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

Selected Topics in Biophotonics (EAD289)

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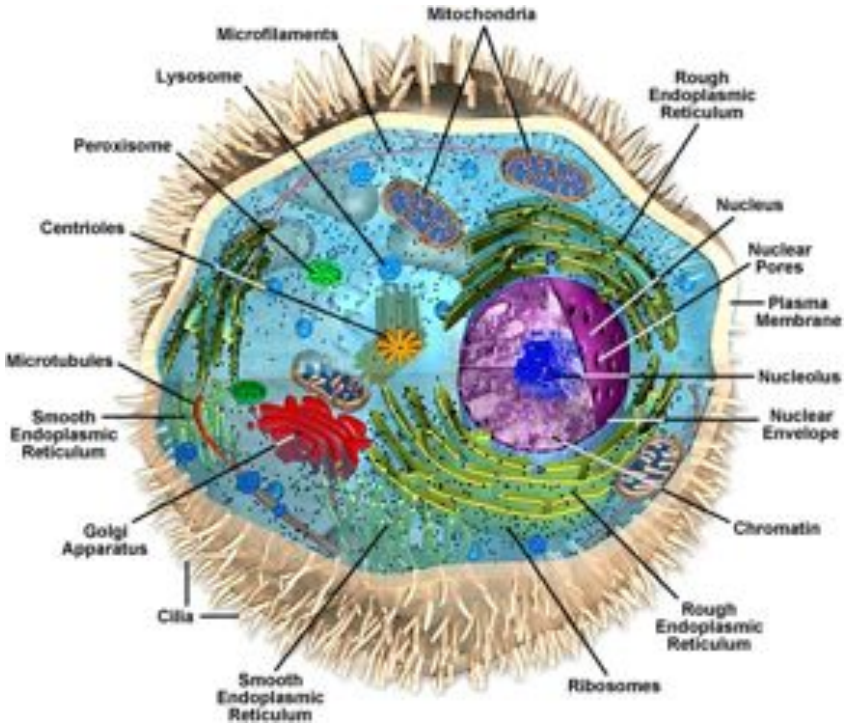
Outline

- Motivation – Why Raman?
- Background theory on Raman spectroscopy
- Spontaneous Raman spectroscopy and 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
- Applications
- Summary

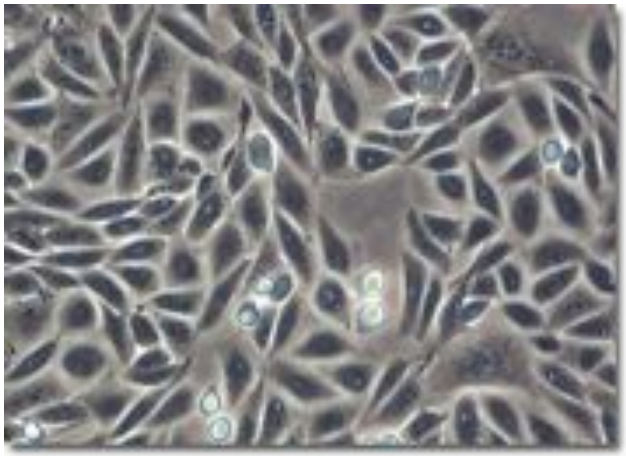
Live cell imaging requires the development of new optical microscopy methods

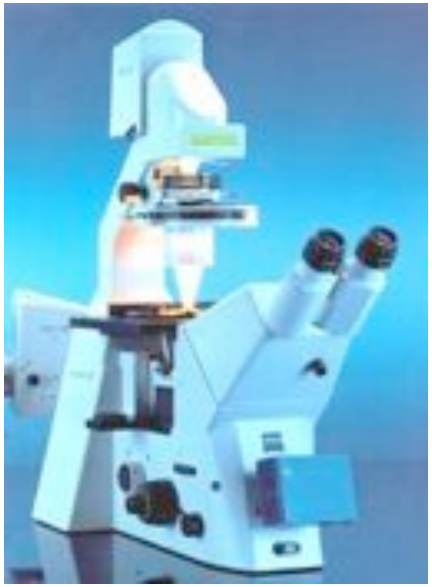
- Specificity
- Sensitivity
- Dynamic live cell imaging
- Long term live cell imaging



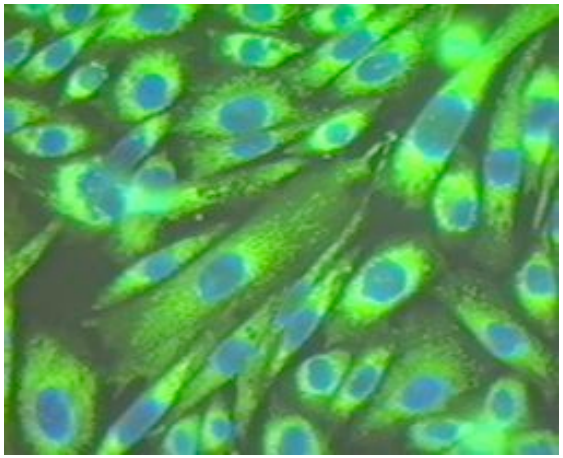
Current state of conventional optical microscopy

Phase contrast





Fluorescence



(+) Low cost
(+) Easy to use

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

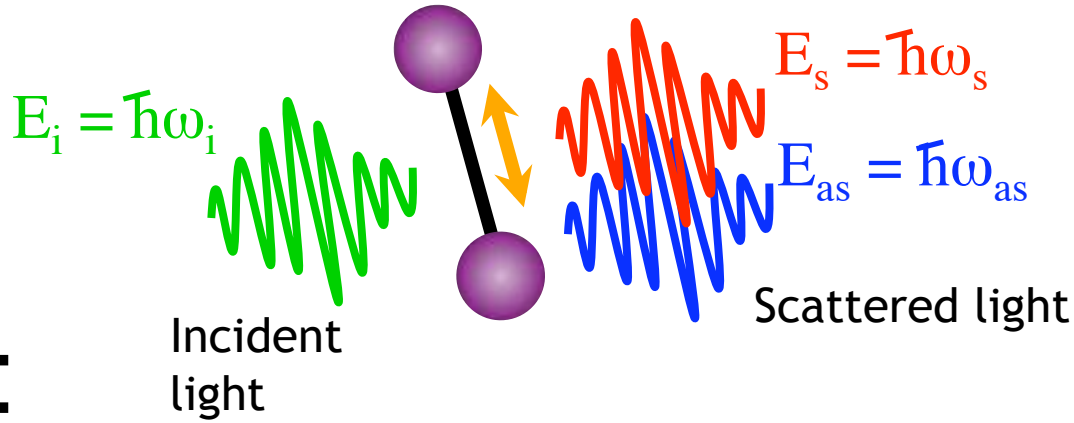
(+) Specific labeling
(+) 3D information with confocal and multiphoton microscopy

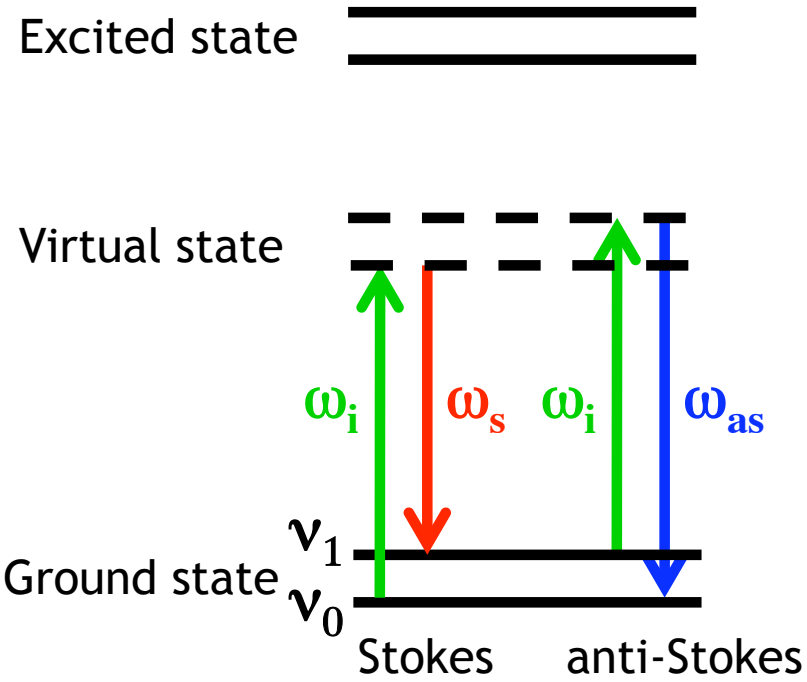
(-) Photobleaching – no long term studies
(-) Toxicity, cell fixation - perturbs cell function

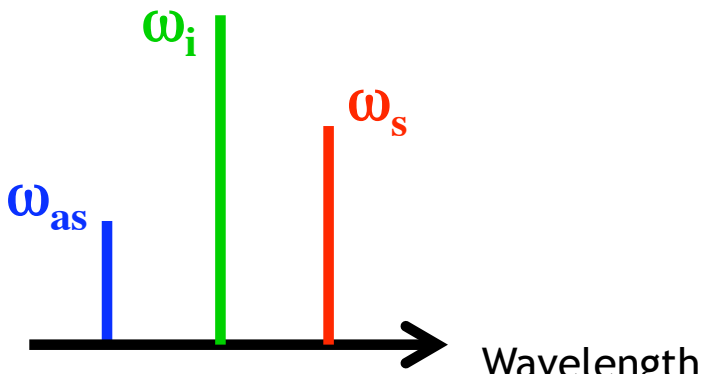
Raman scattering is the interaction of photons and intrinsic molecular bonds

C.V. Raman
1930 Nobel Prize

Molecular vibration in sample







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

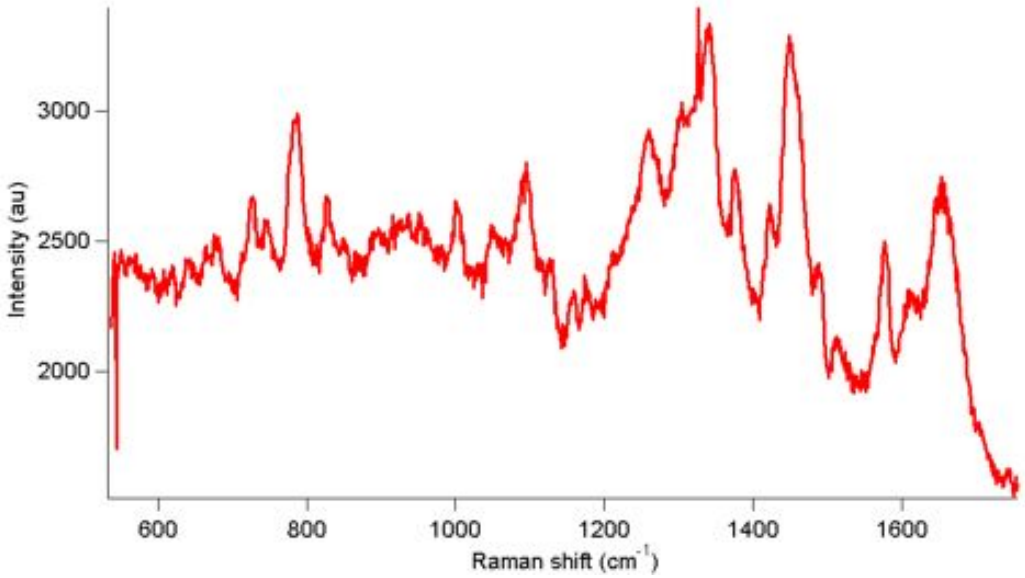
$\alpha = \alpha_o + (d\alpha / dr) r_{max} \cos(\omega_{vib} t)$ Polarizability oscillates at vibrational frequency

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

Rayleigh Anti-Stokes Stokes

Raman spectra of cells provide a wealth of biological information

Single live human T cell

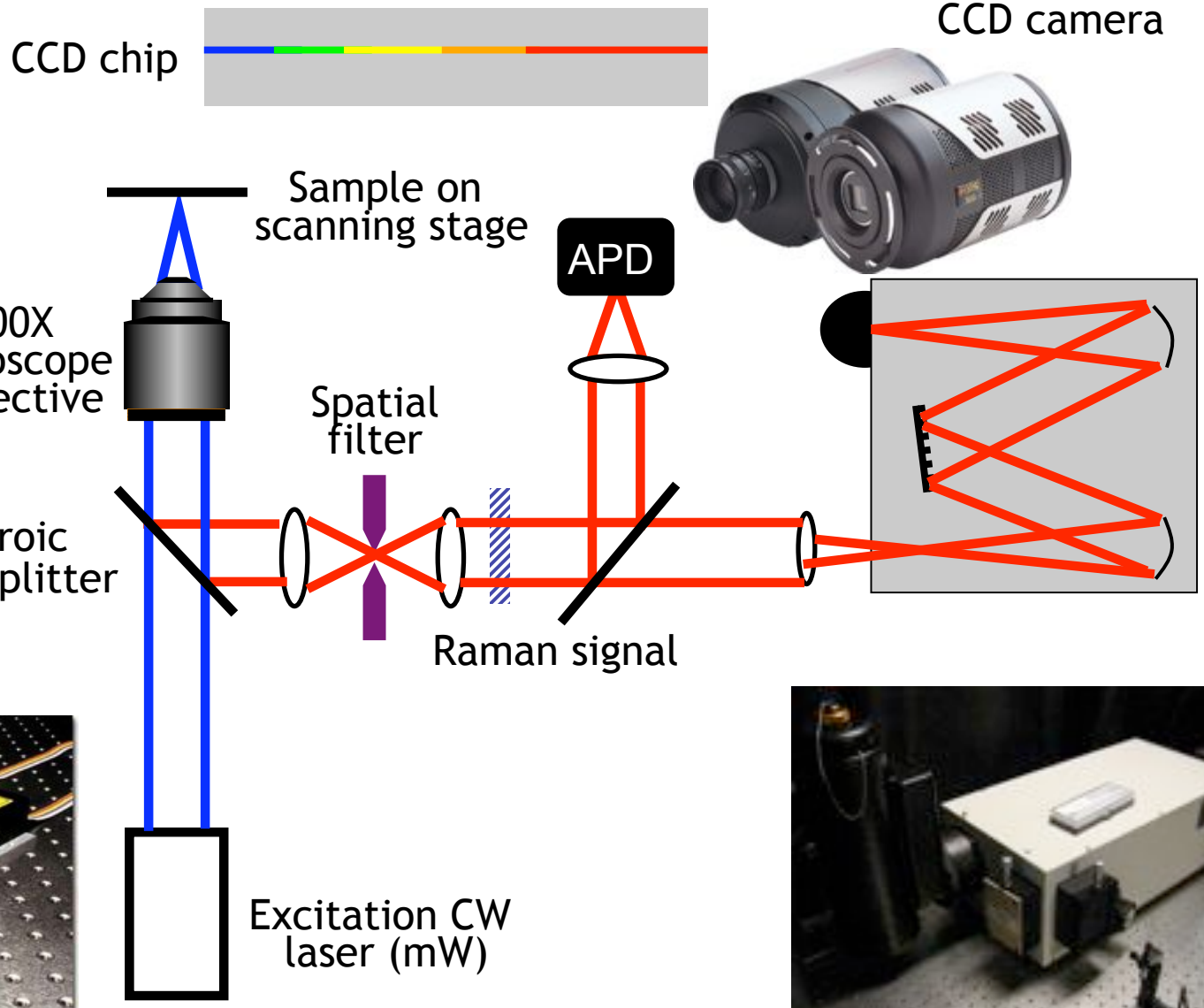


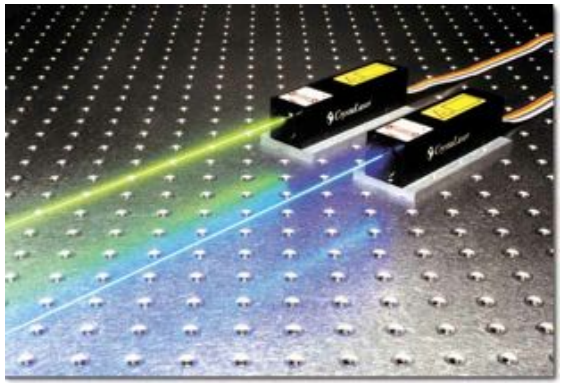
k = 1/λ (cm^{-1}) Wavenumber units

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

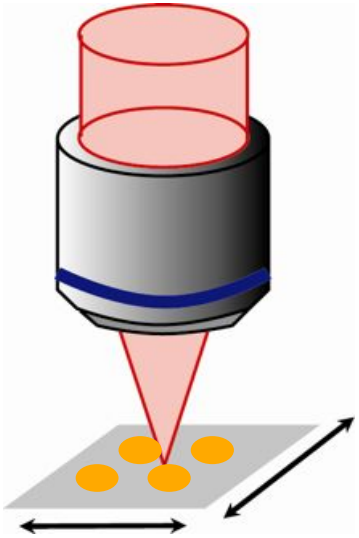
Raman frequency in wavenumber units (cm^{-1})	Assignment[a]
618	p: C-C twist
640	p: C-S str., C-C twist Tyr
666	G, T, Tyr, G bk in RNA
678	G (C-2'-endo-anti)
725	A
746	T
785	U, T, C, bk: O-P-O
831	O-P-O asym. str., Tyr
852	Tyr. ring breath.
893	bk, p: C-C skeletal
1003	Phe, C-C skeletal
1031	Phe, p: 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

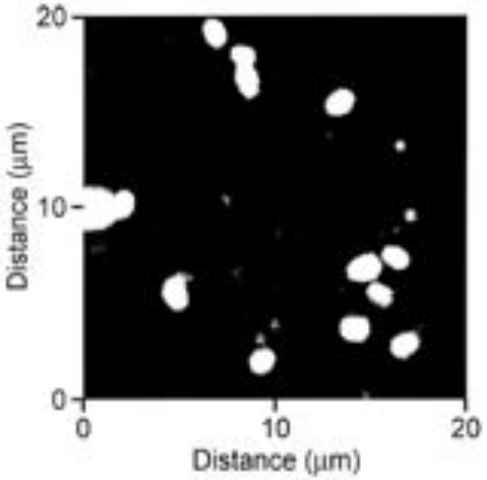
Confocal Raman microscope for microspectroscopy and imaging





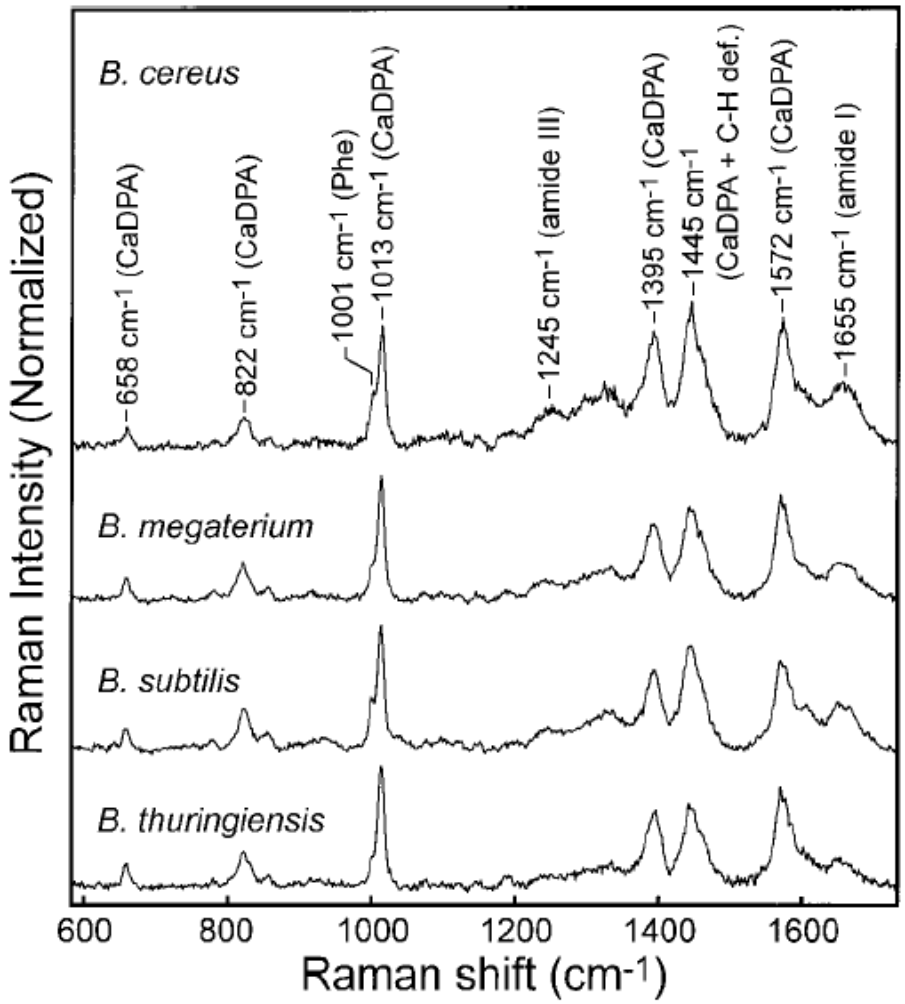
Conventional single cell Raman spectroscopy is usually performed on surfaces



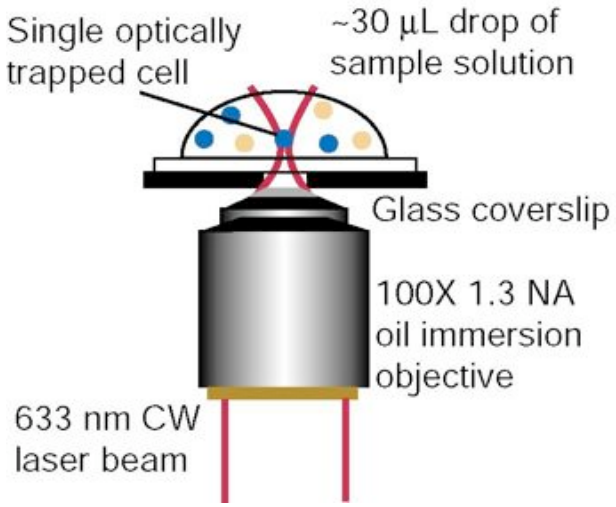


Spores dried on a surface

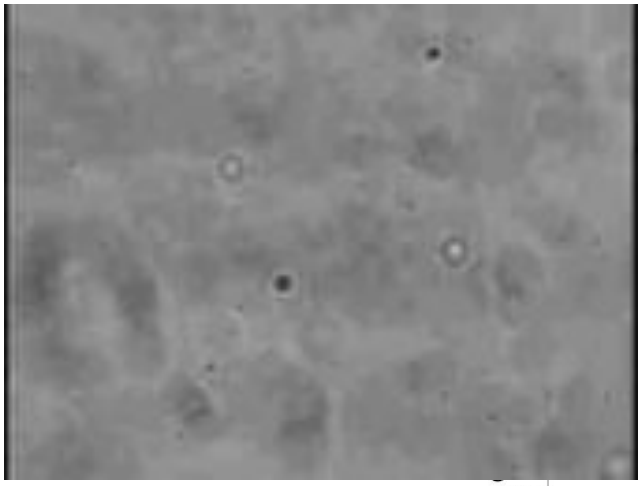
Esposito et. al., Appl. Spec. 57 (7) 868 (2003)

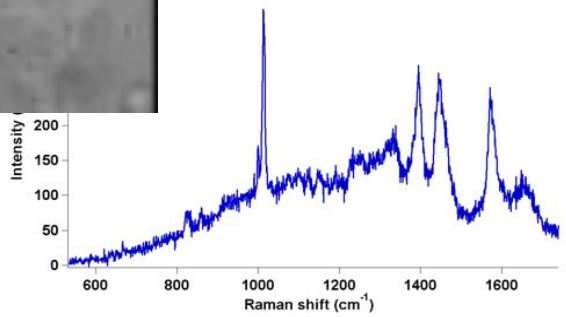


Optical trapping combined with Raman spectroscopy simplifies the analysis of single cells



Optically trapped spore

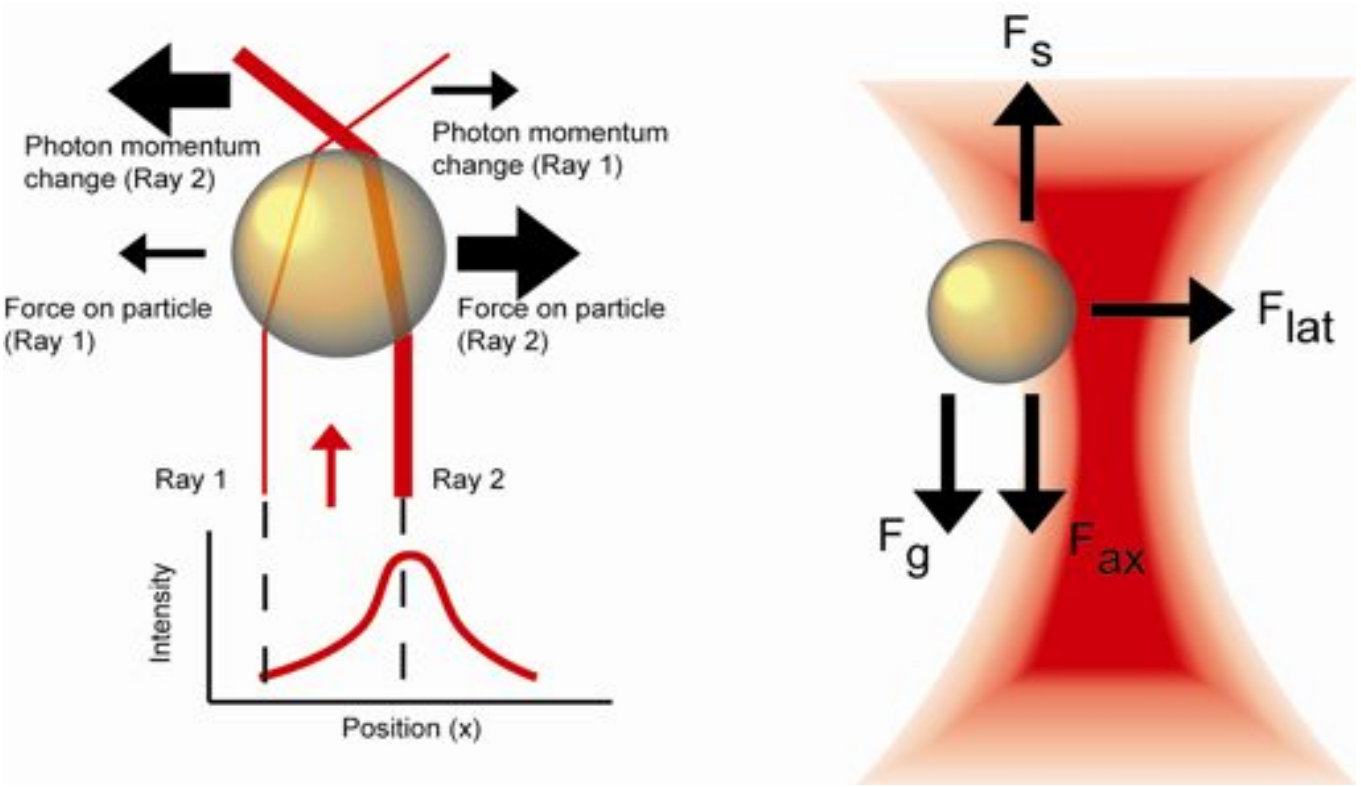




- Advantages:
  - Rapid sampling of many particles in solution
  - Reduces background signals from surfaces
  - Maximizes Raman signals
  - Enables manipulation and sorting of particles

Chan et. al., Analytical Chemistry, 76 599 (2004)

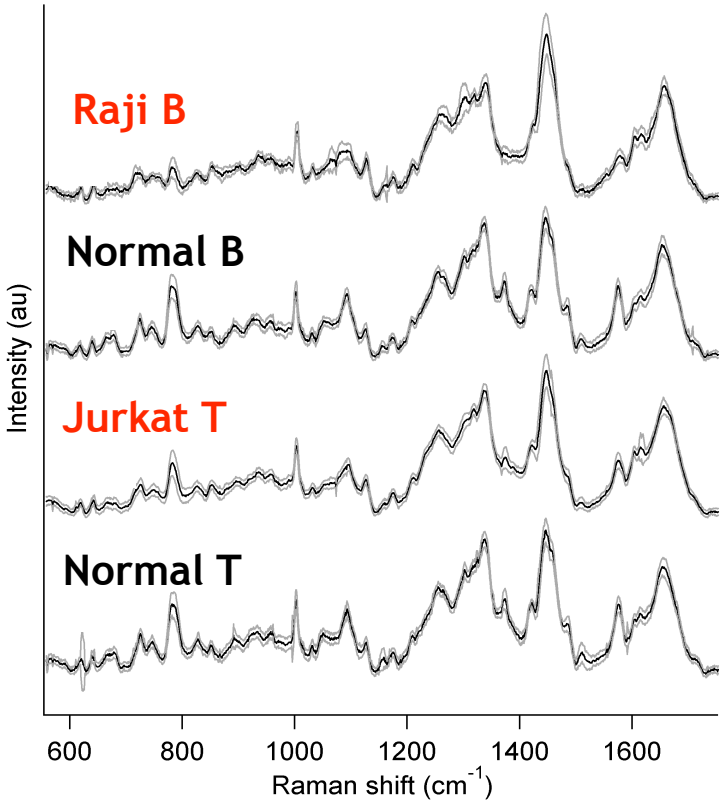
Optical trapping immobilizes a particle within the laser focus



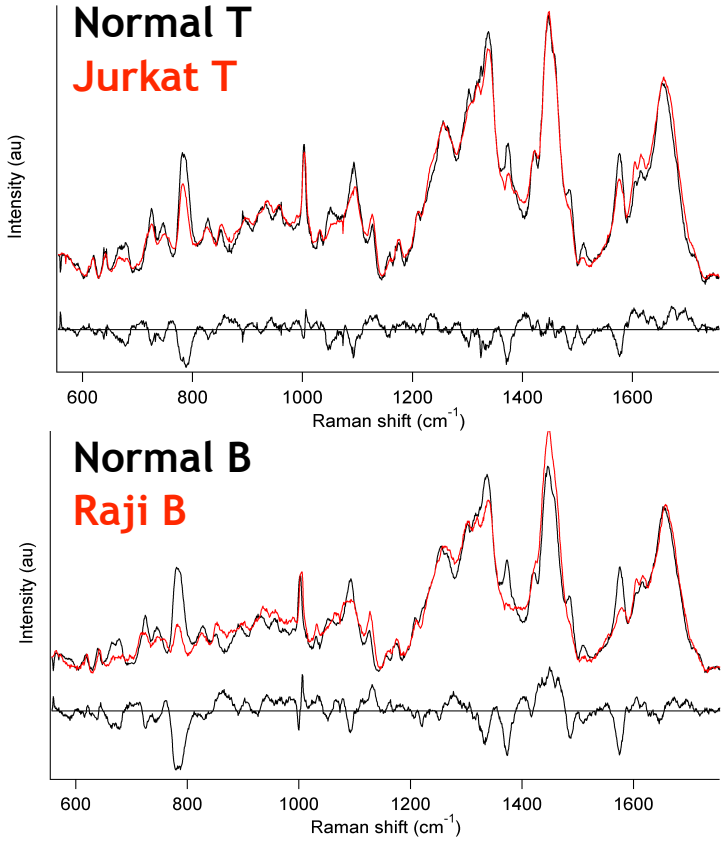
- Tight focusing condition
- High intensity gradients in both axial and lateral directions
- Stable 3-D optical trapping with a single laser beam
- Trapping of organelles and whole cells have been demonstrated

Pediatric leukemia: Normal and malignant cells can be discriminated by their Raman fingerprint

Mean Raman spectra



Difference spectra



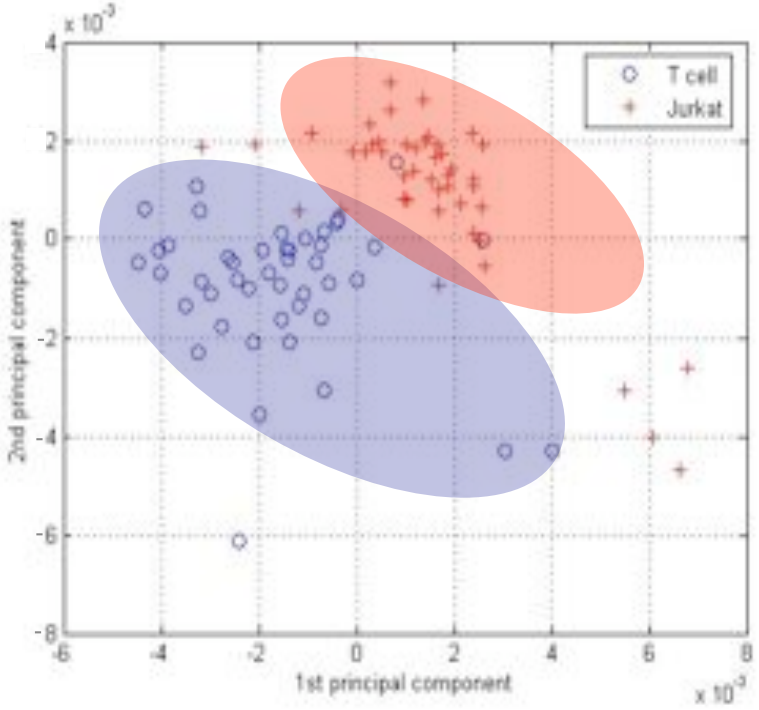
Reproducible within group spectra

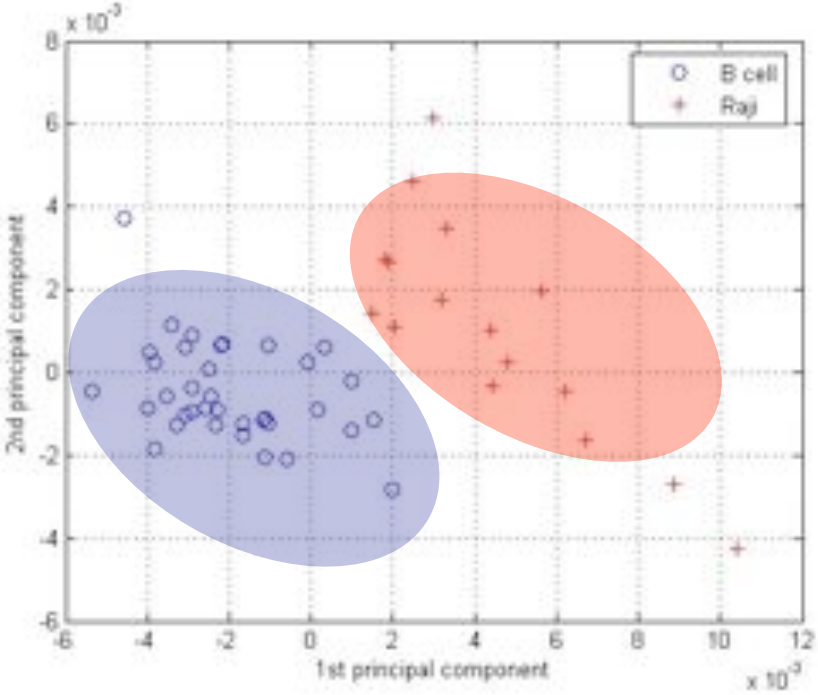
Cancer spectral markers

Chan et. al., Biophysical Journal 90 648-656 (2006)

Multivariate statistical techniques are used to separate and classify cell data

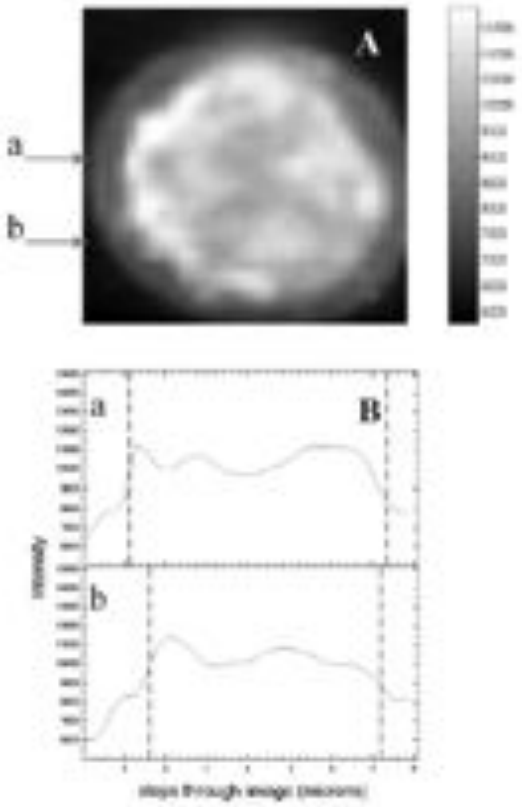
Principal component analysis (PCA)



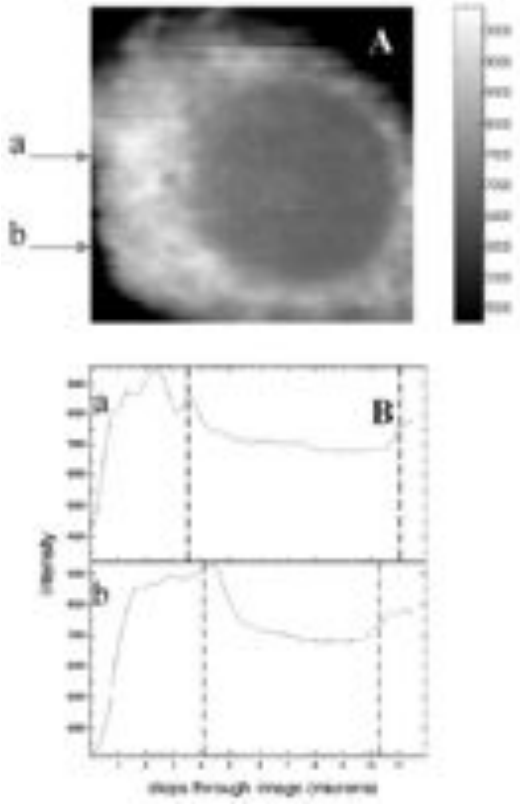


Raman images of formaldehyde fixed human cells

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

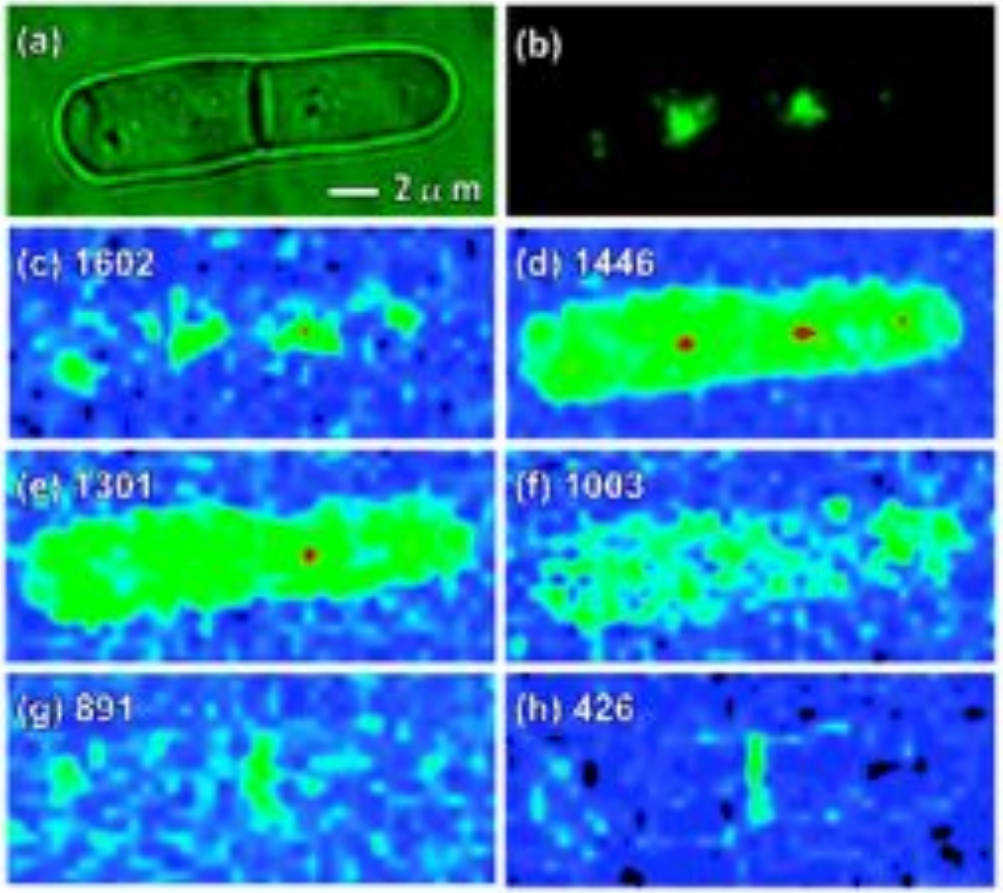
~ 400 nm resolution, 1 hour acquisition time

Uzunbajakava et. al., Biopolymers, V72, 1-9 (2003)

Raman mapping combined with fluorescence microscopy for multi-modal analysis

Raman mapping of chemical components in S. pombe cells

Live S. pombe cell
30 min per image
632 nm laser



GFP mitochondria image

1602 cm^{-1} signal colocalizes with GFP signal

Huang et. al., Biochemistry, V44, 10009-10019 (2005)

Advantages and limitations of spontaneous Raman spectroscopy/imaging

CBST

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

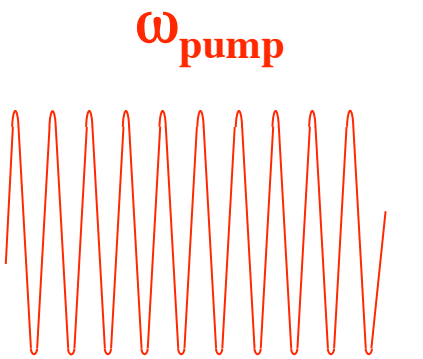
Raman scattering is extremely inefficient (10^{-30} cm^2 cross sections)
1 in 10^8 incident photons are Raman scattered

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

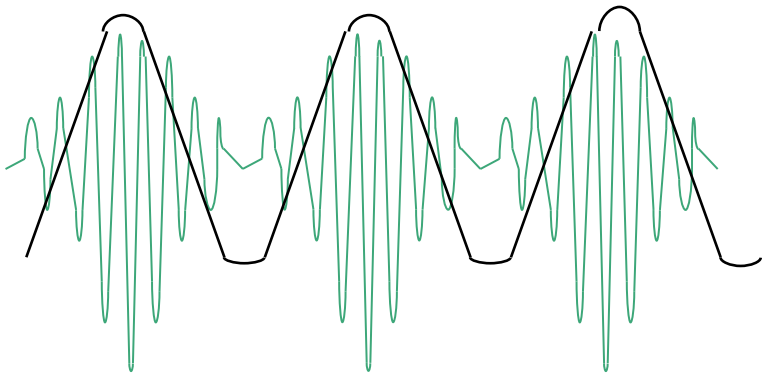
CARS uses two laser frequencies to interact resonantly with a specific molecular vibration

$\omega_{vib} = \omega_{pump} - \omega_{Stokes}$

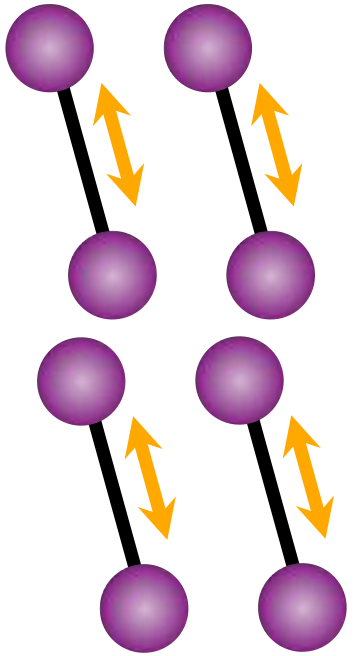


ω_{pump}

ω_{Stokes}

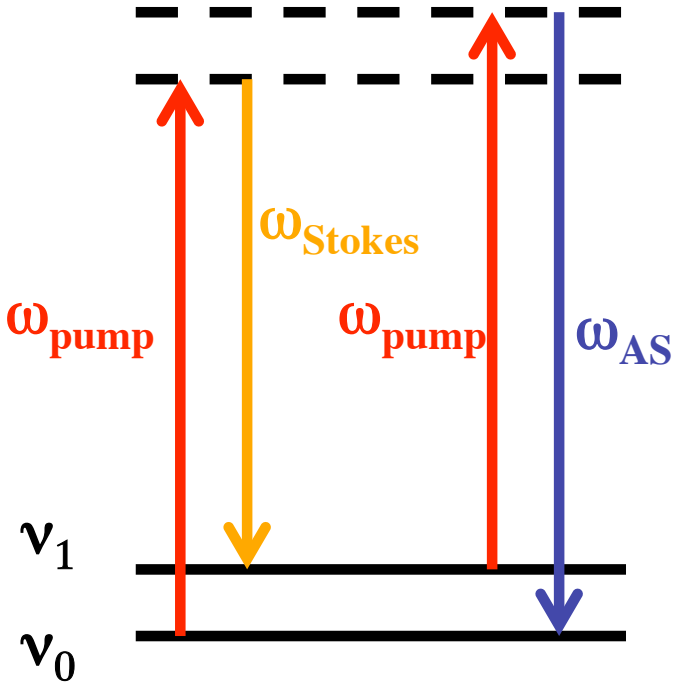


Beating at $\omega_{pump} - \omega_{Stokes}$



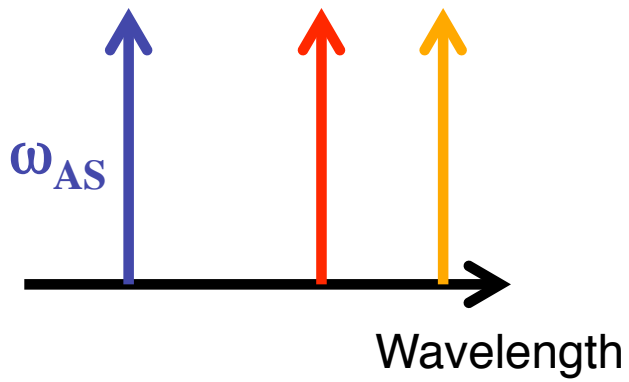
Coherent vibration of specific molecules resonant at ω_{vib}

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



Energy diagram

$$\omega_{AS} = 2\omega_{pump} - \omega_{Stokes}$$



Anti-Stokes

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

Polarization

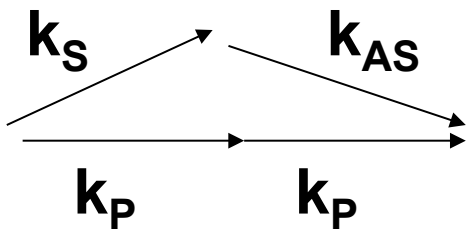
$$P(t) = \chi^{(1)} E(t) + \chi^{(2)} E(t)^2 + \chi^{(3)} E(t)^3 + \ldots$$

Higher order terms becomes important when peak powers are high

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

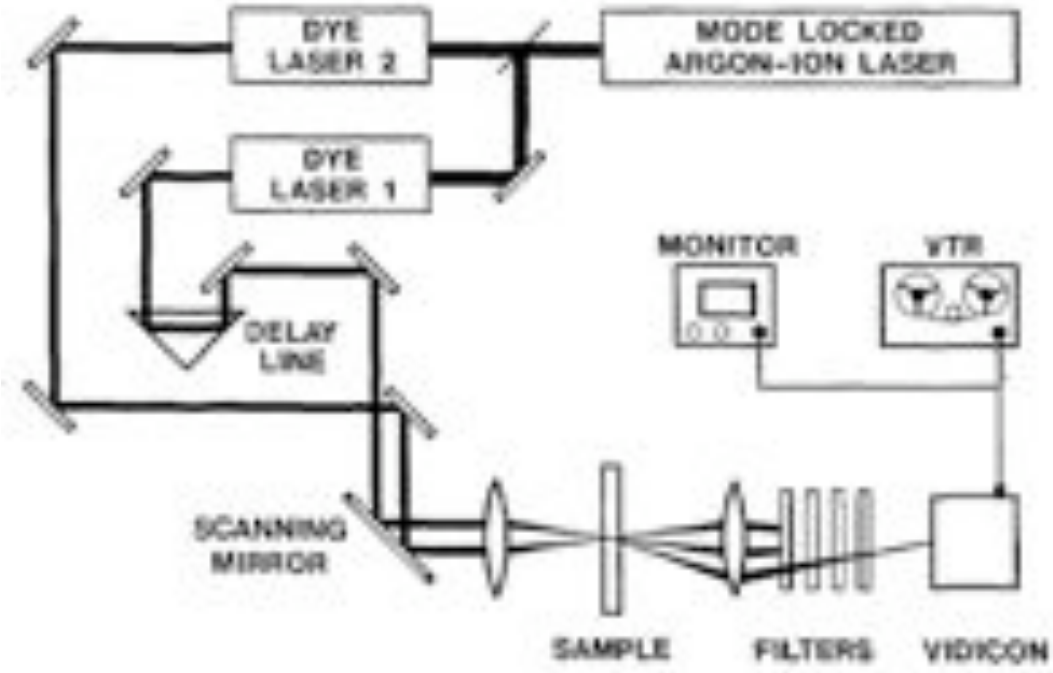
Requires high intensity, pulsed laser sources (ps, fs)

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



Phase matching conditions

First CARS microscope demonstrated in 1982



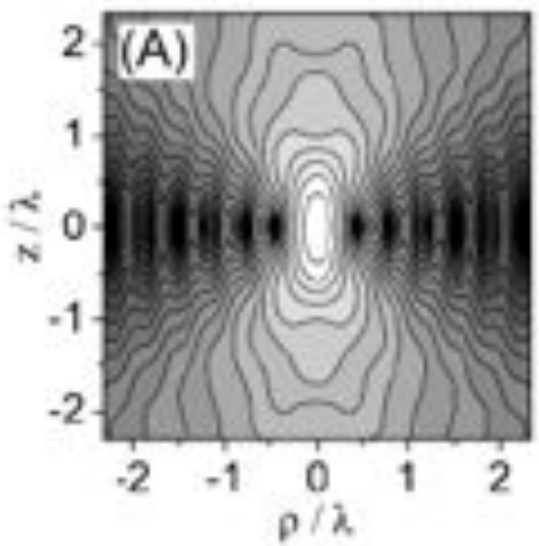
- Drawbacks of this configuration for biological imaging
  - Laser wavelengths at 565-700 nm
  - Phase matching configuration difficult to implement practically

Duncan et. al., Optics Letters, V7, 350-352 (1982)

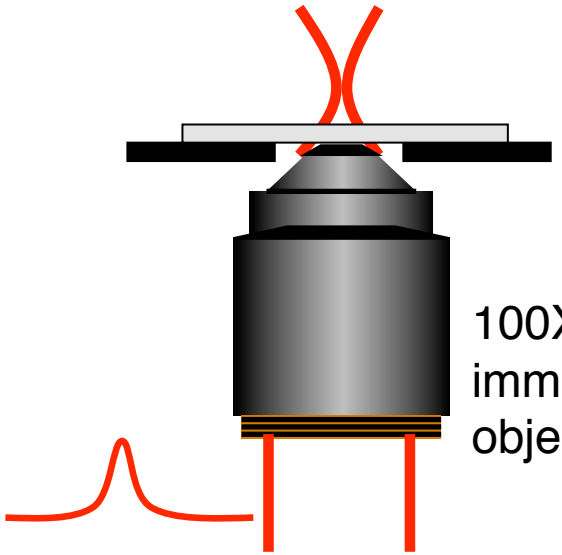
Major improvements developed in 1999 for biological imaging

- 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

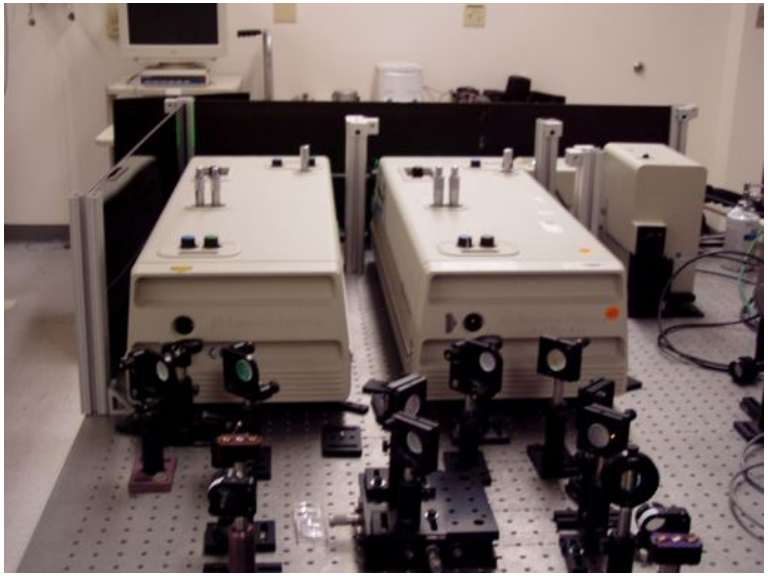


100X, 1.3 NA oil immersion objective

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

Cheng et. al., J. Phys. Chem. B. V108, 827-840 (2004)

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



CW Nd:YVO_4 532 nm pump laser, 10 W

Modelocked Ti:Sapphire laser @ 780-930 nm, 5ps, 80MHz, 600 mW, ω_{pump}

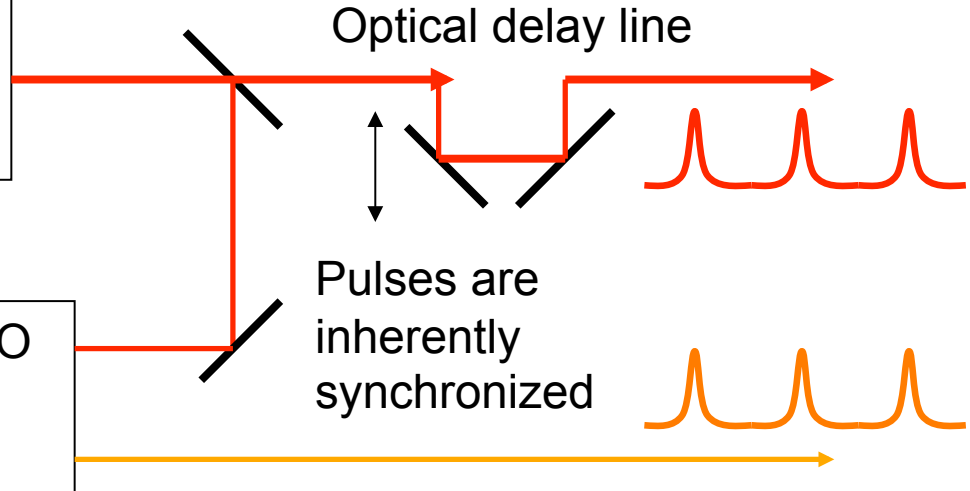
Modelocked Ti:Sapphire laser @ 780-930 nm, 5ps, 80MHz, 600 mW, ω_{Stokes}

Electronic feedback

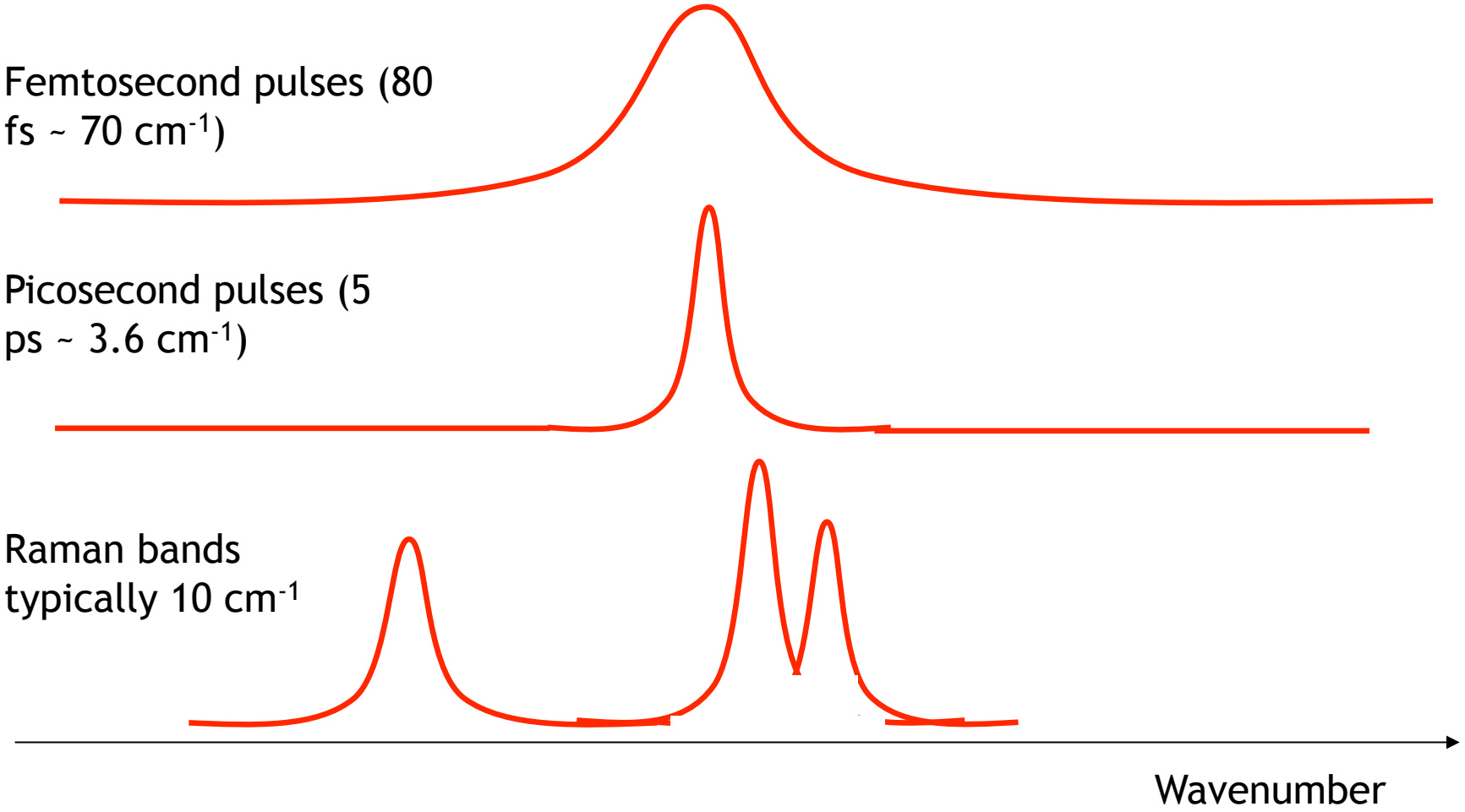
Optical parametric oscillators are another type of system used for CARS microscopy

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}

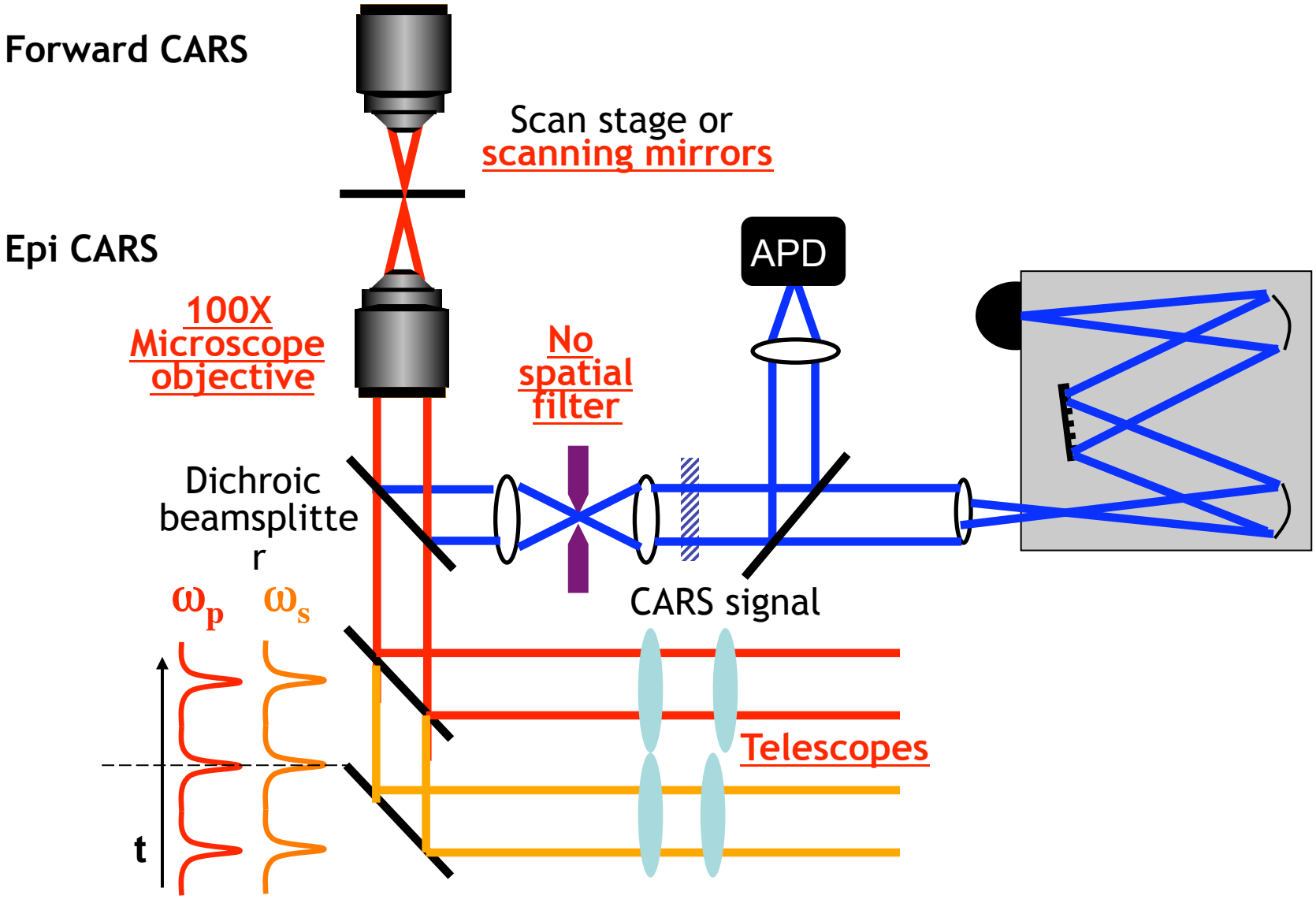


Picosecond or femtosecond pulses, which is better? There are several tradeoffs



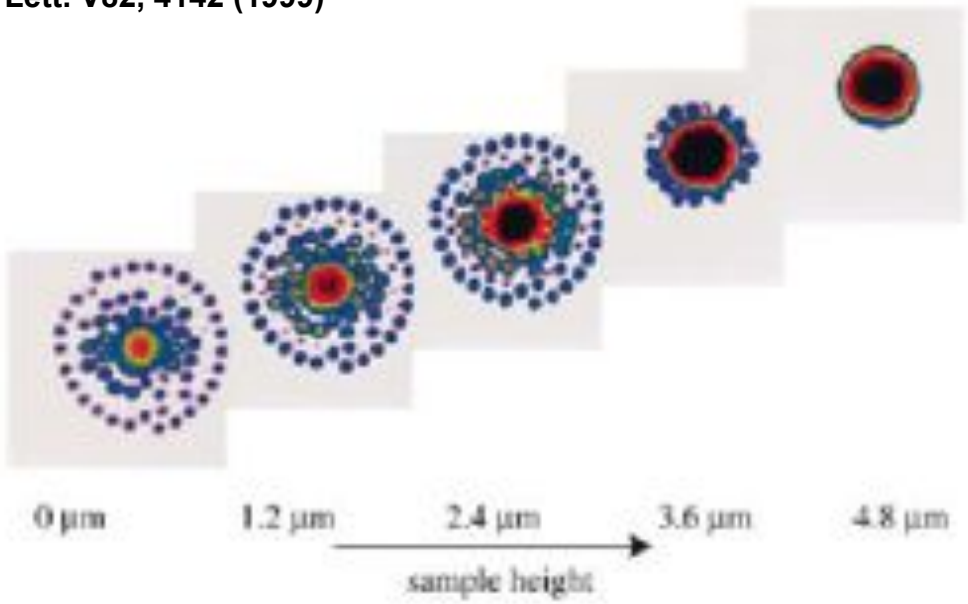
Ps pulses focus all energy to a single Raman band to maximize coherent vibration, at expense of losing peak intensity and multiplex advantage with fs pulses

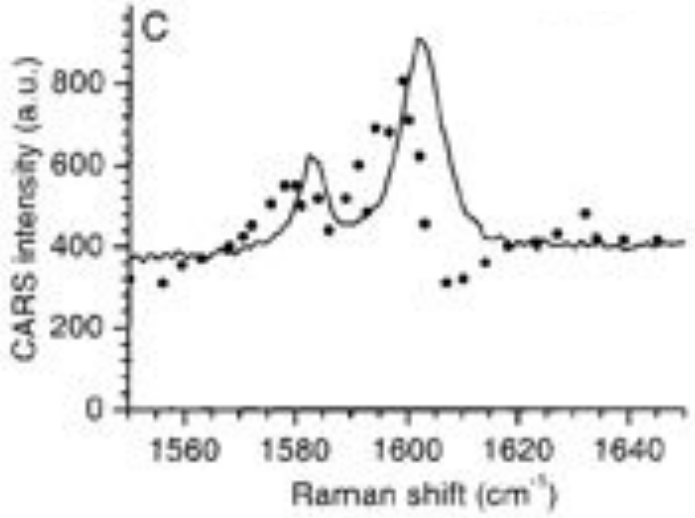
Key components in a CARS microscope setup

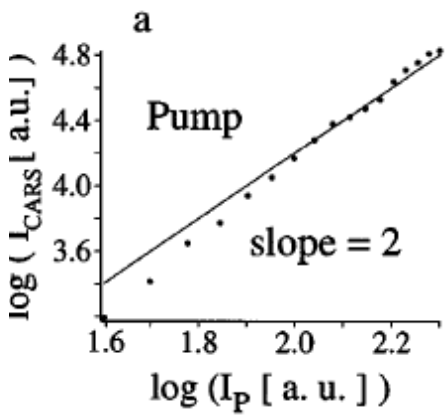


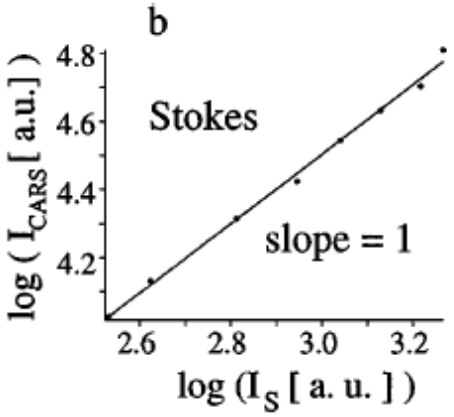
First demonstration on 910 nm polystyrene beads

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

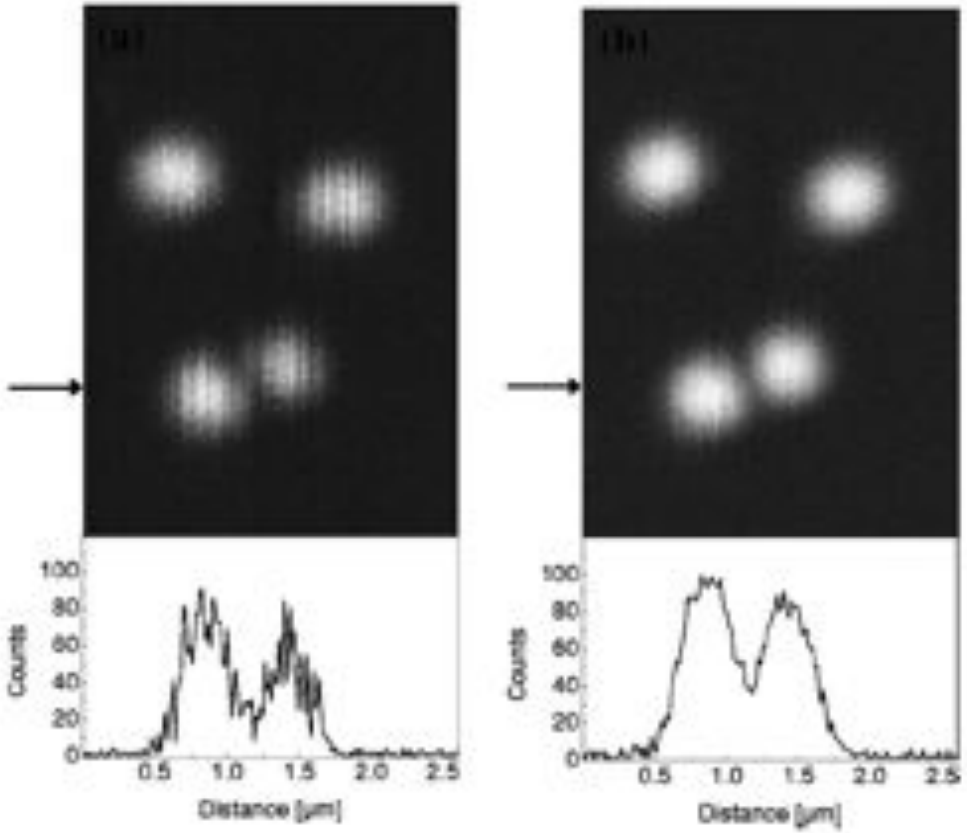








Jitter between two laser trains affects the quality of the CARS image

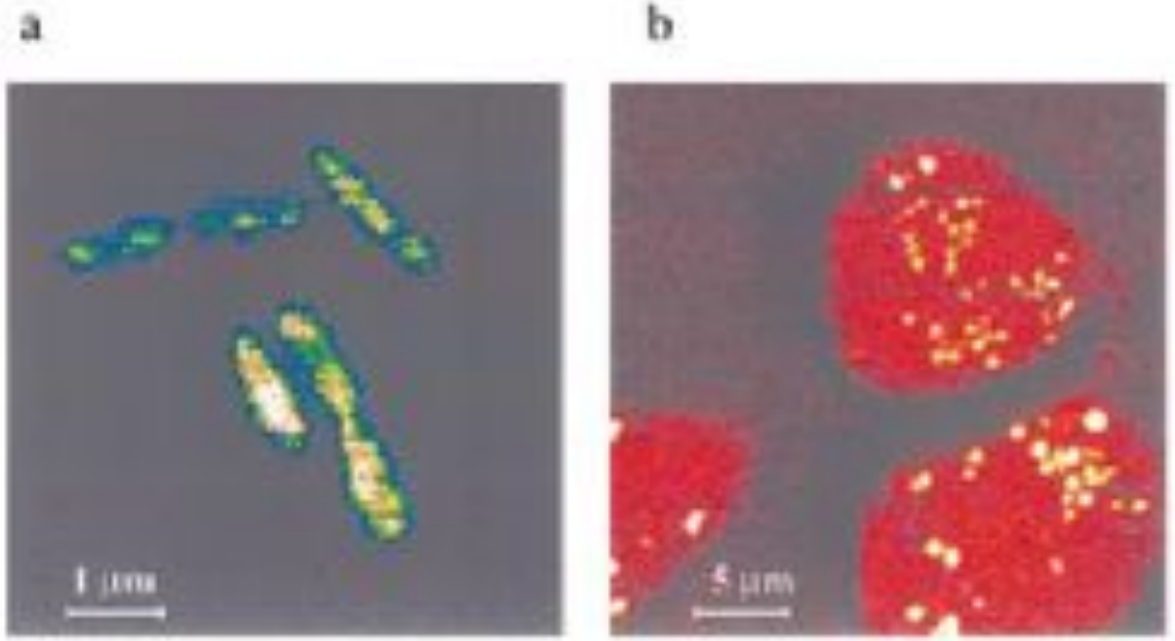


0.5 μm polystyrene beads
0.3 mW, 0.1 mW pump, stokes
22 seconds to acquire image

Jones et. al., Rev. Sci. Instrum. V73, 2843 (2002)

Examples of live cell imaging

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



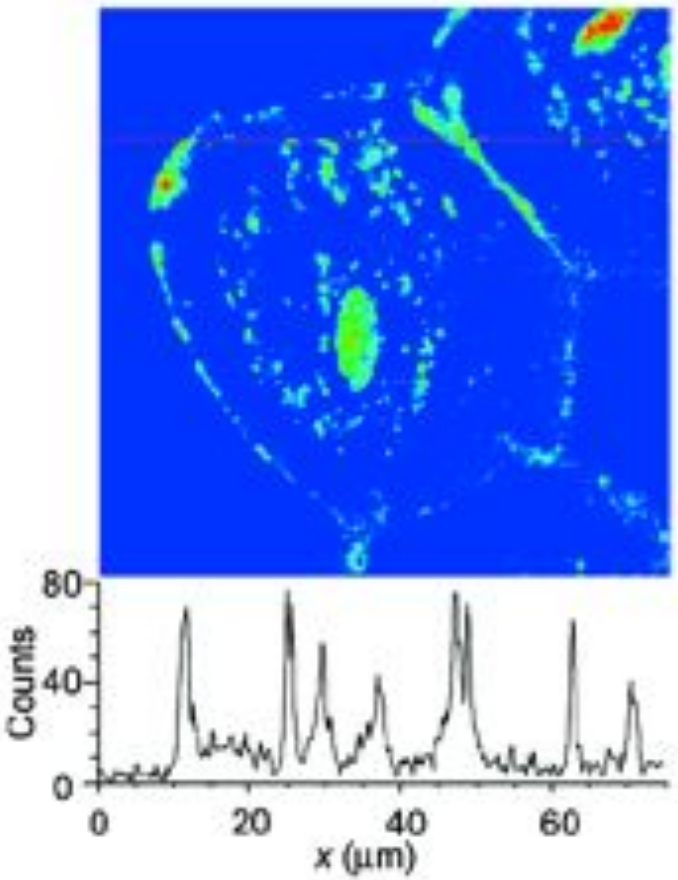
Unstained live bacterial cells. Signal due to cell membranes.

Unstained live HeLa cells. Bright spots due to mitochondria.

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

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

Unstained live human epithelial cell

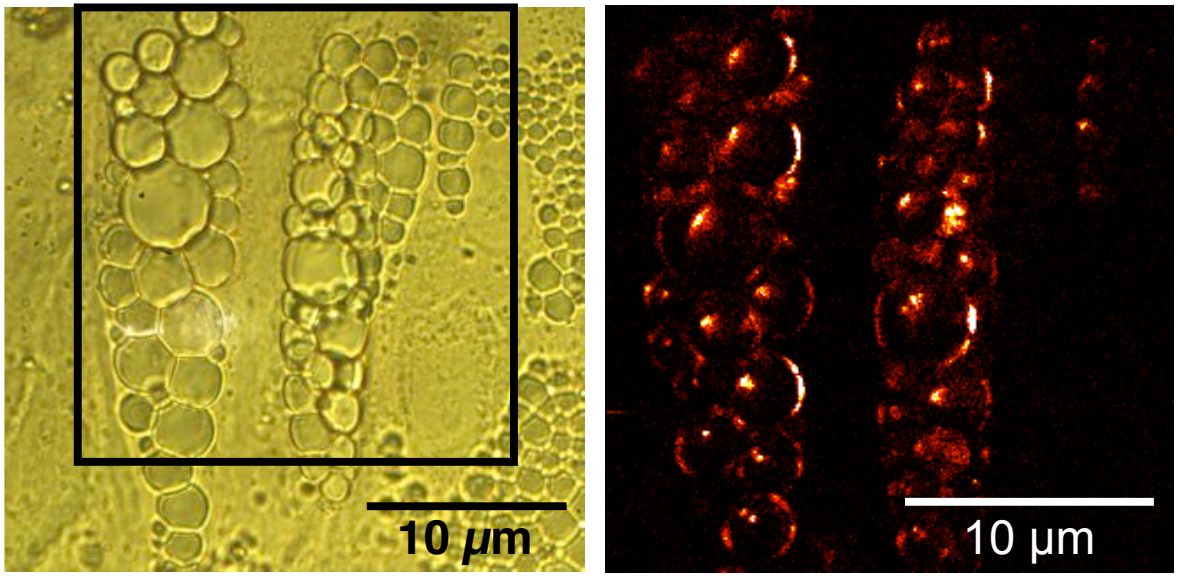


Laser powers - 2 and 1 mW, tuned to 1570 cm^{-1} (protein, nucleic acid) image acquired in 8 min, smallest feature <300 nm

Cheng et. al., J. Phys. Chem. B. V105,1277 (2001)

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

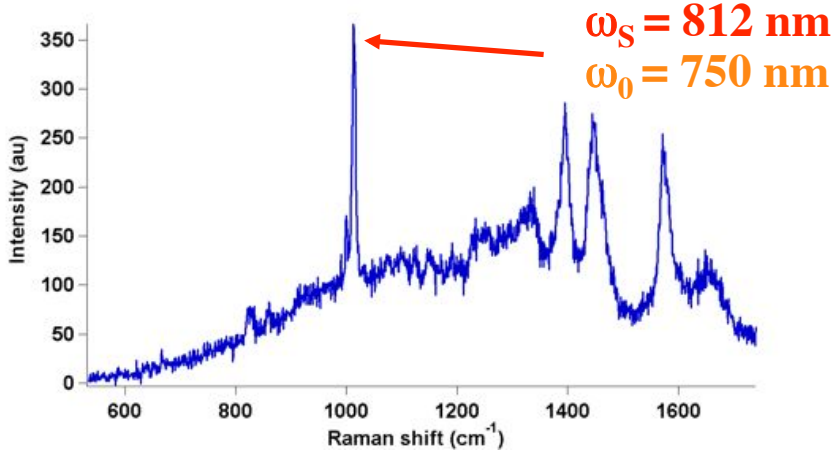
CARS tuned to 2845 cm^{-1} lipid mode



MSC-derived adipocytes (fat cells)

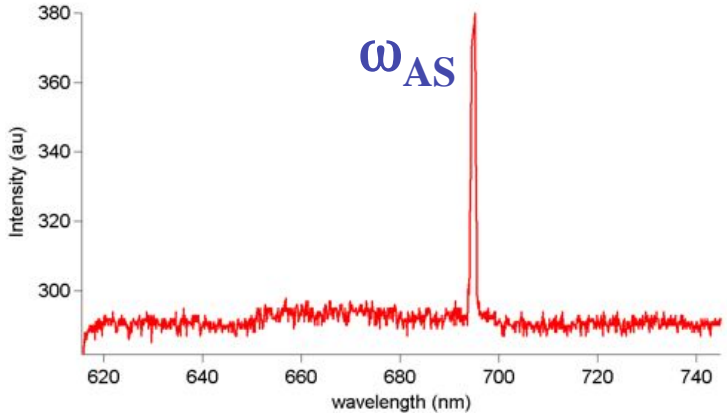
Courtesy: Iwan Schie, Tyler Weeks, Gregory McNerney

Example : CARS imaging of bacterial spores

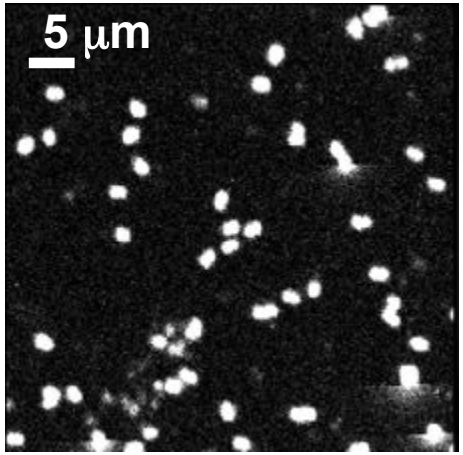


CARS lasers tuned to 1013 cm^{-1} vibration

Raman spectrum of bacterial spore



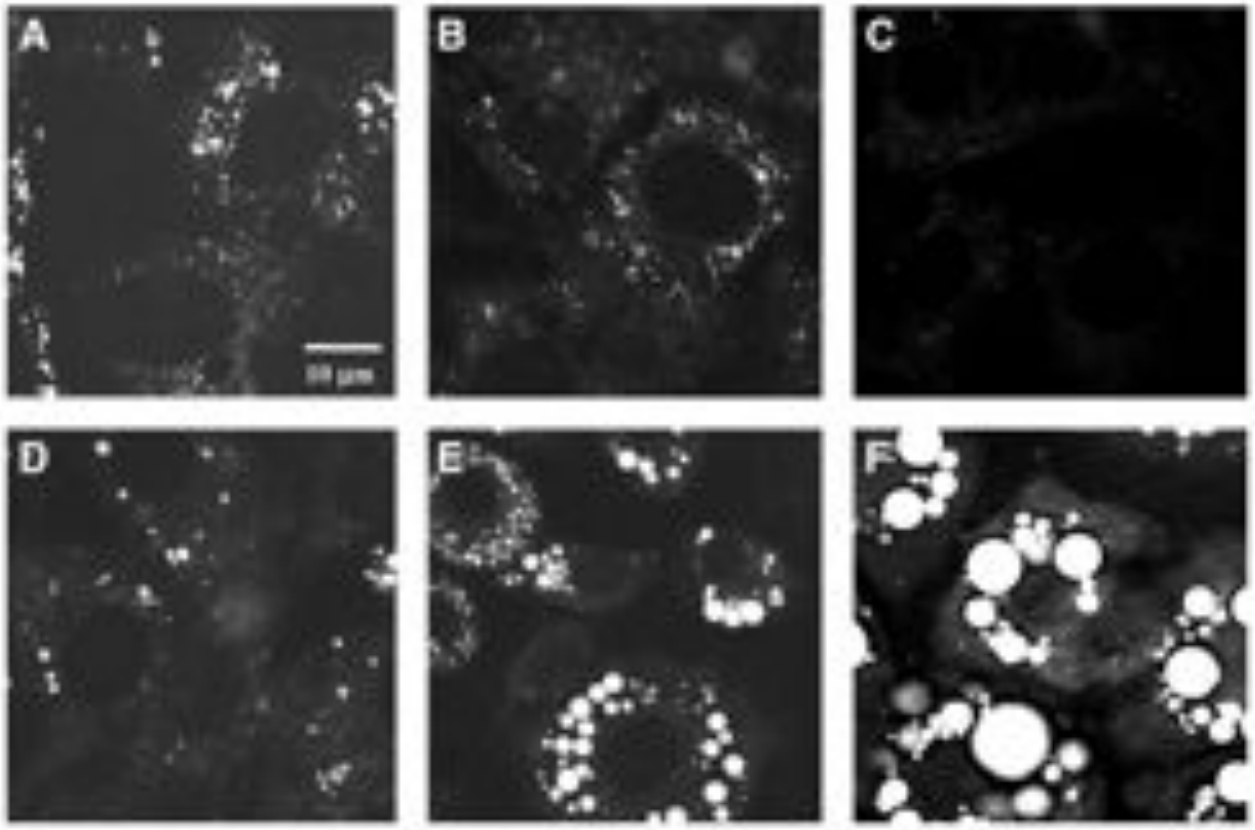
CARS signal at 697 nm



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



0 hr

48 hr

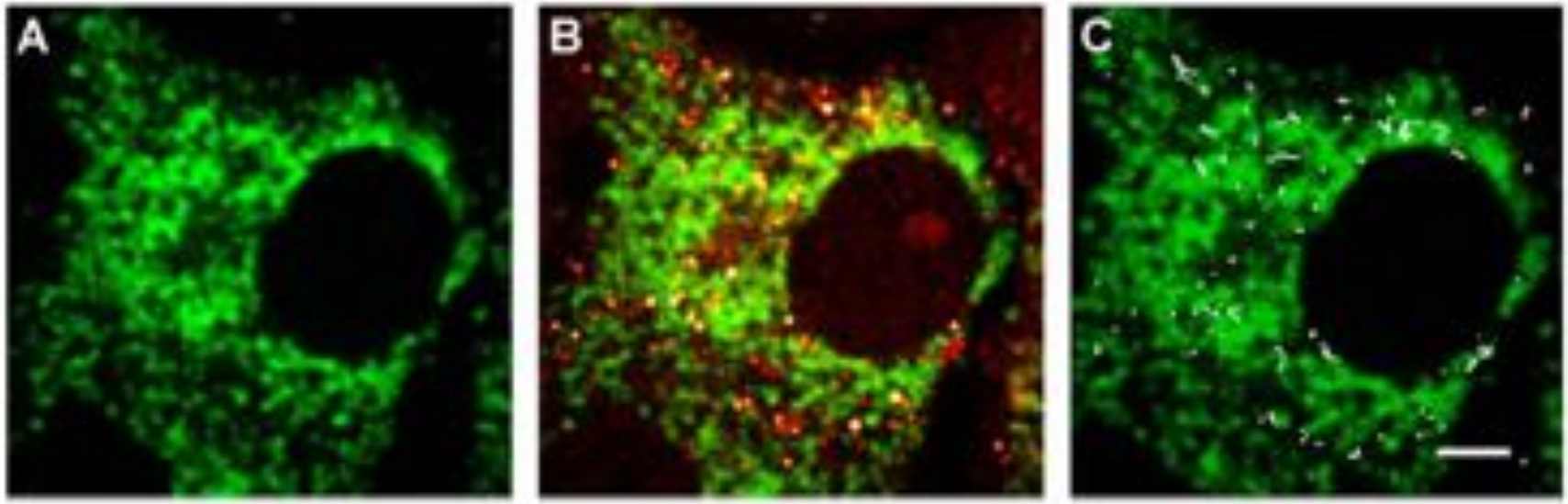
60 hr

192 hr

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

Nan et. al., J. Lipid Res. V44, 2202 (2003)

Tracking trajectories of organelles inside single living cells



Two photon fluorescence - mitochondria

CARS image of lipid droplets overlaid on TPF image

Trajectory of droplets by repeated CARS imaging

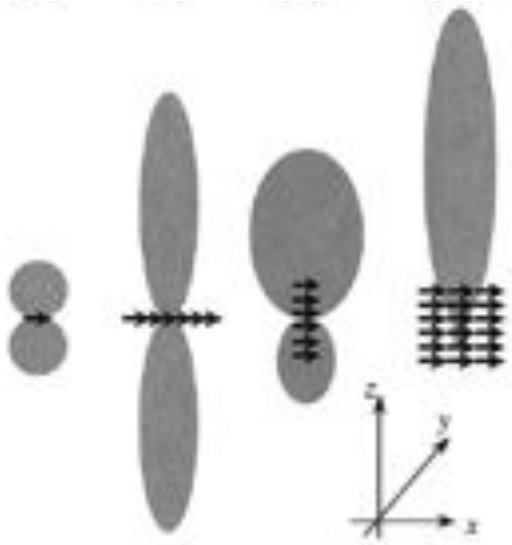
Nan et. al., Biophysical Journal. V91, 728 (2006)

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

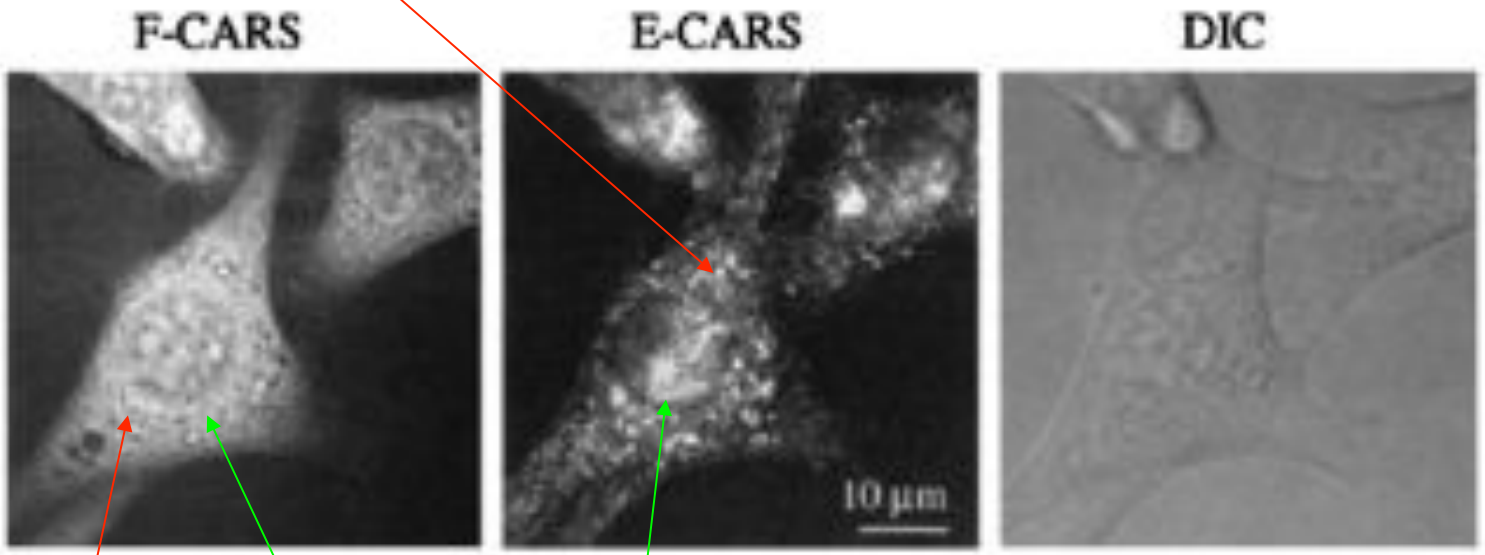
F-CARS detects large scatters, E-CARS detects small scatterers

Volkmer et. al., Phys. Rev. Lett. V87, 0239011 (2001)

Comparison of F-CARS and E-CARS image

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

Small scatterers in cytoplasm visible in E-CARS



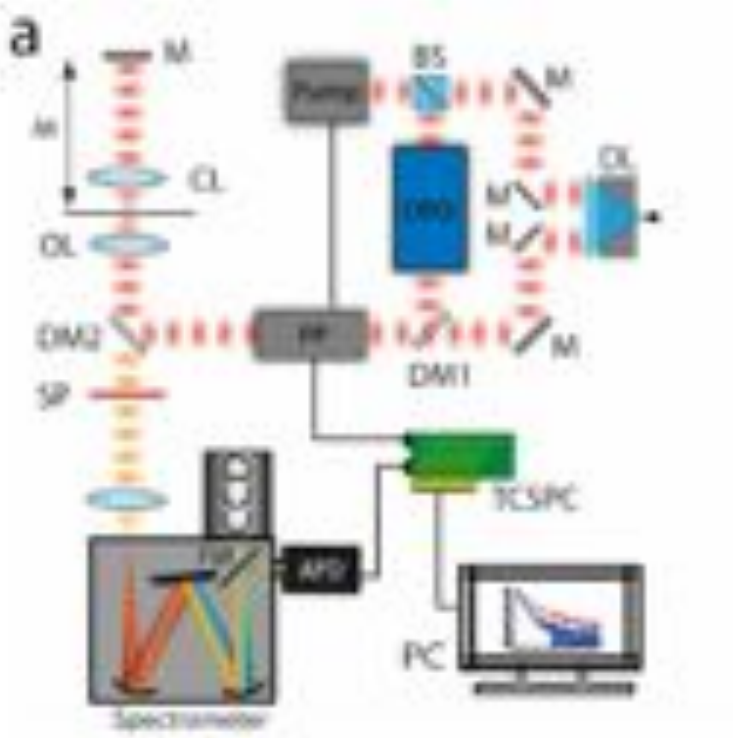
Dark image due to destructive interference in E-CARS

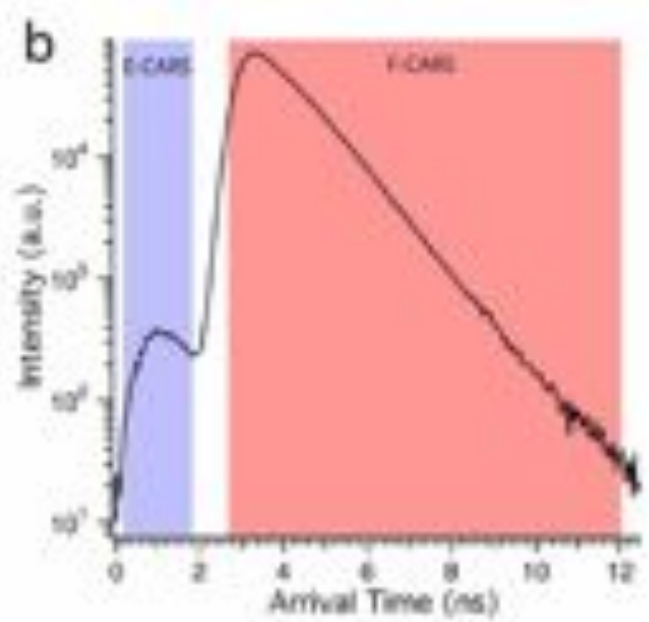
Nuclear membrane edge visible in F-CARS, large axial length

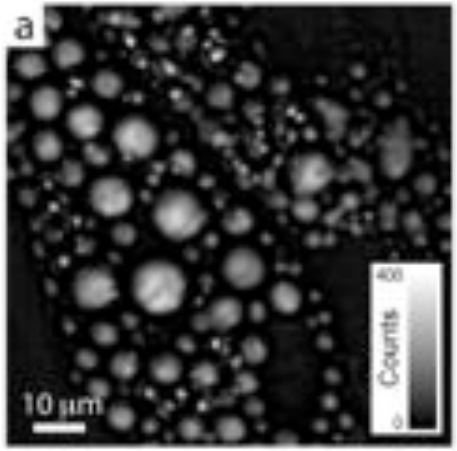
Cytoplasm overwhelmed by solvent signal

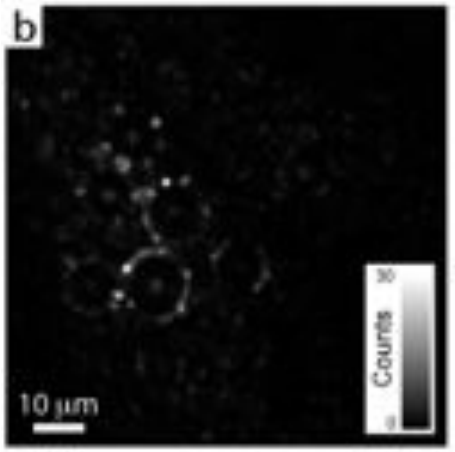
Cheng et. al., Biophys. Journal, V83, 502-509 (2002)

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



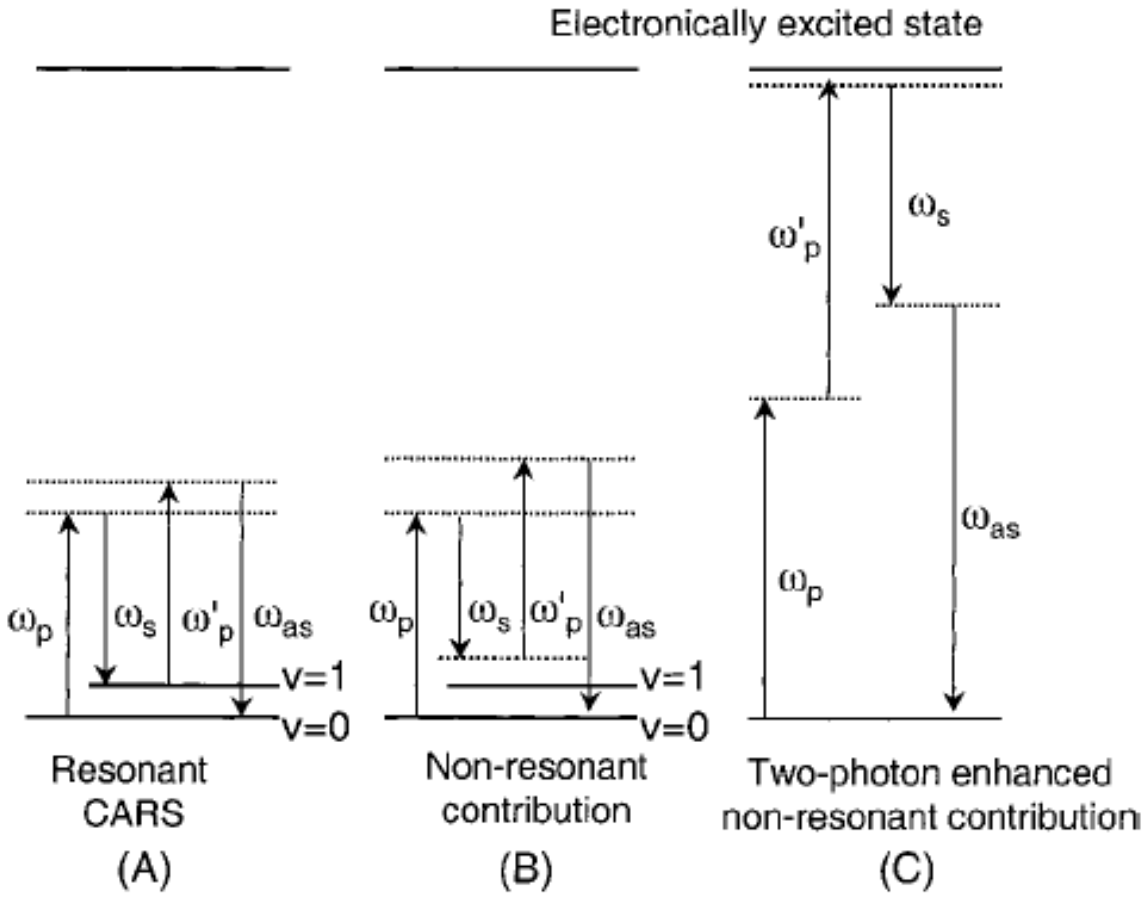






Schie et. al., Optics Express, V16, 2168-2175 (2008)

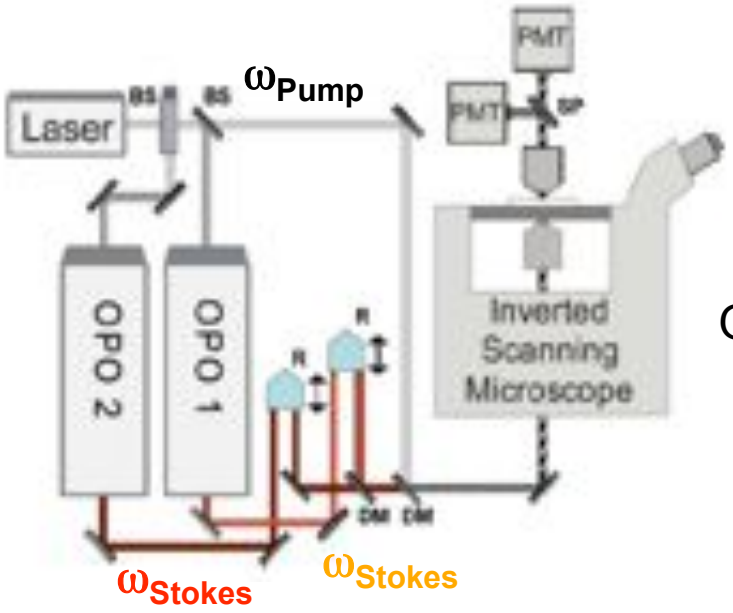
Nonresonant background is a major issue in CARS microscopy



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

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

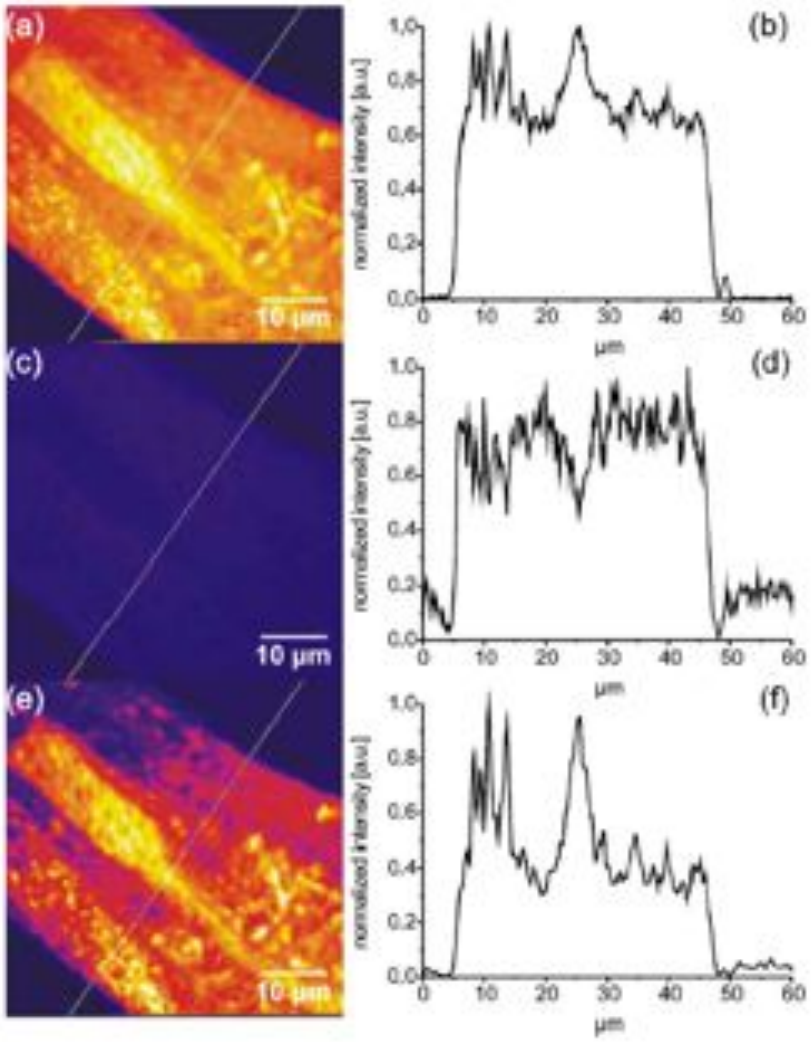
Dual pump CARS microscopy can be used to subtract nonresonant background



On resonance

Off resonance

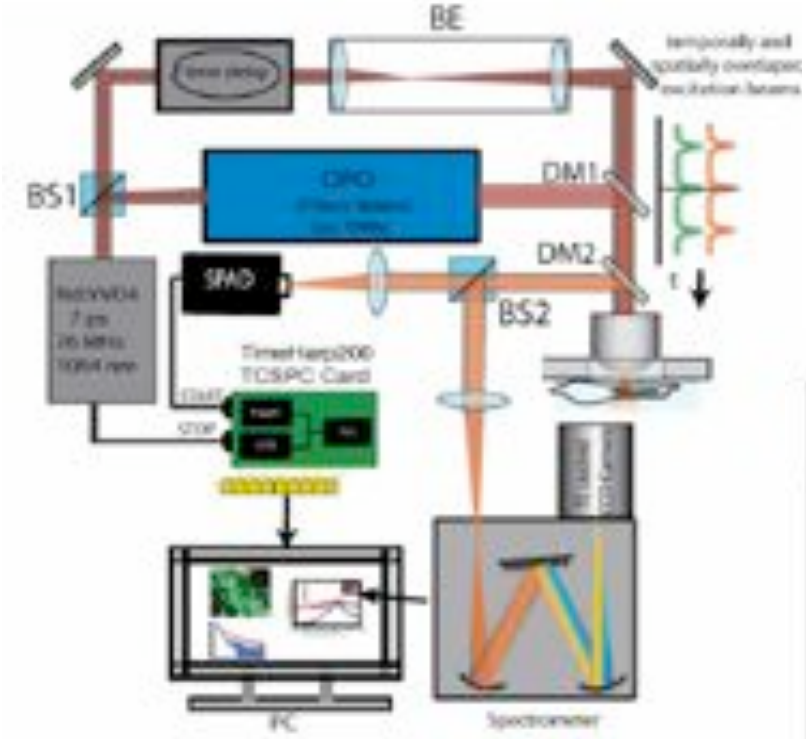
Difference

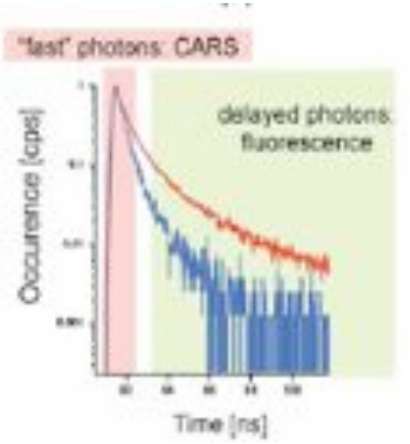


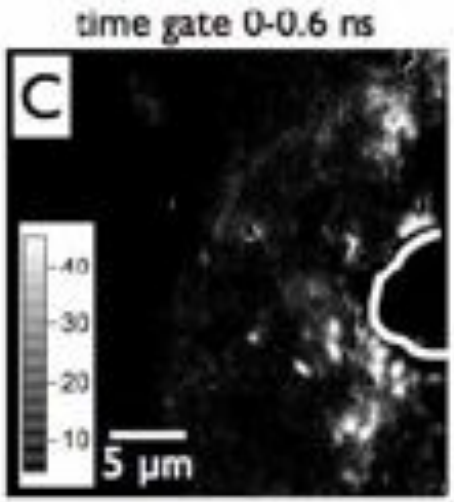
Burkacky et. al., Optics Letters, V31, 3656 (2006)

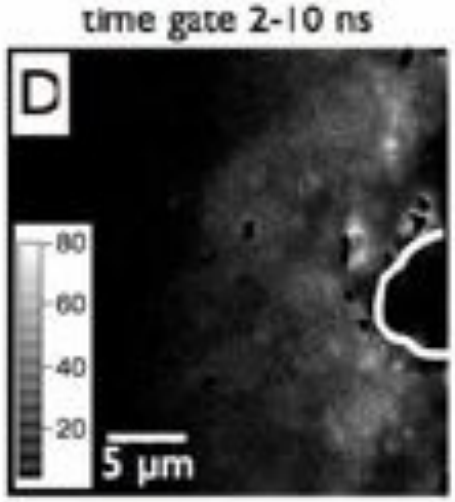
Autofluorescence (2-photon) from the sample may overwhelm the CARS signal

- Raman lifetimes ~ ps
- Fluorescence lifetimes ~ ns

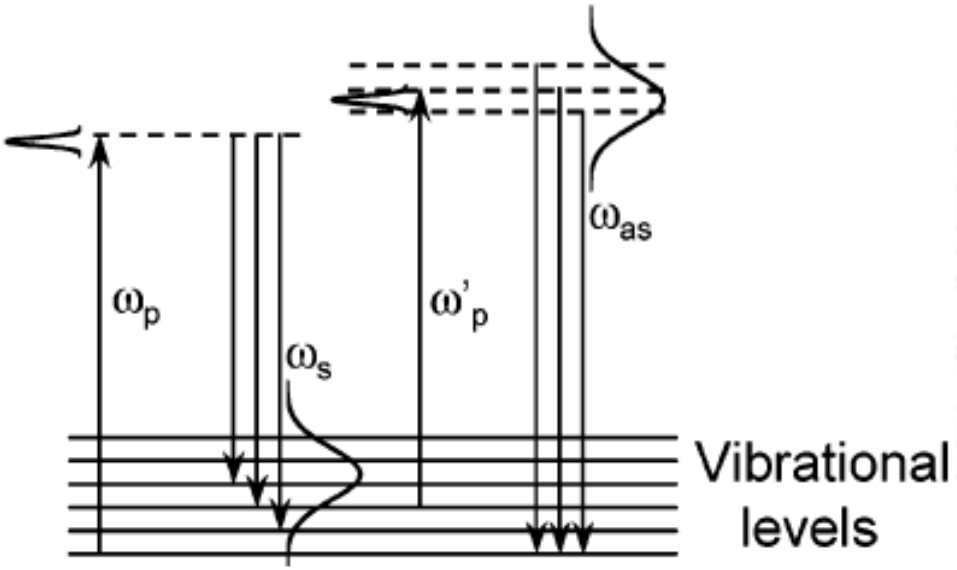




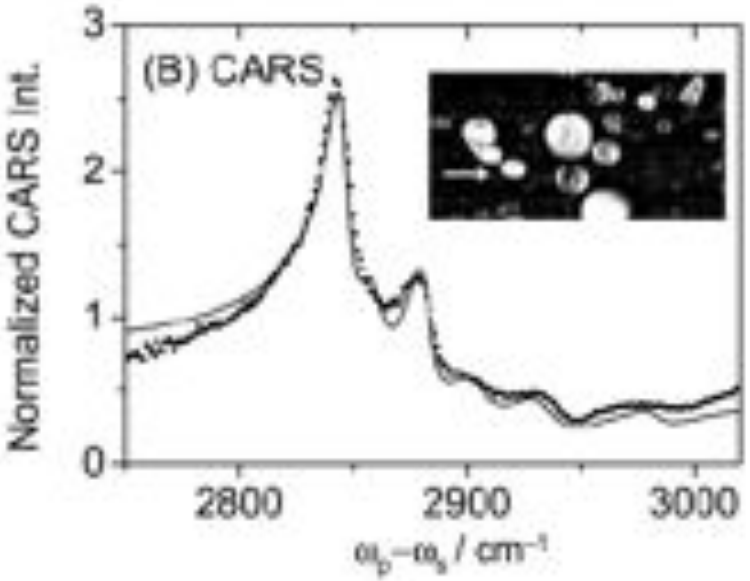




Multiplexed CARS (M-CARS) has been developed for CARS spectroscopy



Population of multiple levels simultaneously (ps-fs combination)



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

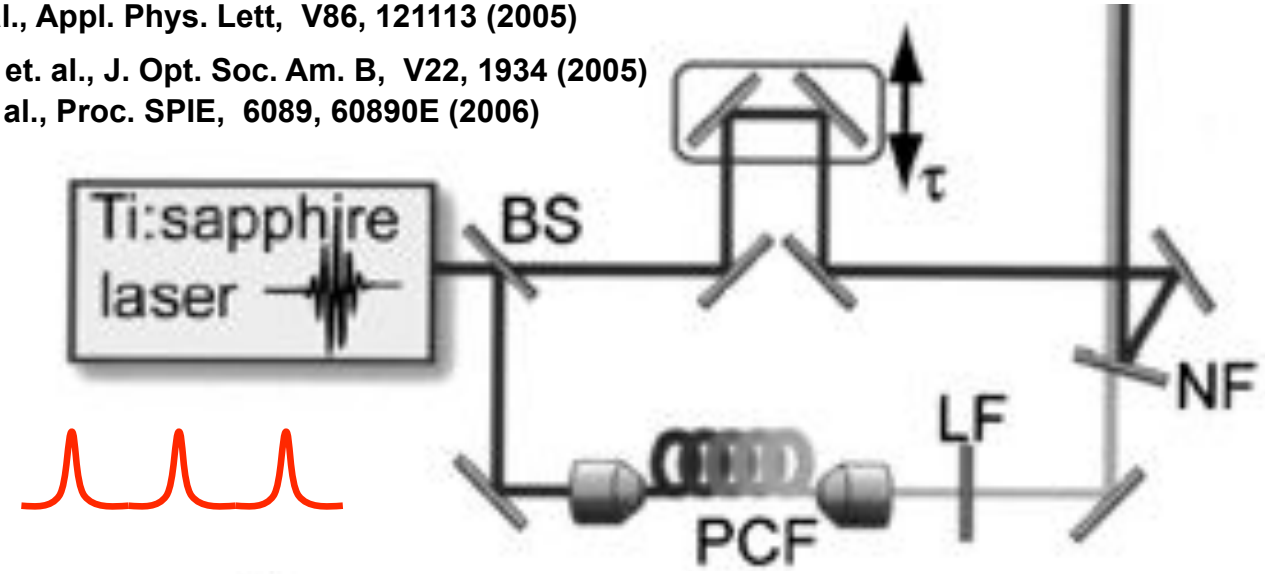
Cheng et. al., J. Phys. Chem. B, V106, 8493-8498 (2002)

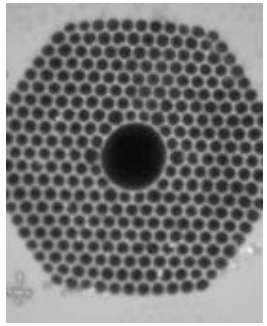
Supercontinuum generation in a photonic crystal fiber can function as a broad source for M-CARS

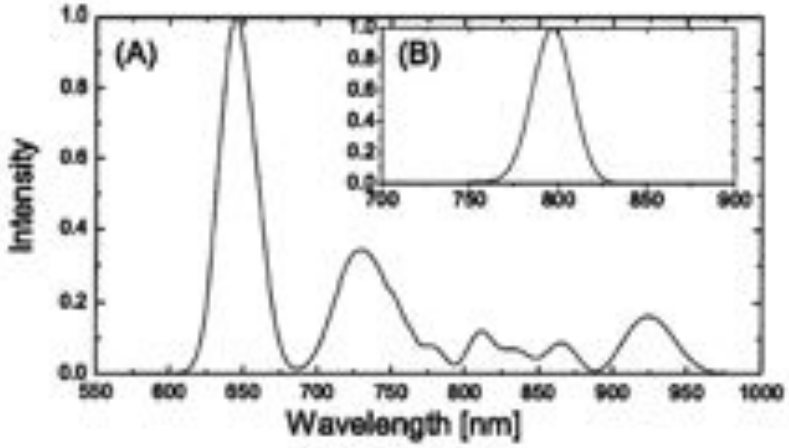
Kano et. al., Appl. Phys. Lett, V86, 121113 (2005)

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

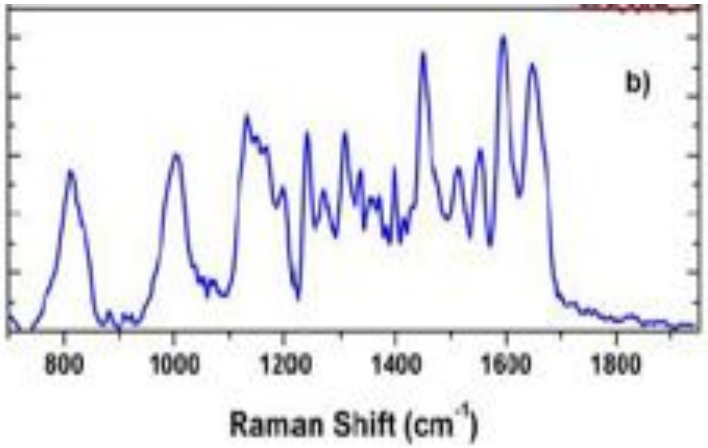
Petrov et. al., Proc. SPIE, 6089, 60890E (2006)







Supercontinuum generation

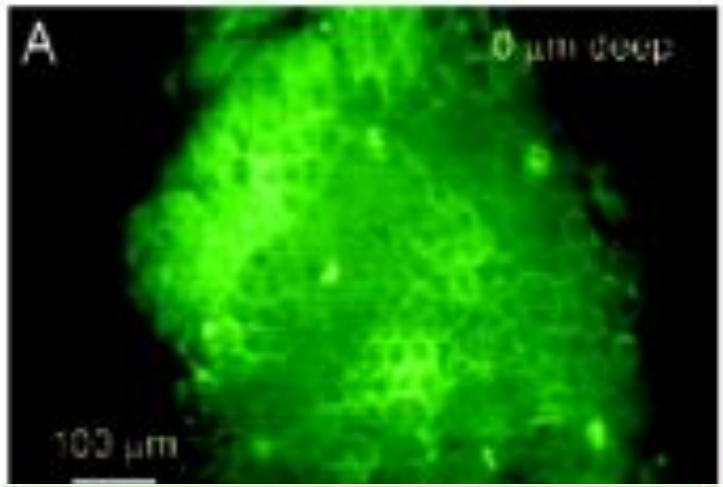


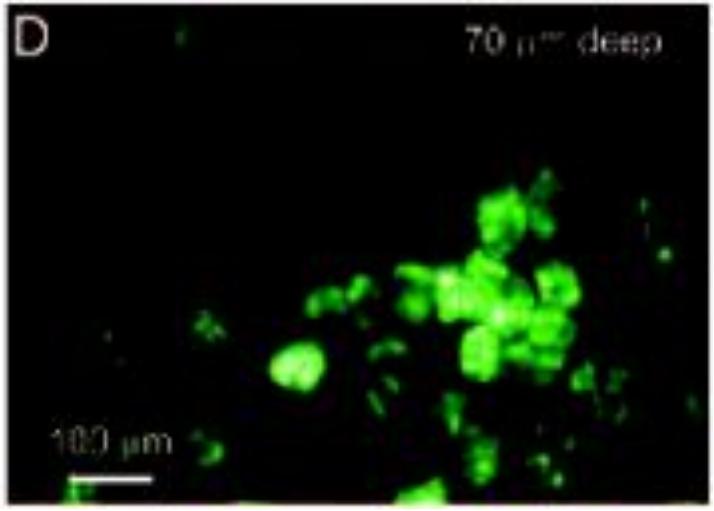
CARS spectrum of bacterial spore – 1 second acq. time

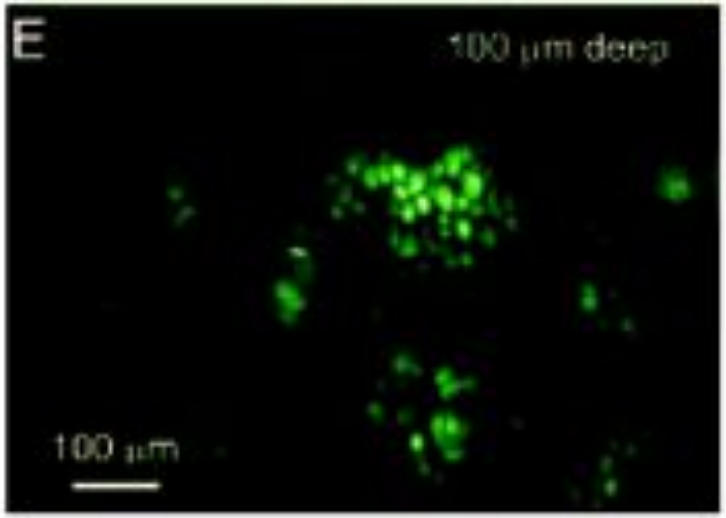
Application : CARS *in-vivo* imaging

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

Stratum corneum







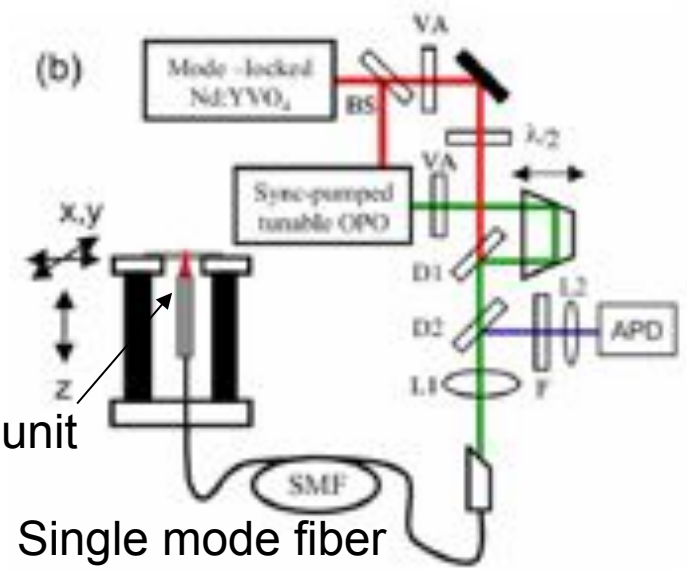
Adipocytes of the dermis

Adipocytes of subcutaneous layer

Evans et. al., PNAS, V102 16807 (2005)

Applications : Fiber-based CARS endoscopy

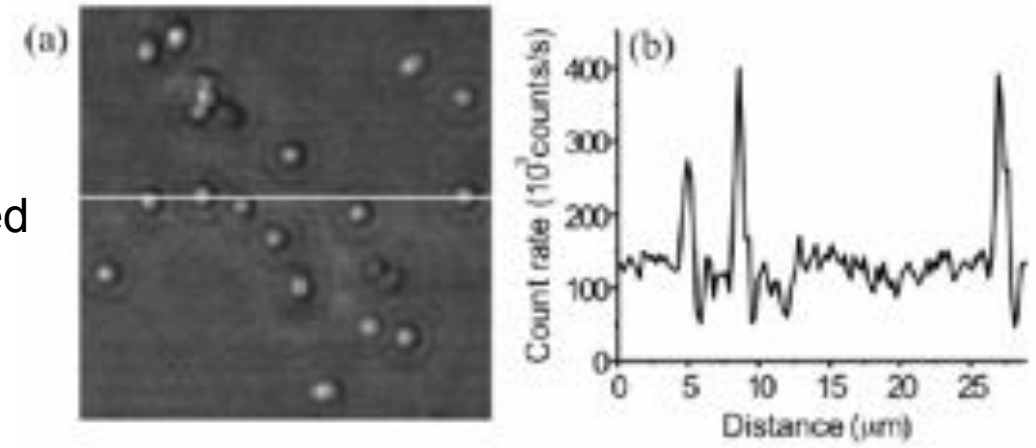
Fiber delivery of two ultrashort laser pulses



Focusing unit

Single mode fiber

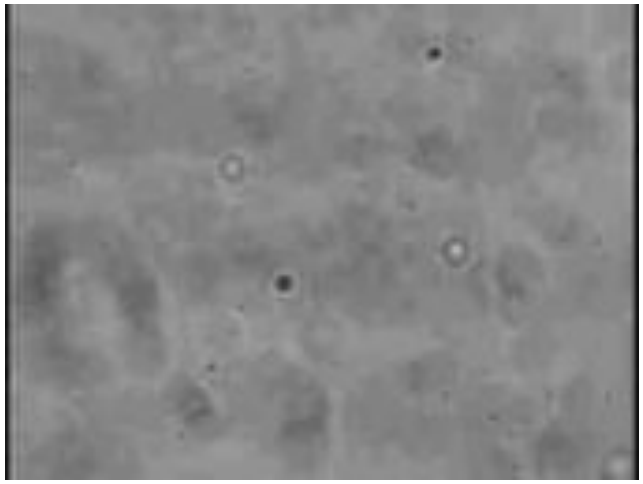
750 nm beads imaged in epi-direction



Legare et. al., Opt. Express, V14 4427 (2006)

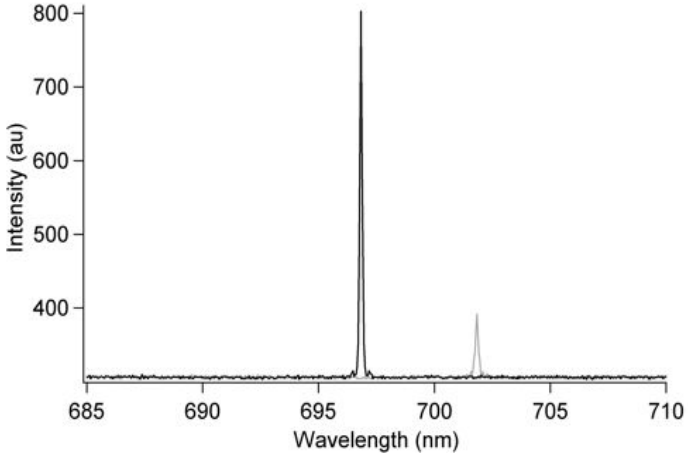
Application : CARS cytometry for rapid, label-less cancer cell detection and sorting

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

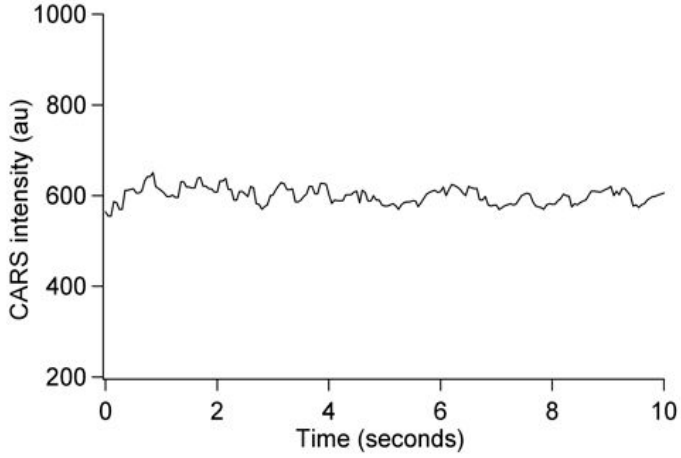


Trapped polystyrene bead using two CARS beams

Potential solution for faster chemical analysis of cells



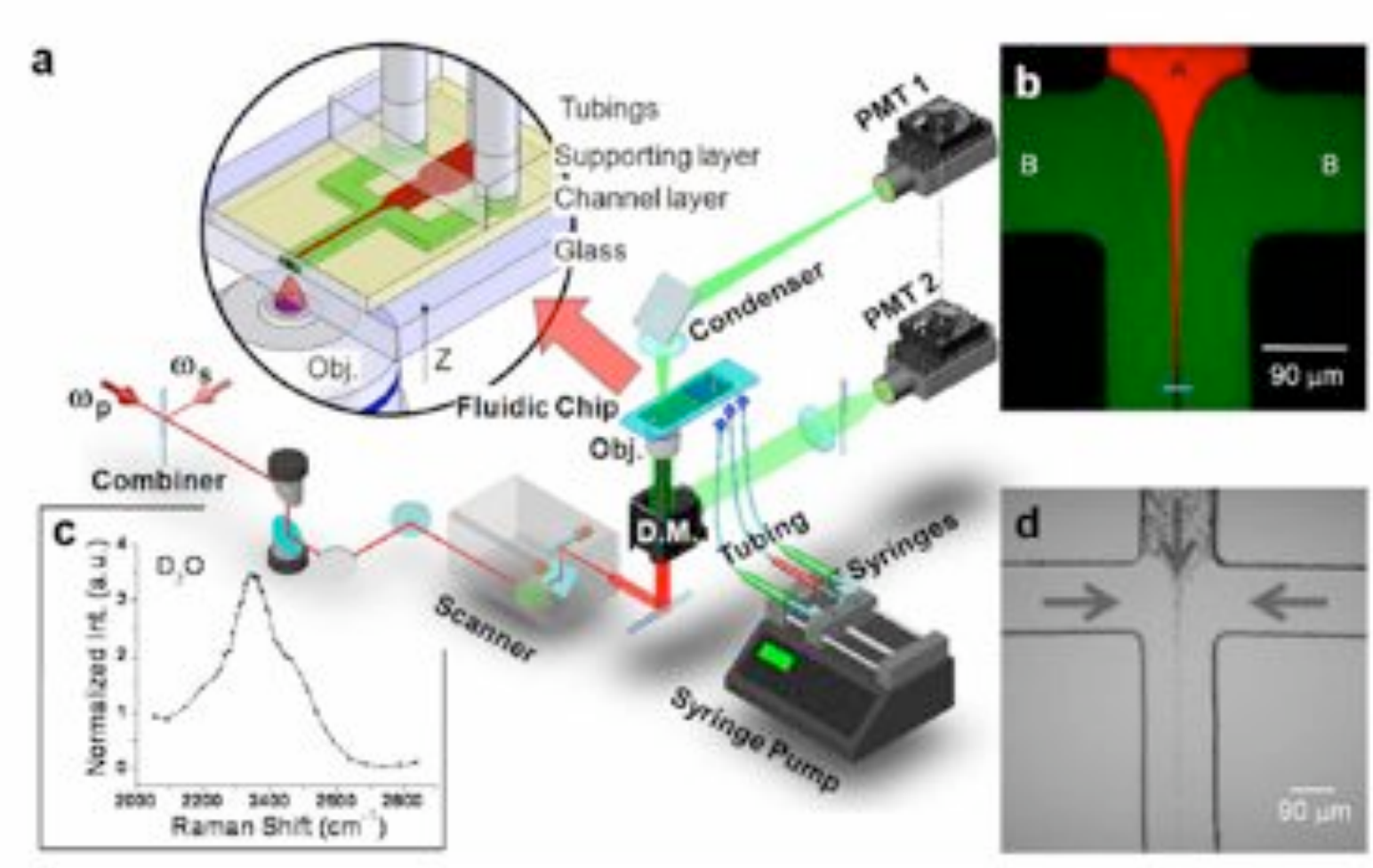
CARS signal from a C=C bond



Microsecond temporal resolution

Chan et. al., IEEE J. Sel. Topics. Quant. Elec. V11 858 (2005)

Application: Microfluidic CARS cytometry



Wang et. al., Optics Express V16 5782 (2008)

Summary

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

Thank you!

chan19@llnl.gov