

# Seeking the sensitivity limit

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CARS workshop 2005

# Better, faster, brighter

## Improving CARS microscopy

- light source
- background suppression
- phase properties
- higher resolution
- faster acquisition
- outlook



# Current status

Spectral bands:	CH, CD, OH, aromatic ring, phosphate, Amide I etc.
Spatial resolution:	0.27 $\mu\text{m}$ lateral, 0.7 $\mu\text{m}$ axial (0.05 $\mu\text{m}$ lateral in near-field)
Scanning speed:	20 frames/sec (E-CARS)
Powers at sample:	1-50 mW for $\sim 5$ ps pulses
Det. Sensitivity:	$10^6$ $\text{CH}_2$ modes in focus (far field)
Limited by:	nonresonant background, signal strength

# Some numbers

## Detection sensitivity

### Lipid bilayer:

E-CARS:

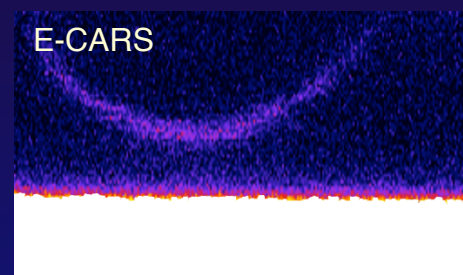
$4.4 \times 10^6$  CH<sub>2</sub> modes in focus

$\sim 8 \times 10^3$  CARS photons/second  
(25 mW pump @ 76 MHz)

$1 \times 10^{-4}$  photons per shot (detected)

$1 \times 10^{-13}$  photon conversion efficiency

### Giant unilamellar vesicle



### Lysed red blood cell

F-CARS:

$10 \times 10^6$  CH<sub>2</sub> modes in focus

$\sim 1 \times 10^7$  CARS photons/second

(25 mW pump @ 76 MHz)

$1.3 \times 10^{-1}$  photons per shot (detected)

$1.3 \times 10^{-10}$  photon conversion efficiency



# We want more photons!



*High sensitivity CARS microscopy and strategies to improve it*

# Optimum light source?

Two synchronized Ti:sapphires

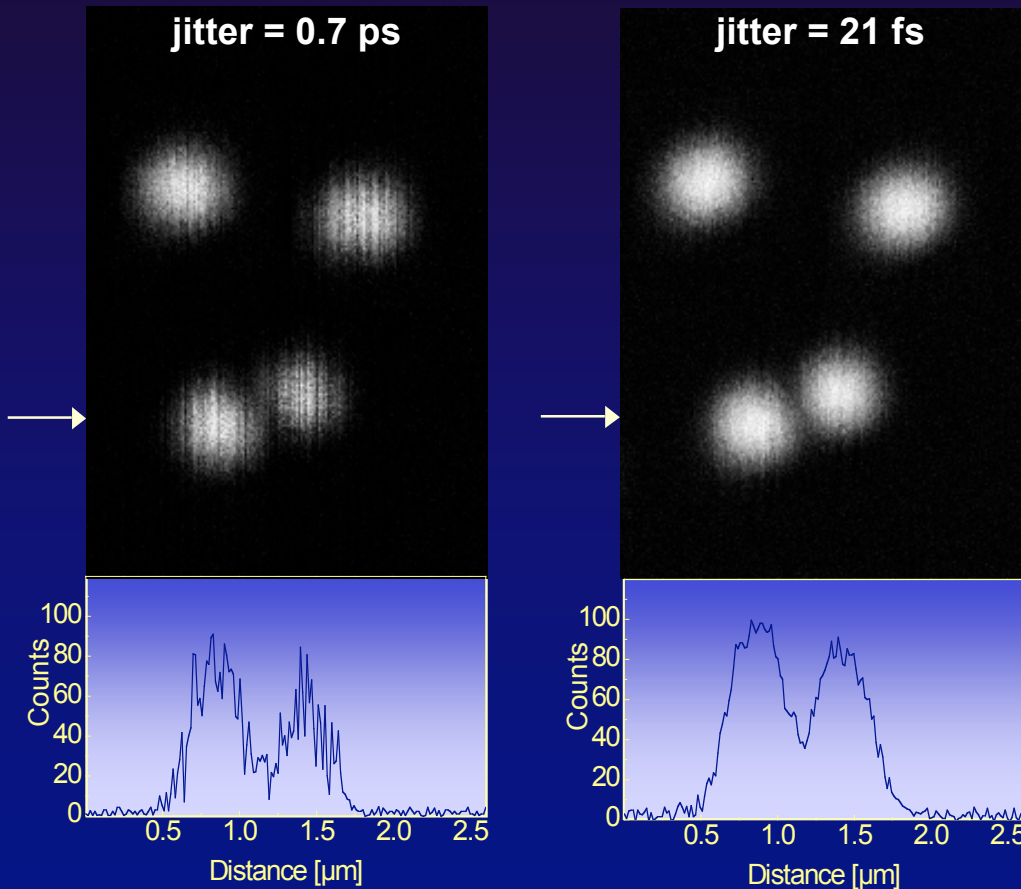
Synchronously pumped OPO

Amplifier systems

Fiber-broadened systems



# Two lasers: Pulse jitter



When using two independent lasers, active synchronization is required

Coherent Synchrolock: 0.25 ps jitter

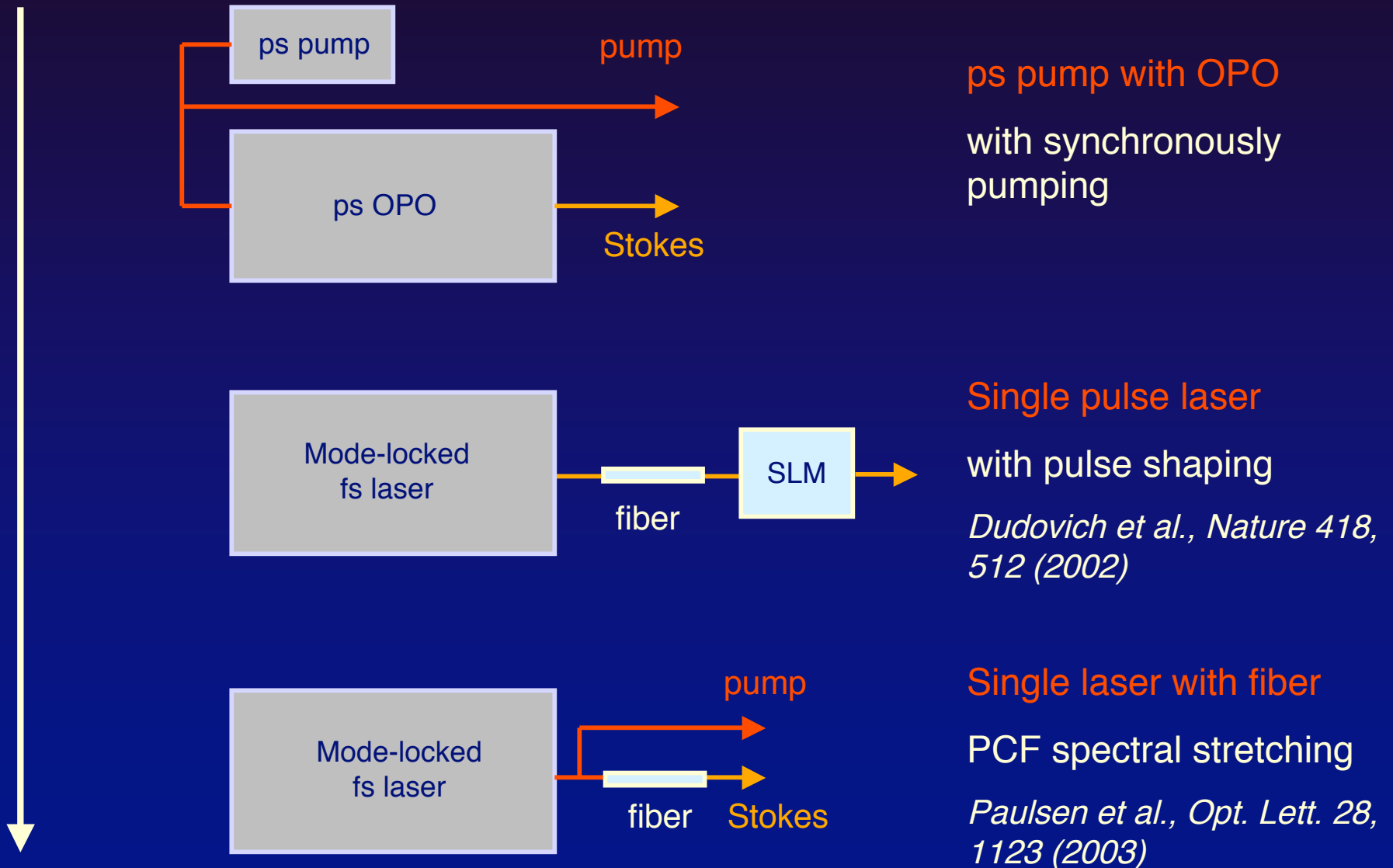
Spectra-Physics Lock-To-Clock: 0.5 ps jitter

*No jitter for synchronously pumped systems!*

*Potma et al., Opt. Lett. 27, 1168 (2002)*

# Cheaper sources?

Price

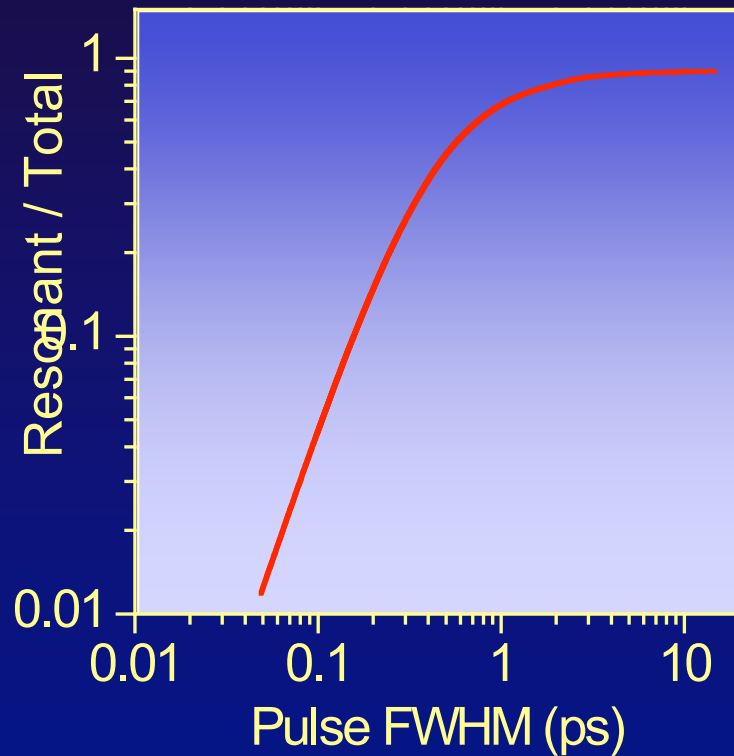


# Light sources and performance

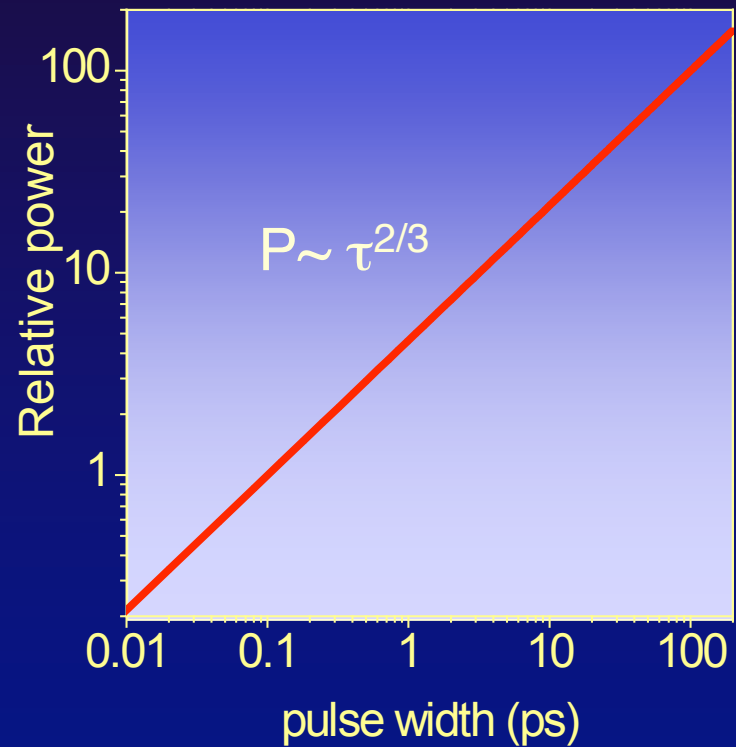
Light source	Signal/background	jitter	tuneability	Scan speed	price
Sync. Locked ps-Ti:sapphires	high	0.5 ps	easy	fast	high
Synchronously pumped ps-OPO	high	none	laborious	fasts	medium
Mode-locked laser with PCF fiber	low	none	limited	fast	low
Regen. amplifier systems with OPA	low	none	easy	slow	high
Super broadband with phase control	medium	none	limited	medium	medium

# Pulse width and power

Signal to background



Excitation power relative to signal at 100 fs



# Controllable parameters

Total collected CARS signal is function of:  
*dwelt time, pulse repetition rate, pulse width*  
*and time averaged excitation power*

$$S_{CARS} \propto \frac{t_{scan}}{(f\tau_{pulse})^2} \langle P(t) \rangle^3$$

Practical limitation:

*photo damage*

# Photo damage

## Two mechanisms for photo damage:

- **linear absorption**: linear heating

*100 mW of 850 nm through 1.2W NA lens gives rise to heating of 0.2 K in aqueous medium (Schole & Hell, Opt. Lett. 23, 325 (1998))*

- **nonlinear absorption**: heating and photo-chemically induced changes

*Multiple (nonlinear) mechanisms, molecule and sample specific*

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According to Hopt & Neher (*Biophys. J.* 80, 2029 (2002))

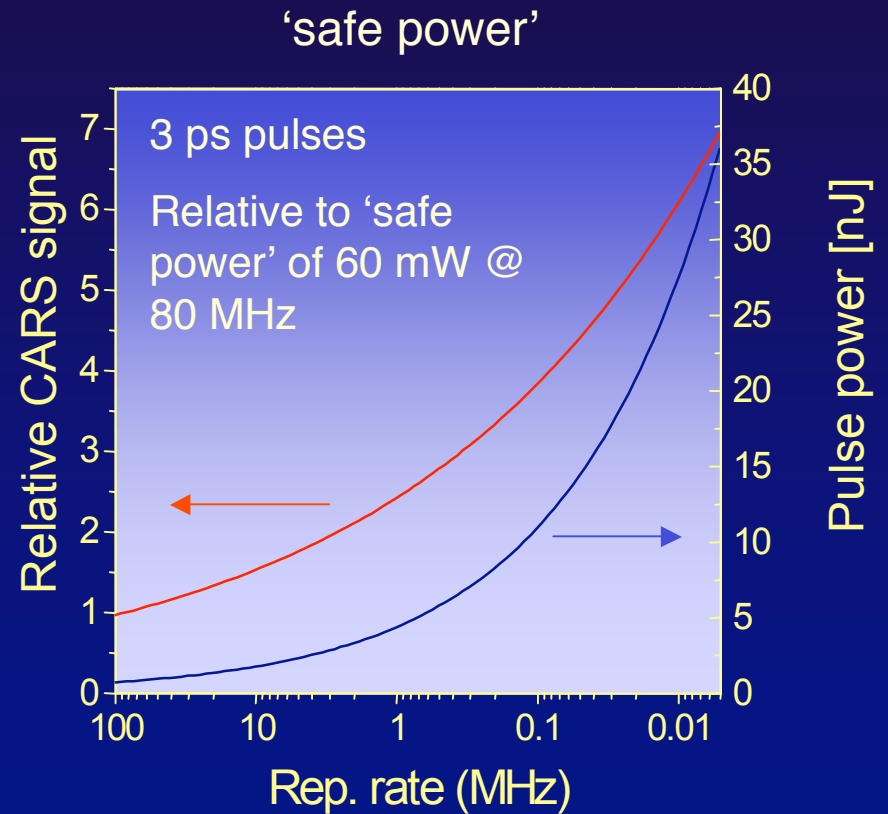
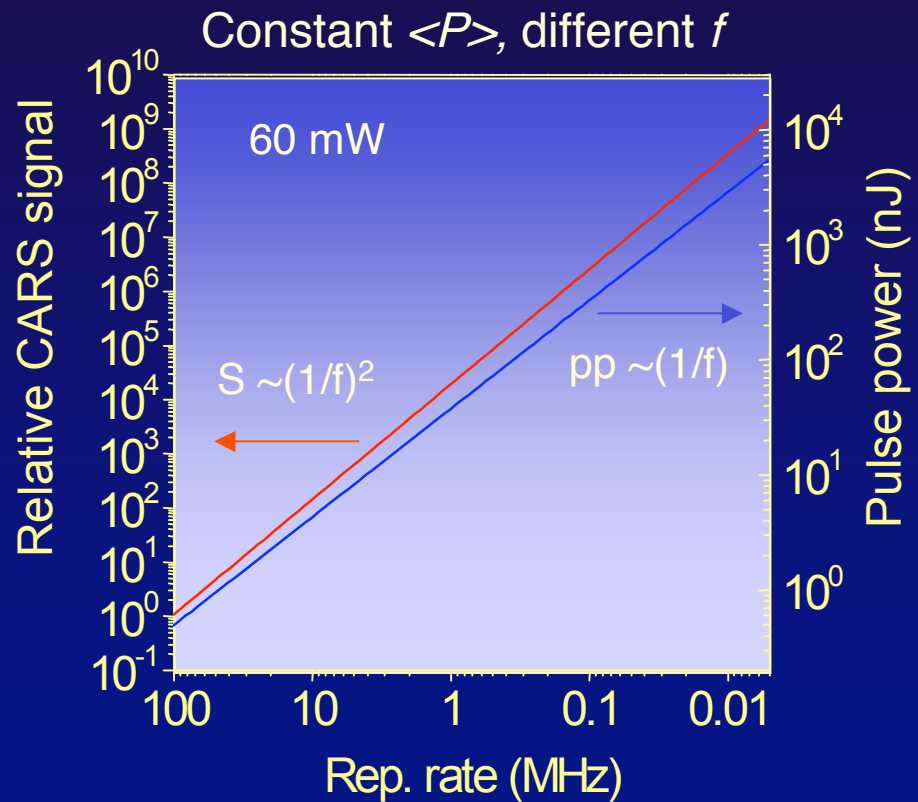
$$\#scans \propto \frac{(f\tau_{pulse})^{1.5}}{t_{scan} \langle P(t) \rangle^{2.5}}$$

*in bovine adrenal chromaffin cell culture:*

# Repetition rate & pulse power

advantage can be taken from lowering rep rate

*if nonlinearity of photodamage < CARS nonlinearity (n=3)*



# Amplified pulses

*Typical pulse power straight from Ti:sapphire is < 12.5 nJ/pulse*

## Use for amplified pulses in biological specimens?

- two-photon microscopy tissue imaging

*(Theer et al., Opt. Lett. 28, 1022 (2003); 200 kHz, 250 nJ/pulse, fs-RegA amplifier)*

- Multifocus CARS microscopy

- Chirped pulse approach for CARS

*(Knutsen et al., Chem. Phys. Lett. 387, 436 (2004); 1kHz, 5  $\mu$ J/pulse, fs-regen. amplifier)*

## Strategies for stronger ps-pulses?

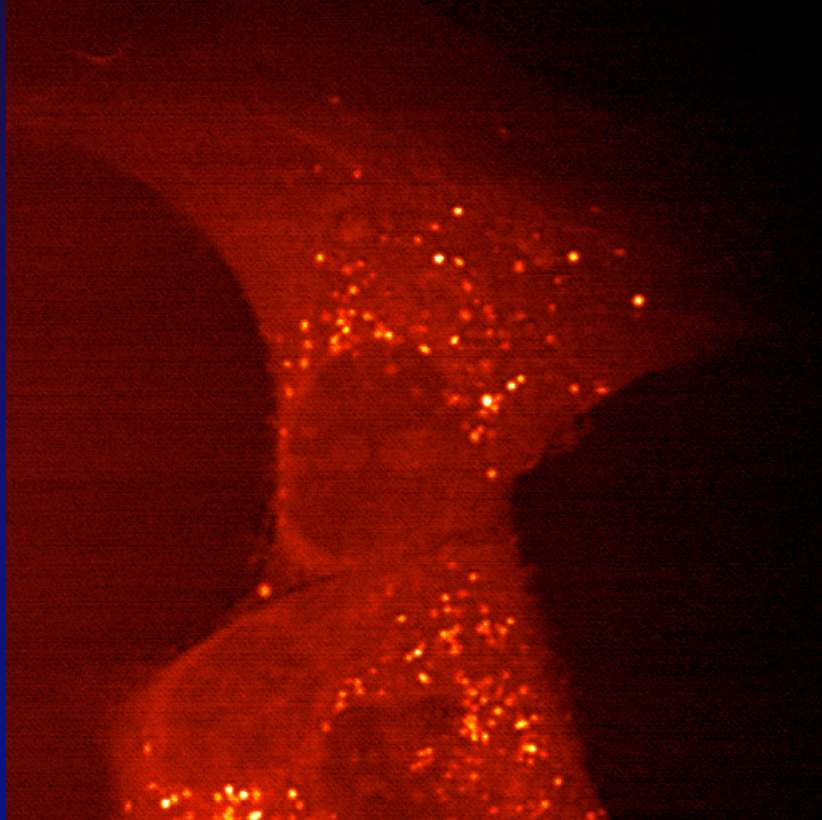
- ps-amplifier through passive external cavity

*Potma et al., Opt. Lett. 19, 1835 (2003); 4MHz-250kHz, 100nJ/pulse, ps-amplifier)*

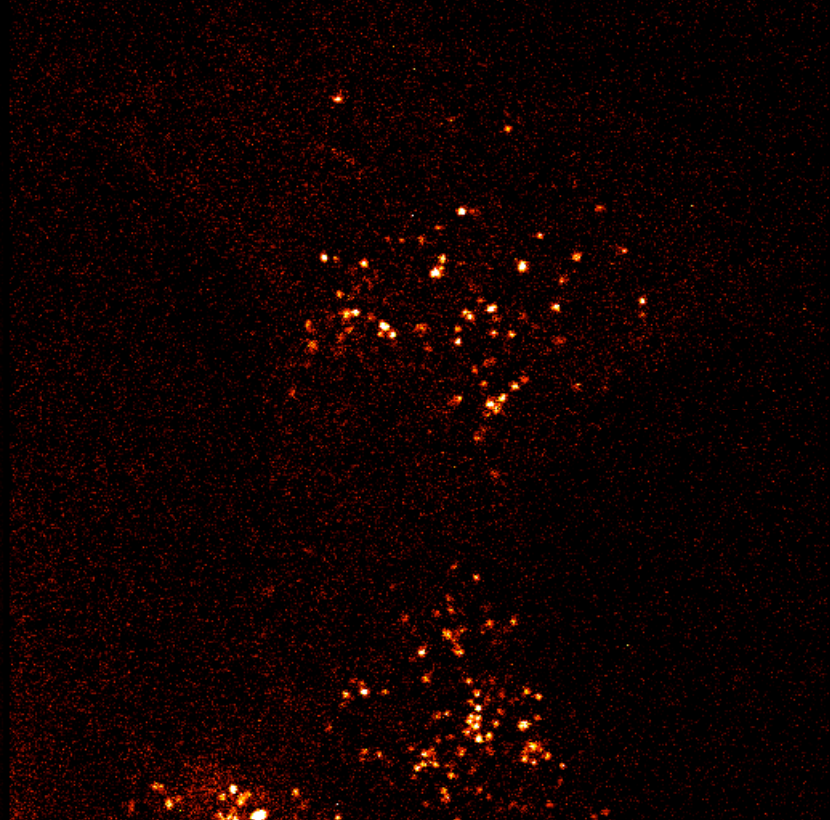
# pulse power & photo damage

Peak energy induced photo damage in L1 fibroblast cells

F-CARS



E-CARS



Pump 18 nJ/pulse (18 mW @ 1 MHz): photo damage

Pump 15 nJ/pulse (30 mW @ 2 MHz): no photo damage

1 frame/s, 2 minutes  
recording time  
(continuous)

# Scanning speed

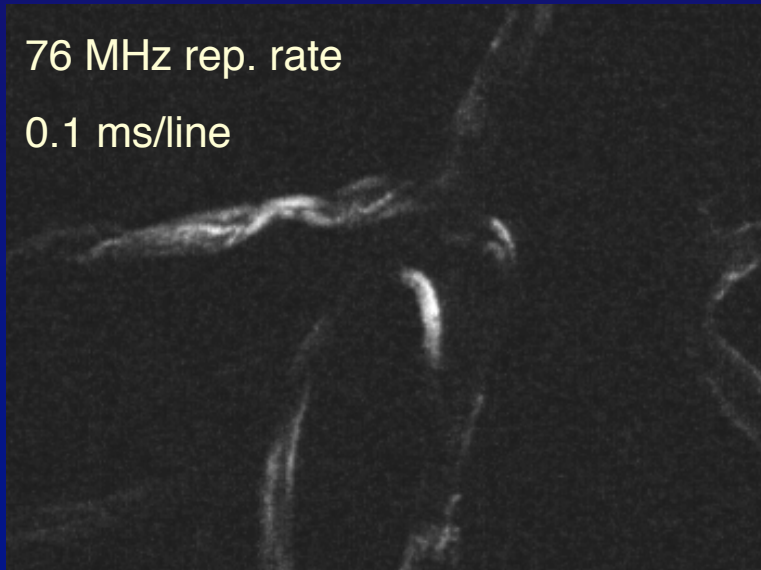
$$S_{CARS} \propto \frac{t_{scan}}{(f\tau_{pulse})^2} \langle P(t) \rangle^3$$

Faster without losing signal:

- higher pulse power, lower repetition rate (f)
- pixel dwell time  $> 1/f$
- NIR average power up to  $\sim 100$  mW, pulse power  $< 10$  nJ

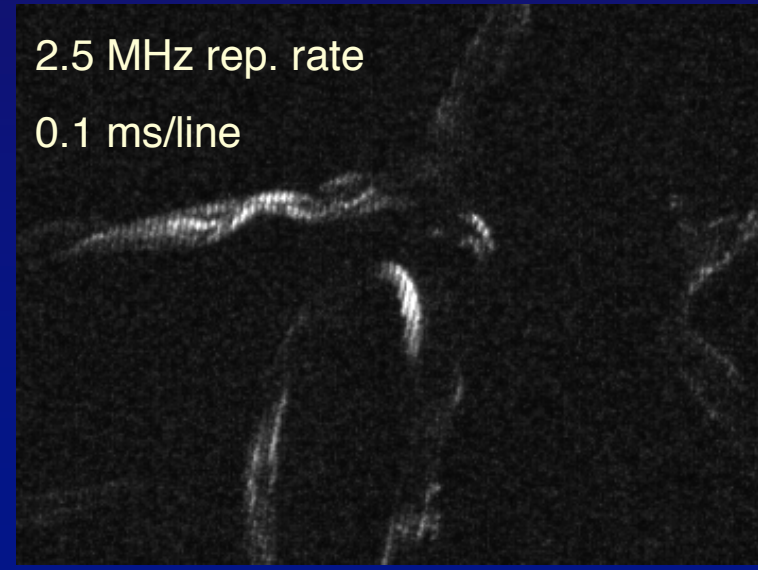
76 MHz rep. rate

0.1 ms/line



2.5 MHz rep. rate

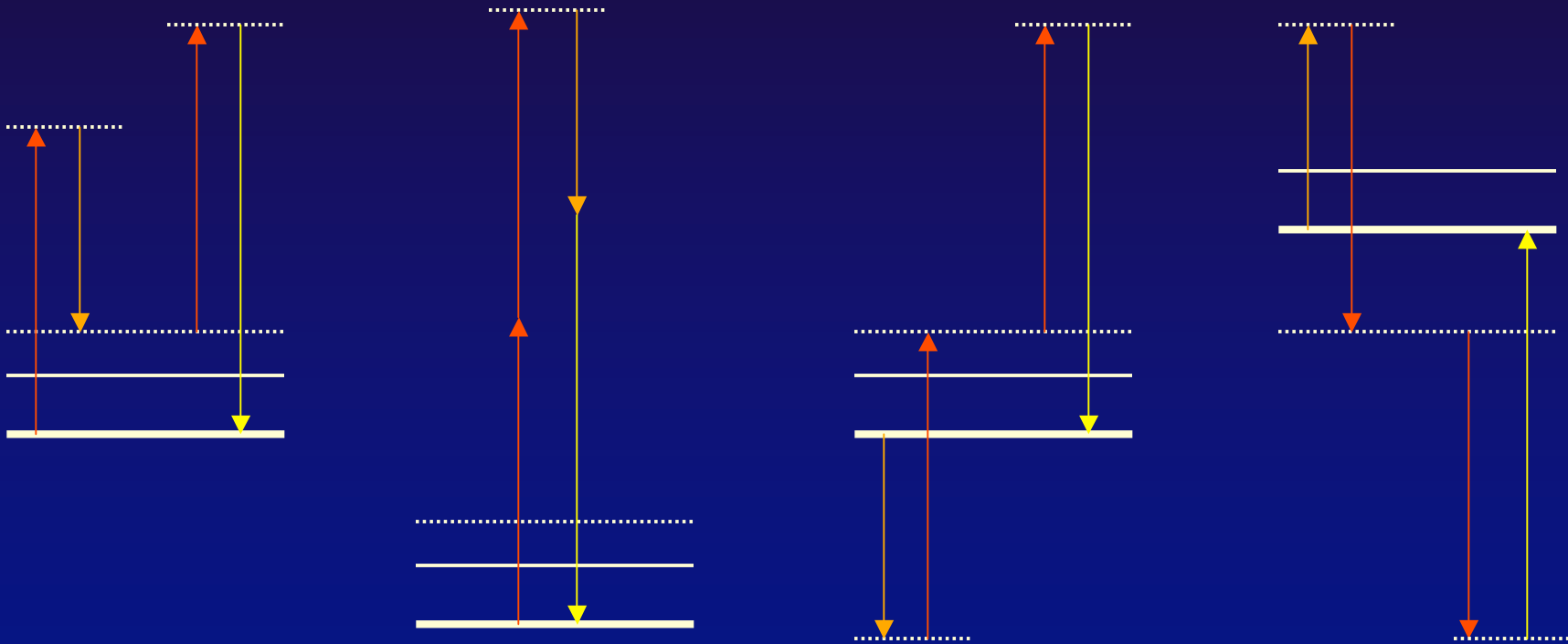
0.1 ms/line



# Nonresonant background:

What is it?

Oscillating electron clouds also radiate at CARS frequency

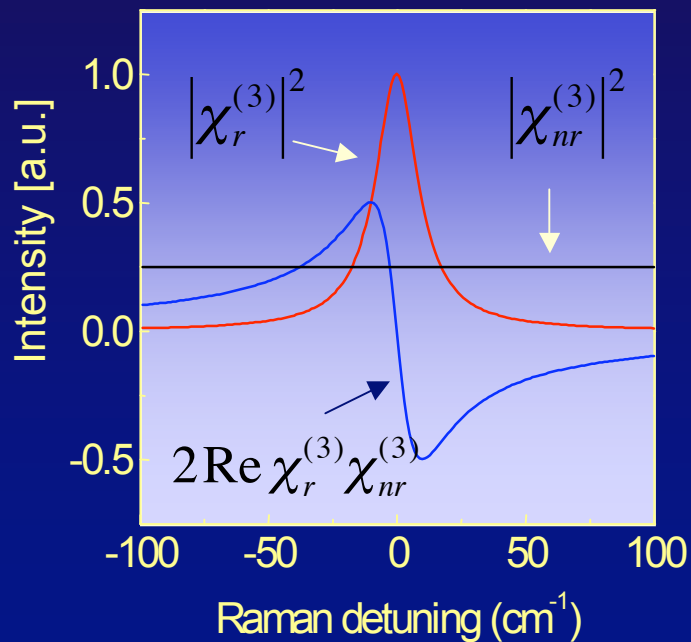


$\chi_{iiii}^{(3)}$  has a total of 384 terms: 32 resonant and 352 nonresonant  
(only 1/12 is resonant)

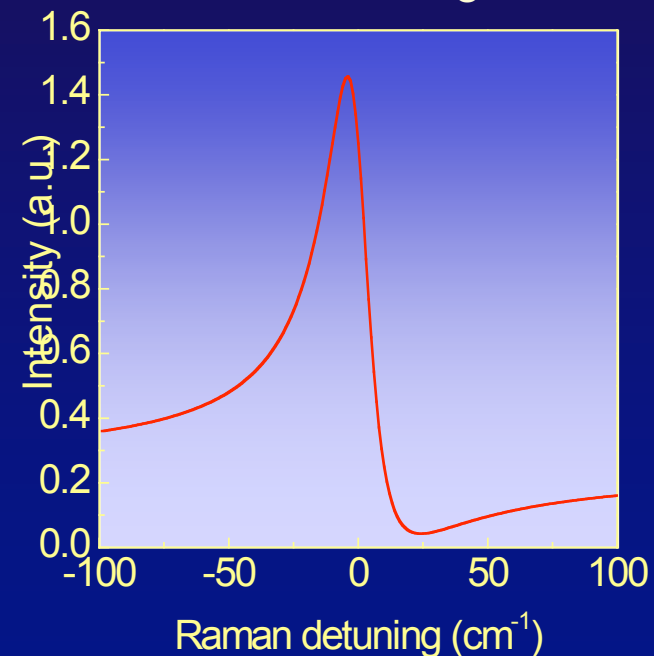
# Not just an addition

$$S_{CARS} \propto |\chi_{nr}^{(3)}|^2 + |\chi_r^{(3)}|^2 + 2\chi_{nr}^{(3)} \operatorname{Re}\{\chi_r^{(3)}\}$$

components

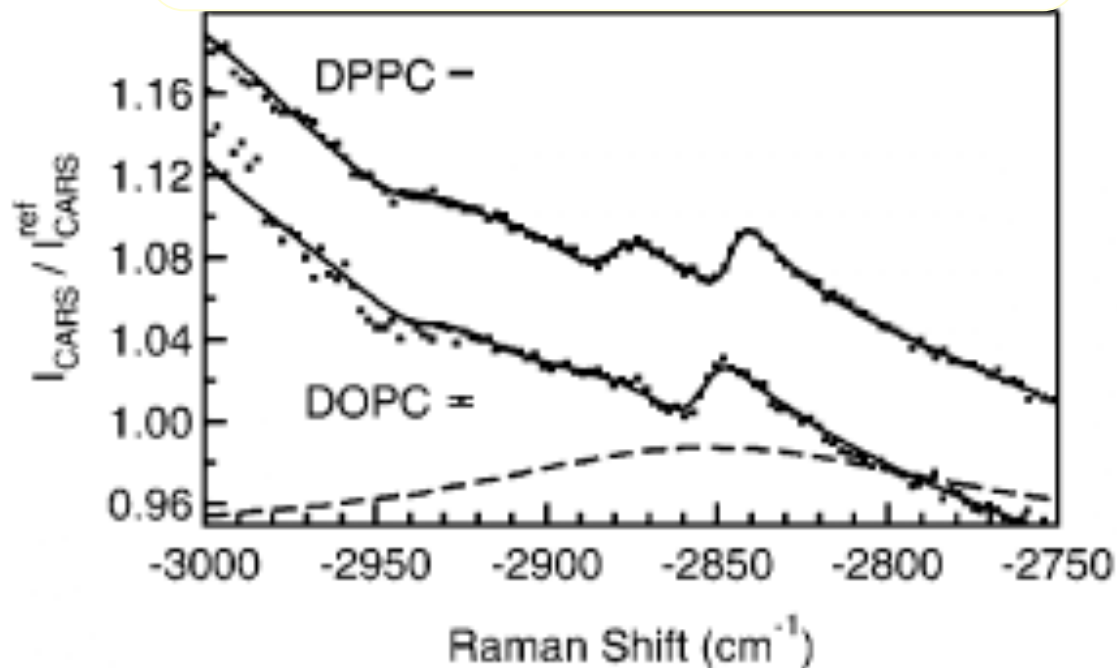


total CARS signal



# Always a problem?

$$S_{CARS} \propto |\chi_{nr}^{(3)}|^2 + |\chi_r^{(3)}|^2 + 2\chi_{nr}^{(3)} \operatorname{Re}\{\chi_r^{(3)}\}$$



Wurpel et al., *J. Phys. Chem. B* 108, 3400 (2004)

# Getting rid of it

## Partially:

- subtraction of off-resonant component
- Epi-CARS for suppressing solvent contribution
- spectral phase control techniques

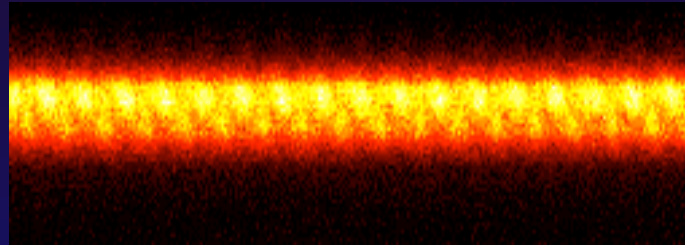
## Entirely:

- Polarization sensitive CARS
- Time-resolved CARS
- Phase sensitive detection

# Poor man's solution

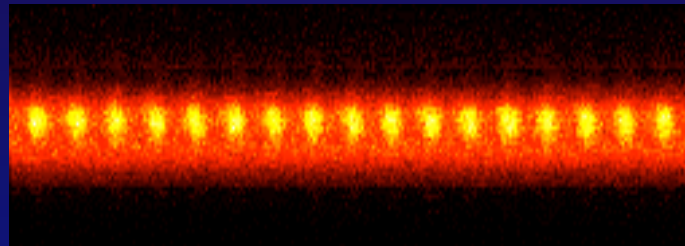
periodic polymer layer

on-resonance



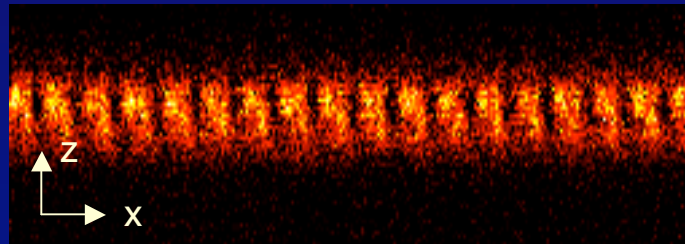
$$|\chi_{nr}^{(3)}|^2 + |\chi_r^{(3)}|^2 + 2\text{Re} \chi_r^{(3)} \chi_{nr}^{(3)}$$

off-resonance



$$|\chi_{nr}^{(3)}|^2$$

on-resonance  
-  
off-resonance

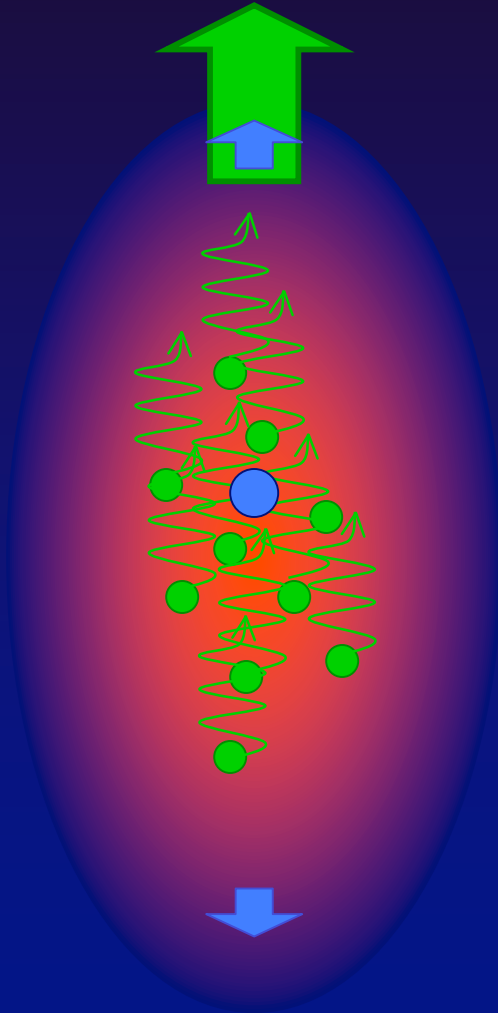


$$|\chi_r^{(3)}|^2 + 2\text{Re} \chi_r^{(3)} \chi_{nr}^{(3)}$$

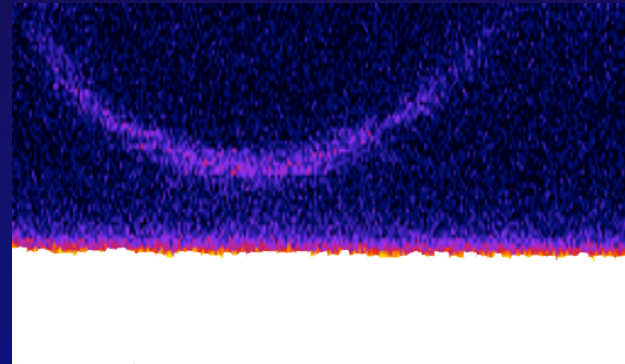
*Tert-butoxyl skeletal mode @ 891 cm<sup>-1</sup>*

# What E-CARS does

E-CARS detects  $\chi^{(3)}$  discontinuities



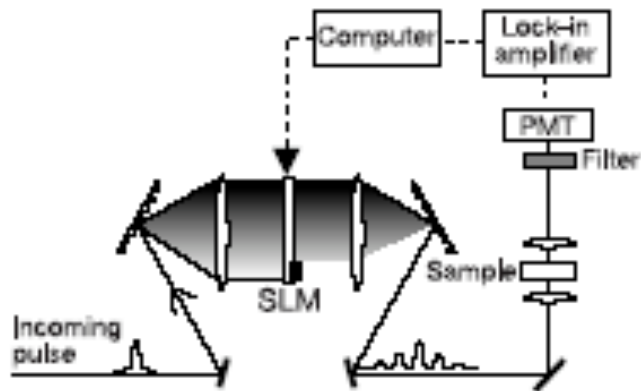
$$|\chi_{nr,solvent}^{(3)}|^2 + |\chi_{nr,object}^{(3)}|^2 + |\chi_r^{(3)}|^2 + 2\text{Re} \chi_r^{(3)} \chi_{nr(solvent+object)}^{(3)}$$



$$|\chi_{nr,object}^{(3)}|^2 + |\chi_r^{(3)}|^2 + 2\text{Re} \chi_r^{(3)} \chi_{nr,object}^{(3)}$$

- Solvent background is completely rejected
- For objects  $> \lambda_{\text{CARS}}/4$ , destructive interference reduces the signal in E-CARS

# Coherent control methods



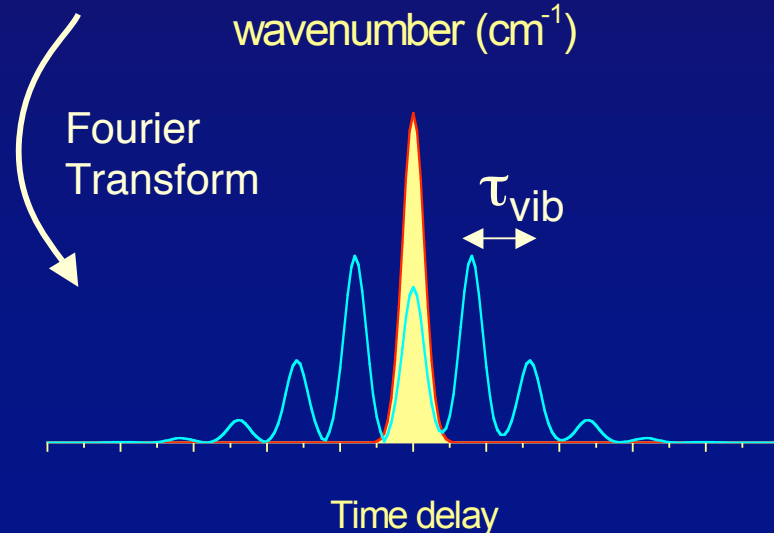
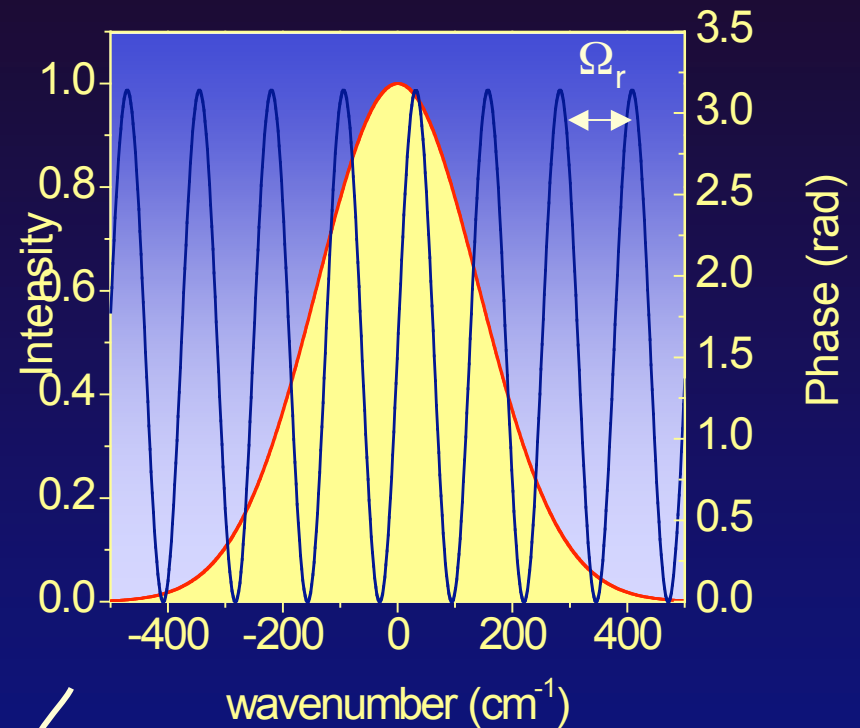
spectrum is phase-modulated:

$$E(\omega) = |E_0(\omega)|e^{i\Phi(\omega)}$$

Constructive interference at the Raman frequency:

$$\Phi(\omega) = \Phi(\omega - \Omega_r)$$

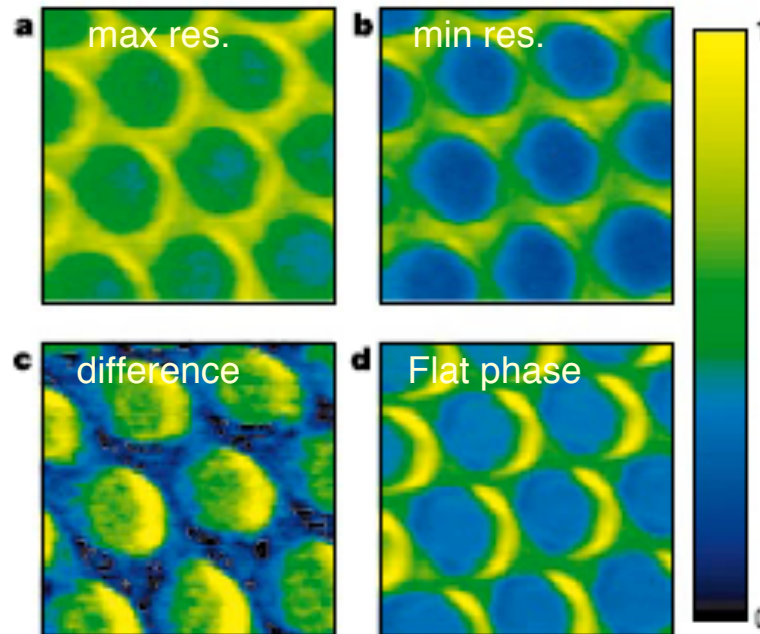
*Dudovich et al., Nature 418, 512 (2002)*



# Background suppression

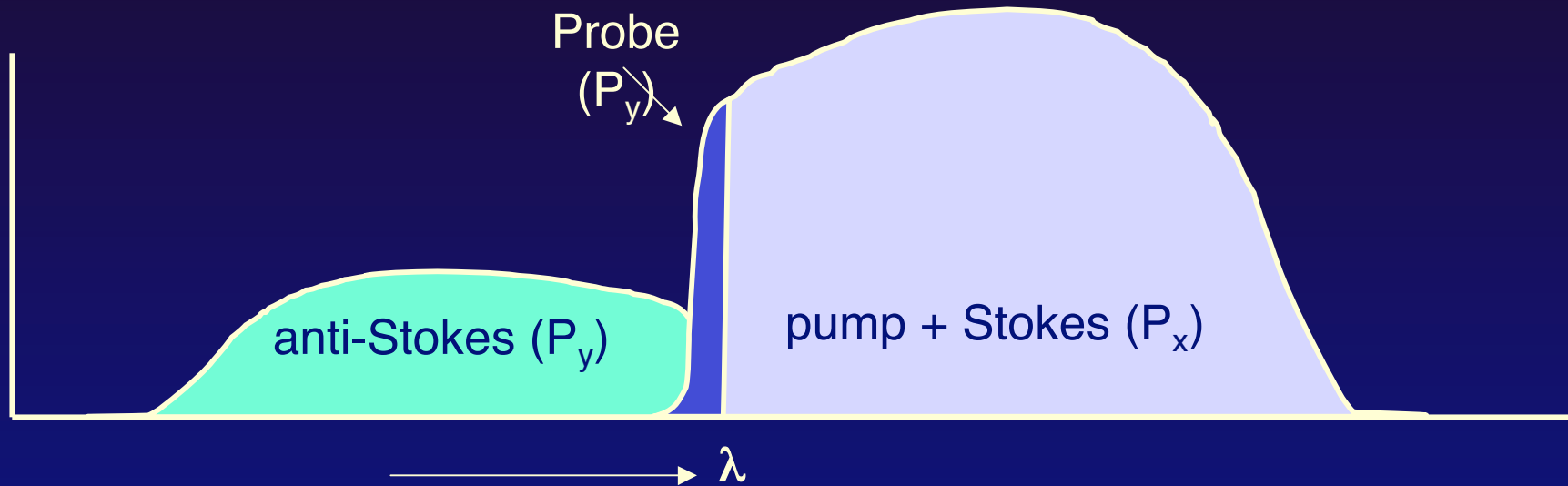
with spectral phase shaping

Glass capillaries filled with  $\text{CH}_2\text{Br}_2$



- spectral resolution limited by SLM
- nonresonant background at  $\Omega_r$  cannot be suppressed

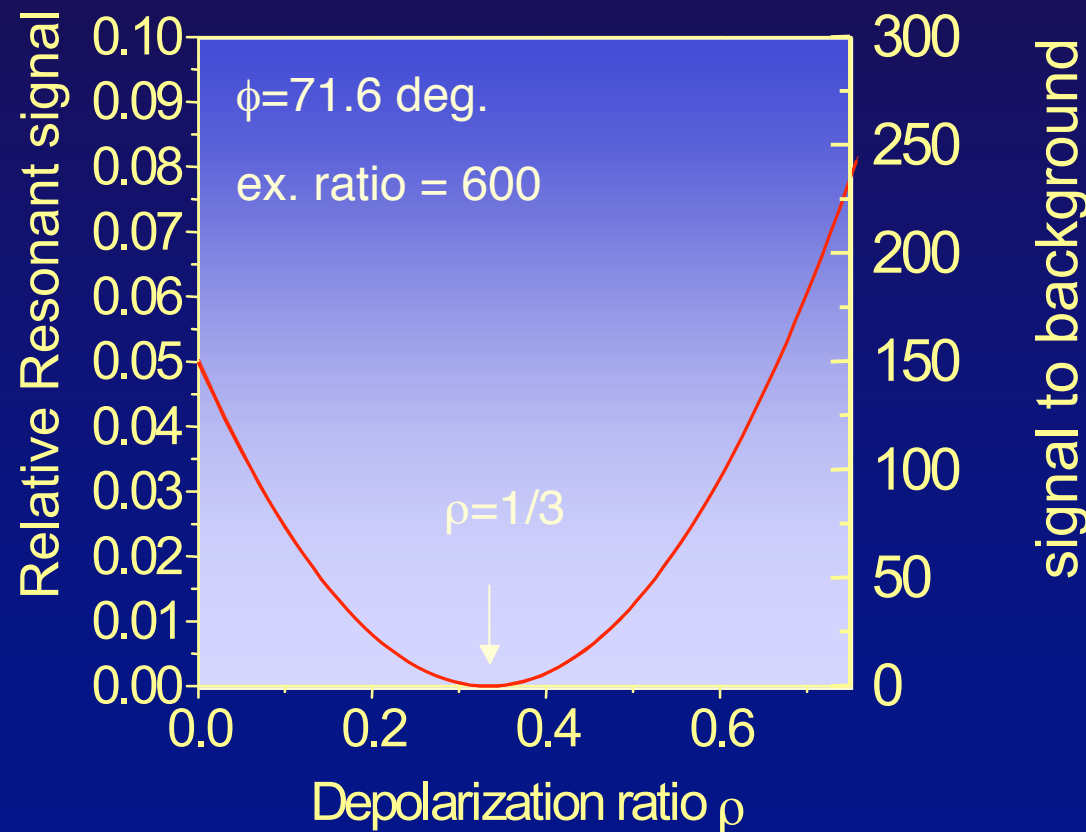
# Single pulse + polarization



- High spectral resolution
- background suppression through phase shaping

# Narrowband P-CARS

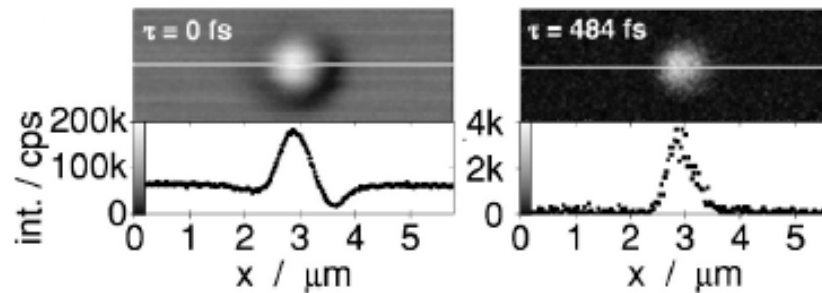
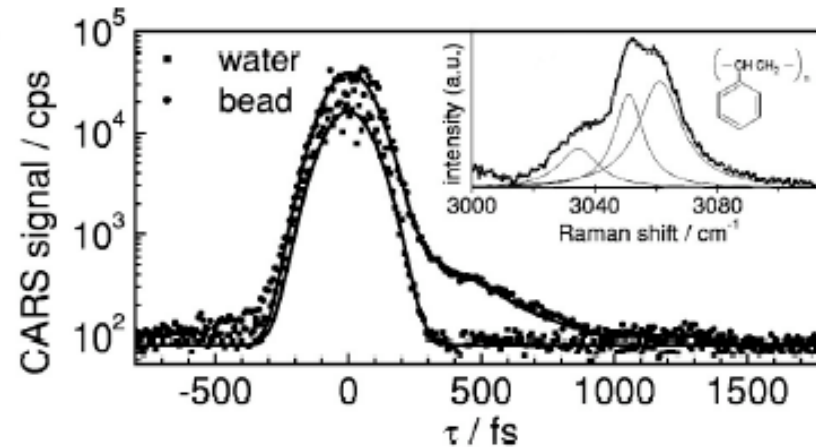
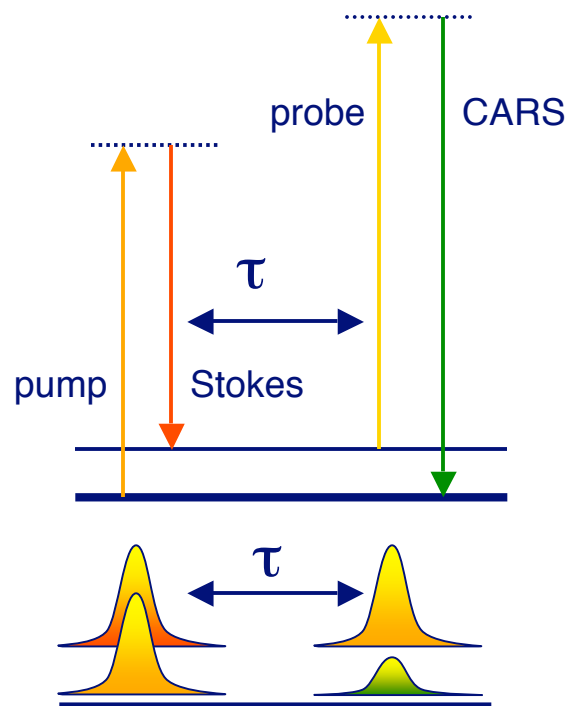
- P-CARS rejects all the nonresonant background
- Improves signal/background, decreases absolute signal strength
- Limited by inherent depolarization and birefringence



CH<sub>2</sub> stretch  
@ 2845 cm<sup>-1</sup>:  
 $\rho = 0.21$

# Time-resolved CARS

Probing the Raman free induction decay



- complex 3-color configuration
- signal at longer time delays is small

Volkmer et al., *Appl. Phys. Lett.* 80, 1505 (2002)

# Phase: spatial and spectral

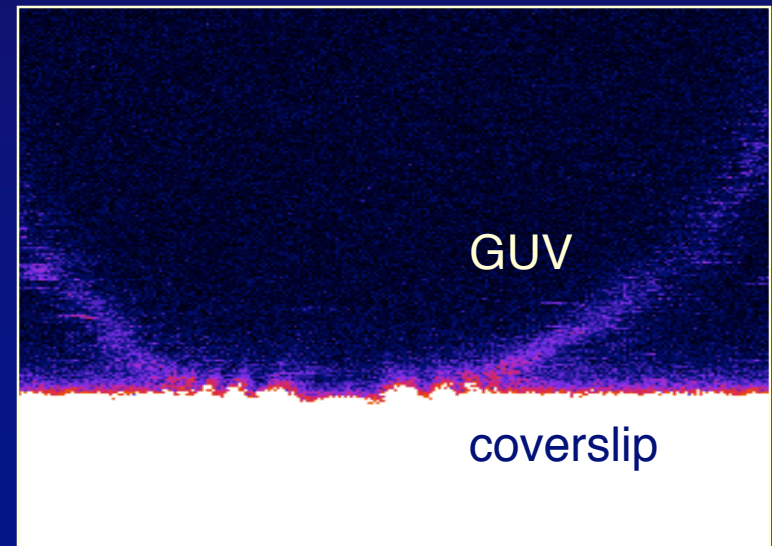
Both spectral and spatial phase play a role in CARS signal formation

$$E_{CARS} = A(\omega, r)e^{i\phi(\omega, r)}$$

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## Spatial phase effects

- destructive interference in E-CARS
- interference between back-reflected ( $\chi^{(1)}$ ) forward signal and pure epi-signals ( $\chi^{(3)}$ )
- Additional components in CARS correlation spectroscopy



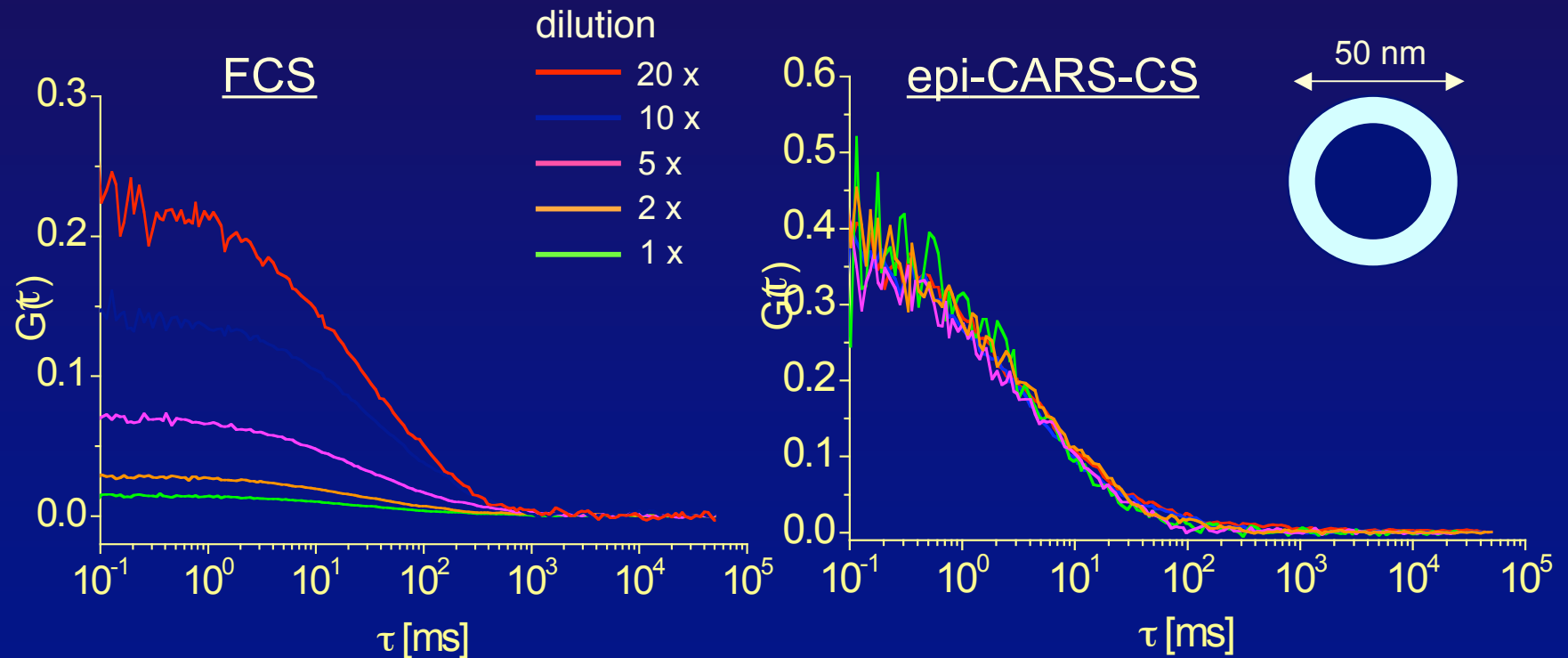
# Spatial phase effects

in epi-CARS correlation spectroscopy

$$G_b(\tau) = \frac{2\sqrt{2}}{\langle N \rangle} \left(1 + \frac{2\tau}{\tau_D}\right)^{-1} \left(1 + \frac{2r_0^2\tau}{z_0^2\tau_D}\right)^{1/2} + \exp\left(-\frac{2k_{as}^2 r_0^2 \tau / \tau_D}{1 + r_0^2 \tau / z_0^2 \tau_D}\right) \left(1 + \frac{2\tau}{\tau_D}\right)^{-2} \left(1 + \frac{2r_0^2\tau}{z_0^2\tau_D}\right)^{-1}$$

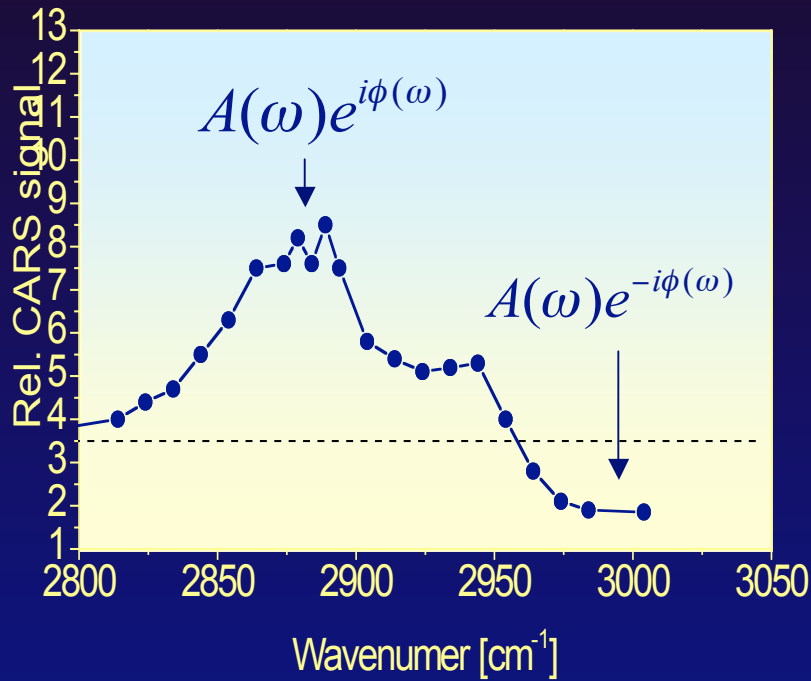
FCS:  $\langle N \rangle \ll 1$

Coherence effect: Fast decay

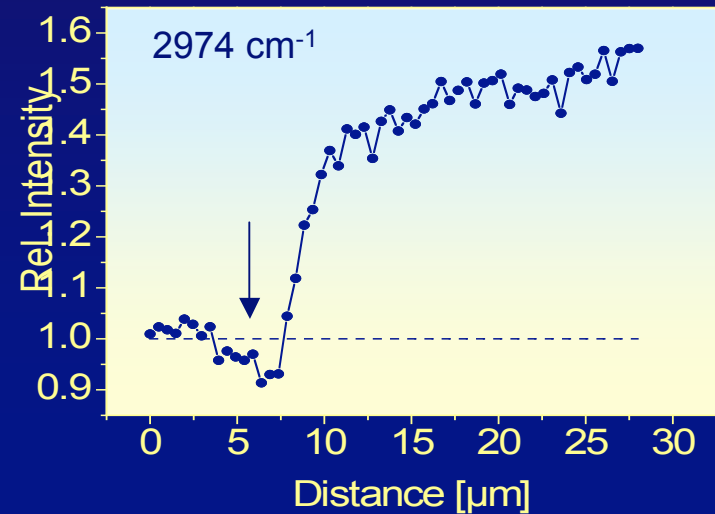
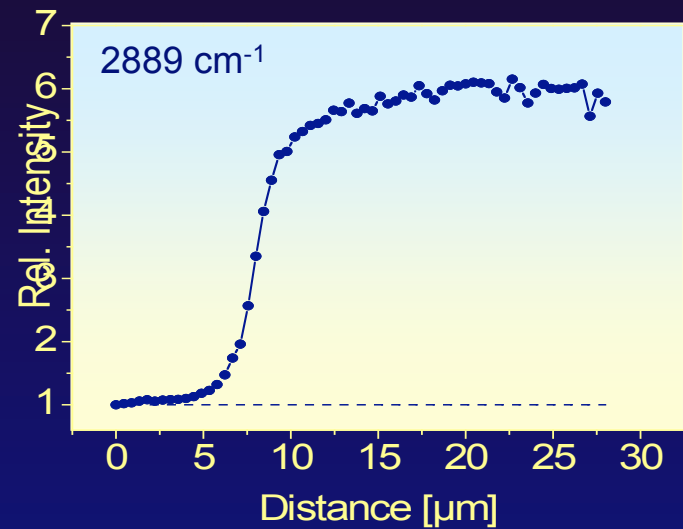


# Spectral phase effects

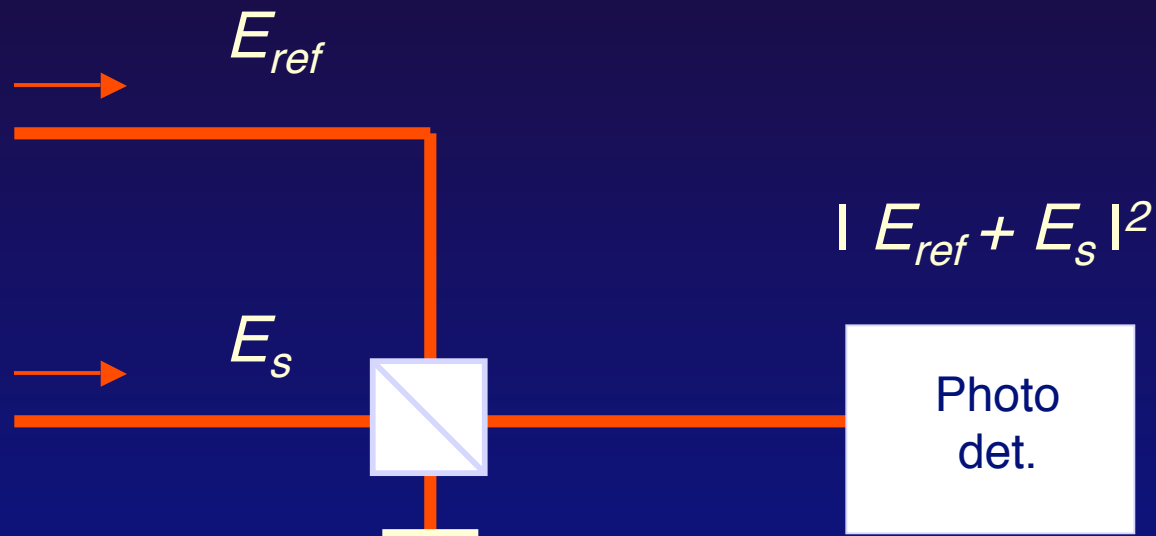
Glycerol solution



Linear Z-scan



# Using phase: CARS interferometry



$$|E_s|^2 + |E_{ref}|^2 + 2E_s E_{ref} \cos \phi$$

# What interferometry offers

- Linear in concentration
- Linear in  $\text{Im} \{\chi^{(3)}\}$ , proportional to spontaneous Raman
- Linear amplification through varying  $E_{\text{ref}}$

*at the expense of added complexity*

# Faster acquisition

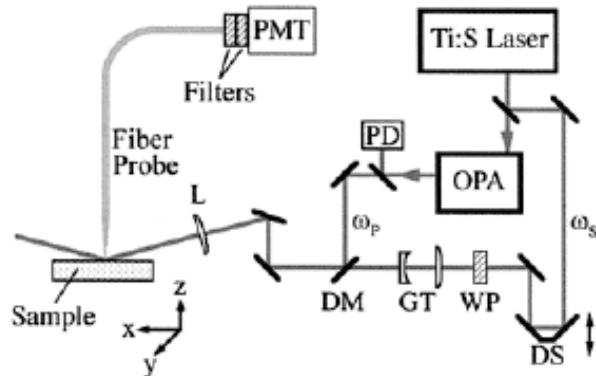
- **Point-illumination**
  - resonant Galvos
  - spinning polygon
  - multifocal: scanning array
  - multifocal: microlens spinning disk
  
- **Wide-field illumination**

*Retaining image brightness with higher acquisition times always requires higher illumination powers at the sample*

# Higher resolution

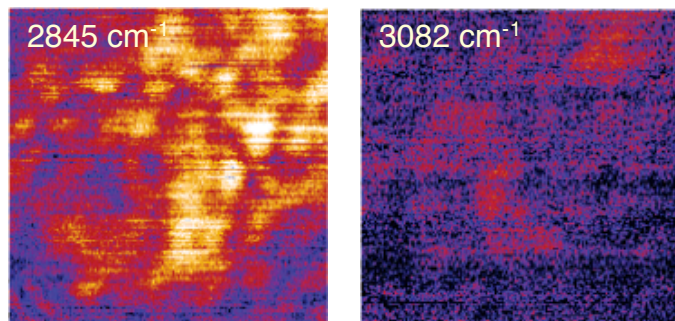
near field methods

## Fiber probe detected



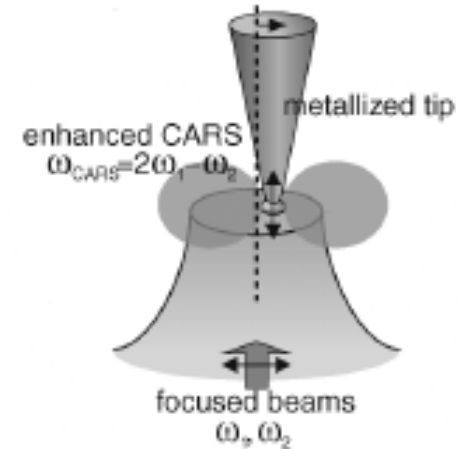
Resolution  $\sim 130$  nm

Human hepatocyte ( $4.3 \times 4.3 \mu\text{m}$ )



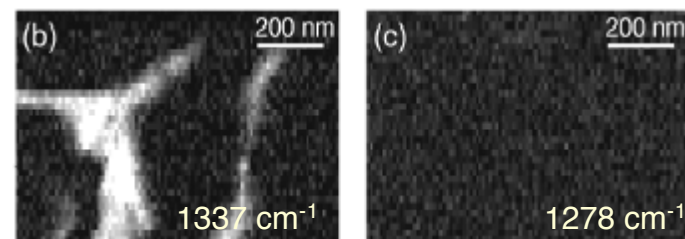
Schaller et al., *J. Phys. Chem. B* 106, 8489 (2002)

## Tip enhanced NSOM



Resolution  $< 50$  nm

Bundle of DNA on coverslip



Ichimura et al., *Phys. Rev. Lett.* 92, 220801 (2004)

Suitable for thin ( $< 1 \mu\text{m}$ ) samples on surfaces

# Recap

## Brightest signals, fastest acquisition:

High rep rate (1-100 MHz) ps light source (< 50 mW)

Point-scanning F-CARS mode: > 1 photon/shot

> 20 frames/s

## Economic rejection of solvent background:

E-CARS (signal reduction  $1-10^3$  relative to F-CARS)

## Highest signal to background:

P-CARS with signal/background > 100

## Highest spatial resolution:

Tip-enhanced near field CARS microscopy with resolution > 50 nm

# Outlook

CARS is becoming a versatile technique for biological imaging.

Room for improvement of light source: tuneability, pulse width, rep. rate and cost.

Opportunities for improving signal strength and sensitivity with phase sensitive methods .

More insight into photo damaging mechanisms would be helpful for CARS and nonlinear imaging in general.

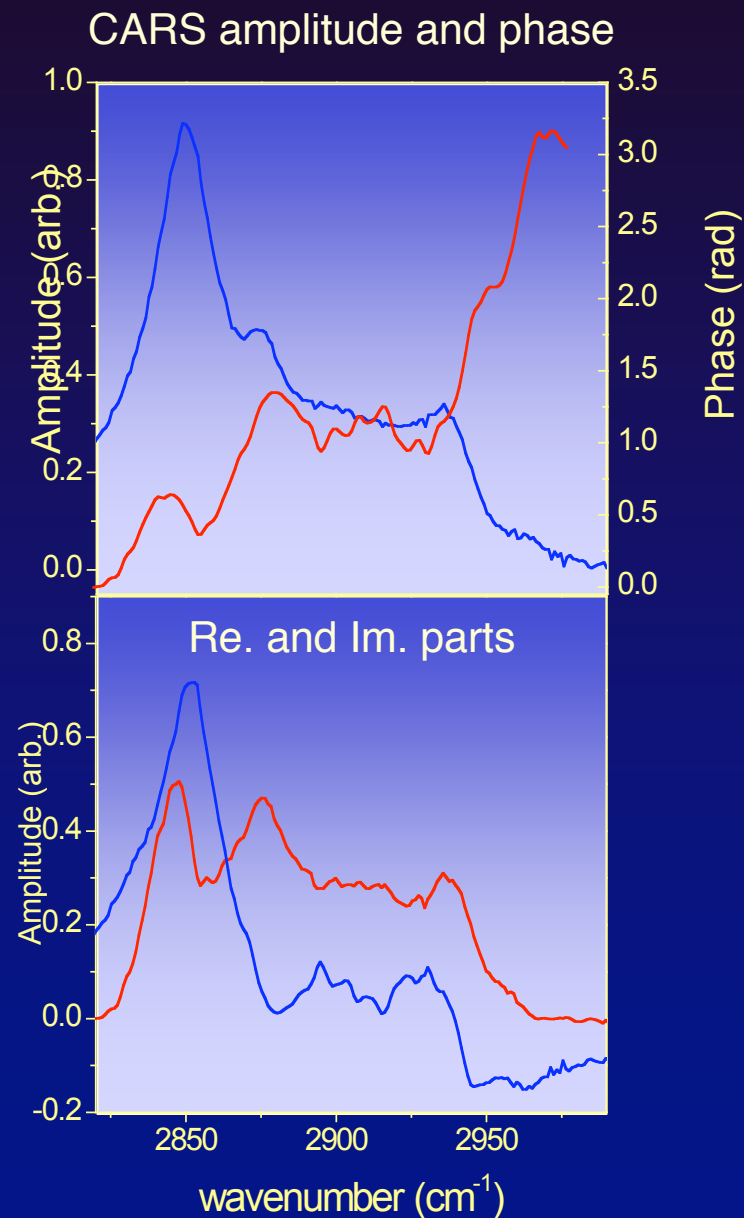
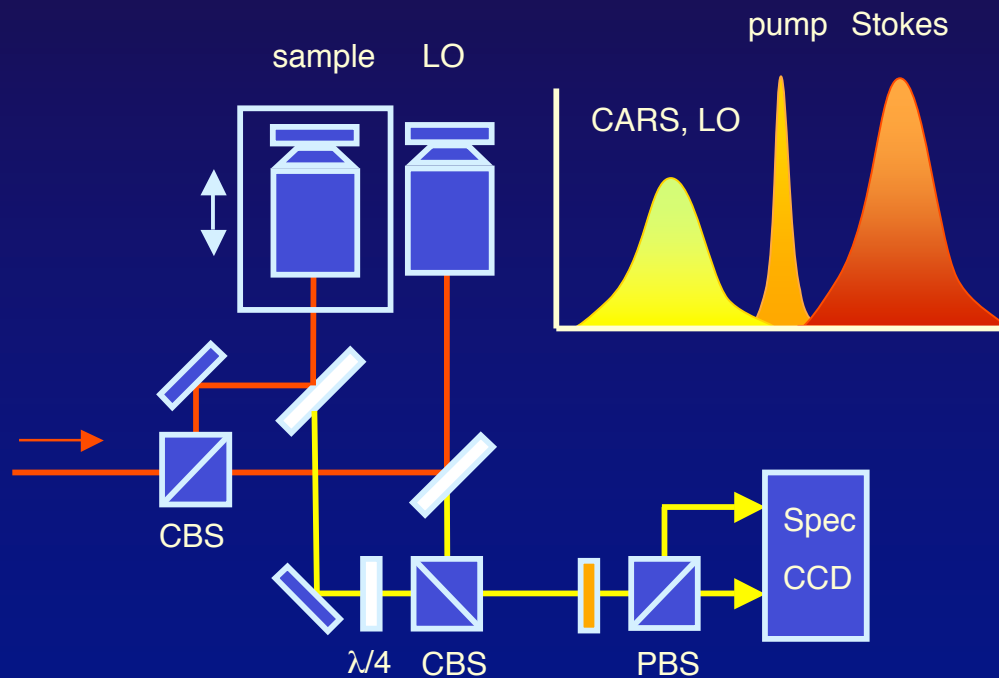
Thanks!



# Broadband interferometry

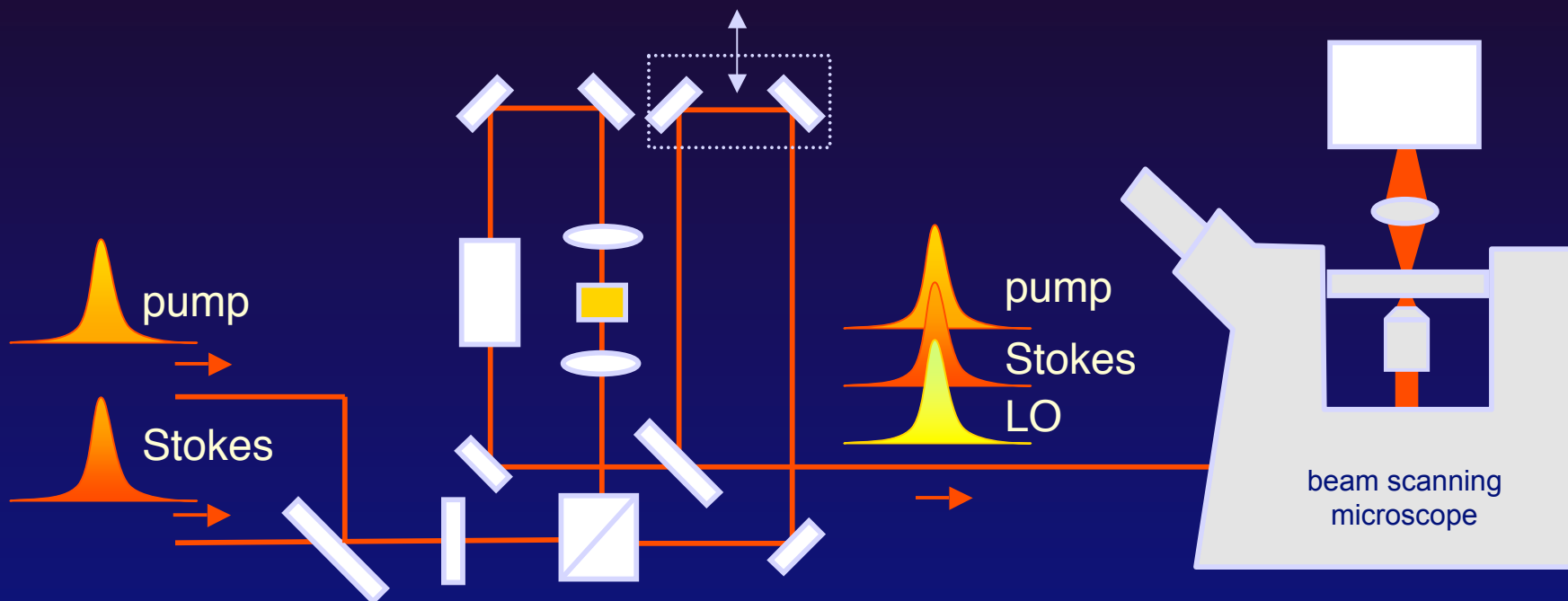
## CARS spectral interferometer

*Resolves spectral phase and amplitude*



*Evans et al., Opt Lett. 29, 2923 (2004)*

# Narrowband interferometry



regular

imaginary

real

