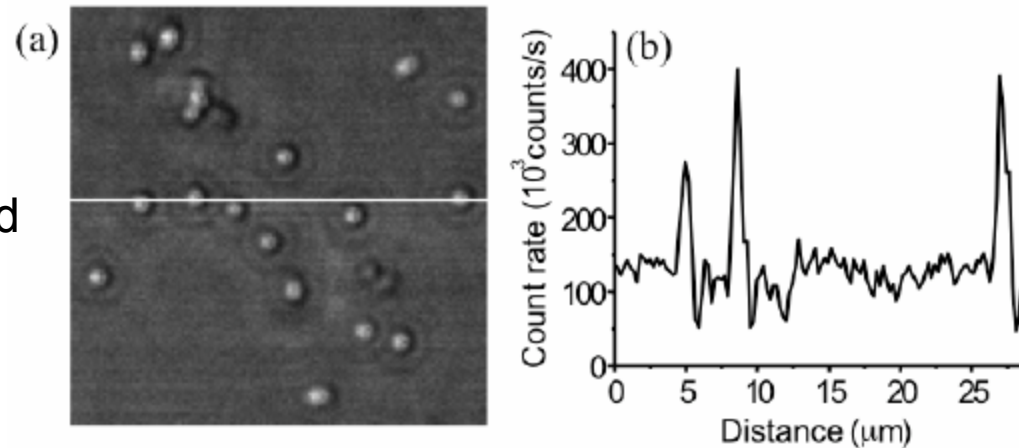
Adipocytes of subcutaneous layer

Future applications : CARS endoscopy

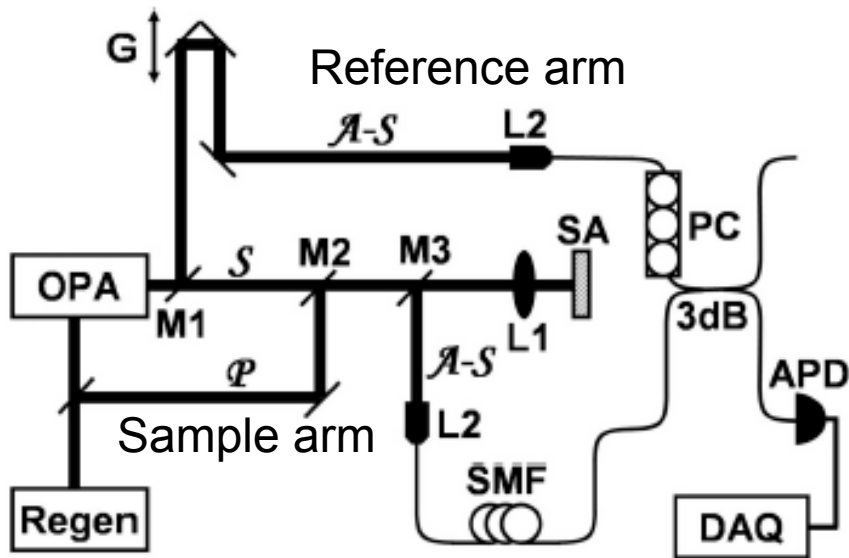
Fiber delivery of two ultrashort laser pulses



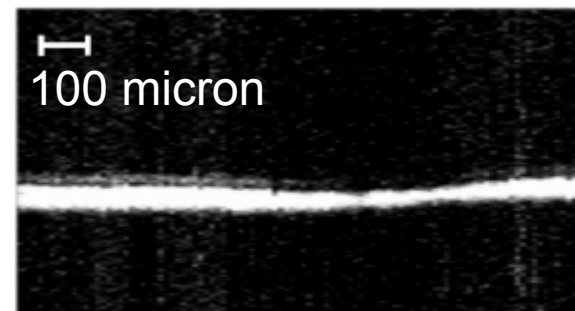
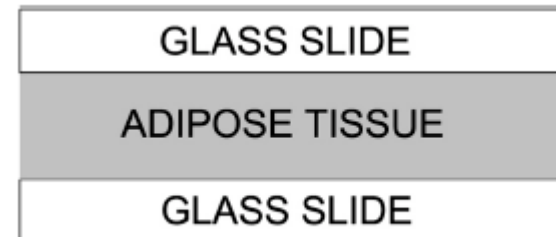
750 nm beads imaged in epi-direction



Future applications : Optical coherence tomography with chemical contrast (CARS-OCT)



Axial resolution limited by coherence length of light source $\sim 32 \mu\text{m}$ for ps pulses



Summary



- CARS microscopy is a new technique for live cell imaging with chemical contrast without using tags.
- Inherent Raman signals do not photobleach, enabling long term cell studies
- Motivation for CARS development due to limitations of spontaneous Raman spectroscopy (signal strength, resolution)
- There are many forms of CARS (F-CARS, E-CARS, P-CARS, M-CARS) being developed since 1999.
- Future directions
 - CARS-based flow cytometry for single cell sorting
 - In-vivo CARS
 - Fiber based CARS for endoscopy
 - CARS optical coherence tomography