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Coherent Raman microscopy for biomedical applications

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CRIMSON

Introduction and motivation (I)

- **Optical microscopy** is an extremely powerful investigation tool thanks to its ability of visualizing morphological details in cells and tissues on the sub-micrometer scale

- It provides a much **higher spatial resolution** compared to magnetic resonance imaging, and at the same time it does not require the sample to be fixed, as in electron microscopy.

- **Fluorescence microscopy** using exogenous (dyes or semiconductor quantum dots) or endogenous (GFPs) markers offers a superb sensitivity, down to the single molecule limit.

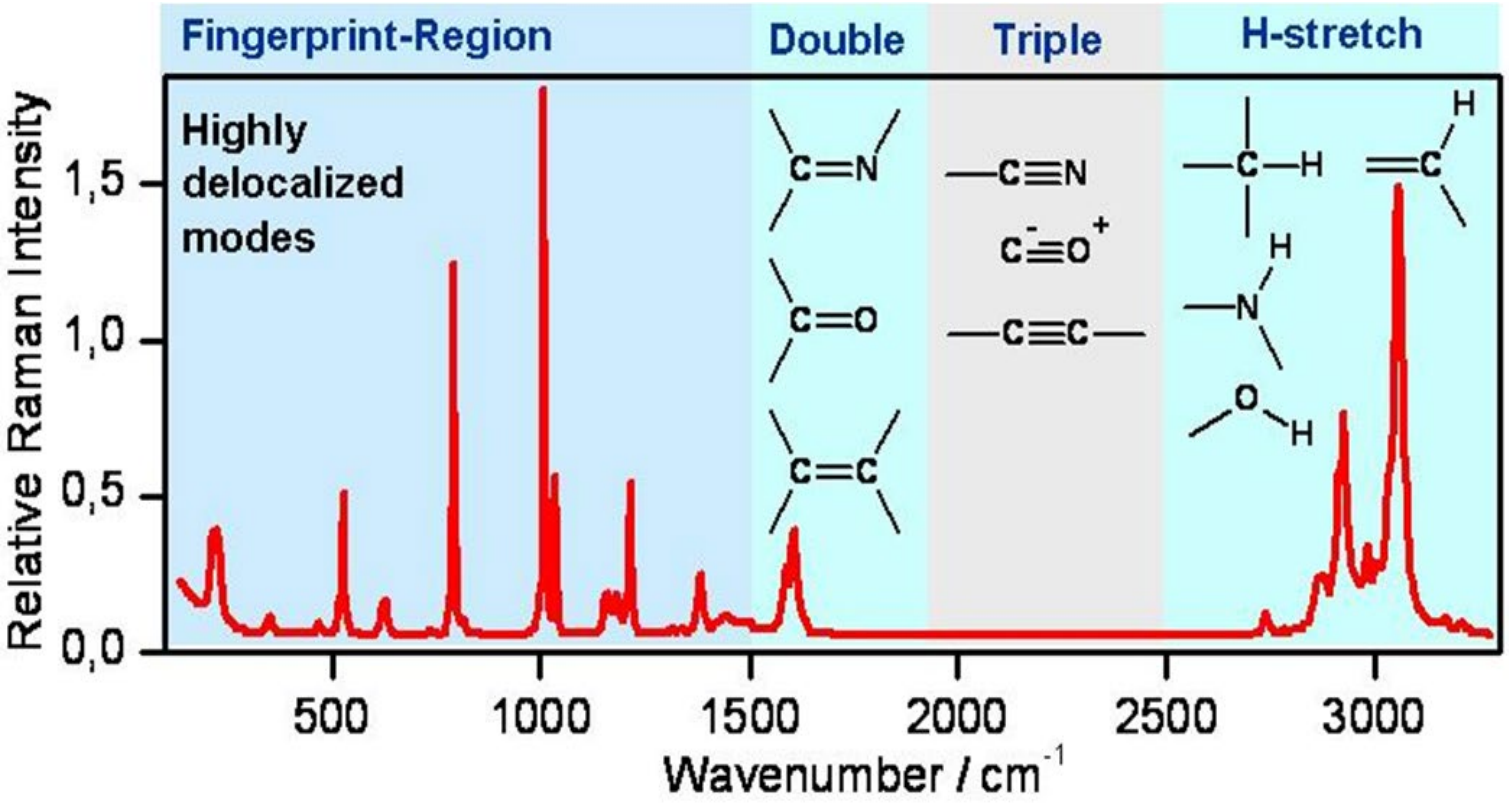
However…

Introduction and motivation (II)

- The addition of **fluorescent markers** cannot be implemented within certain cells or tissues, or it can give strong perturbation to the investigated system
- For **small molecules** (signaling peptides, neurotransmitters, metabolites and drugs) the dimension of the marker is comparable to or even bigger than that of the molecule itself, so that it heavily interferes with its biological function
- In many **clinical applications** involving live tissue imaging, staining with fluorophores is not possible or not desired

$\Rightarrow$ there is a great need for **intrinsic, label-free imaging methods** that do not require the addition of any fluorescent molecule.

Vibrational fingerprinting of molecules (I)



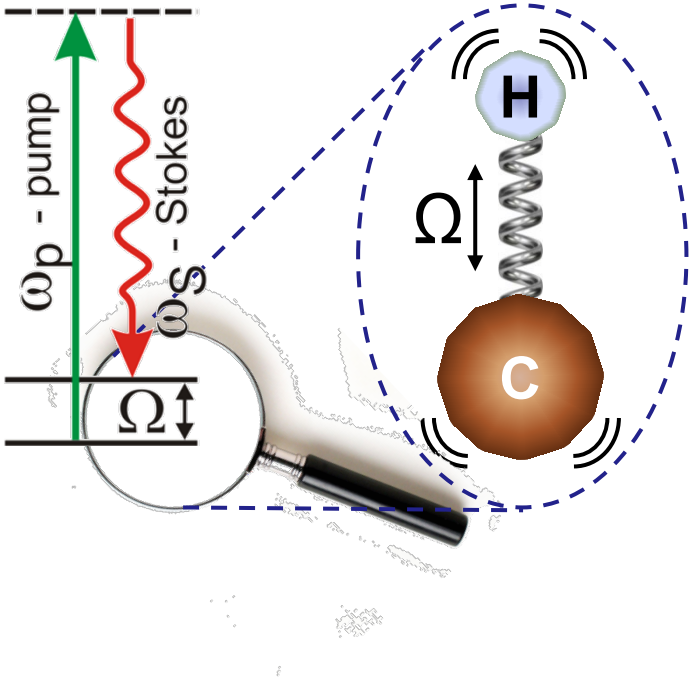
Every component of a biological specimen (cell or tissue) is characterized by a vibrational spectrum that reflects its molecular structure and provides an endogenous and chemically specific signature for its identification.

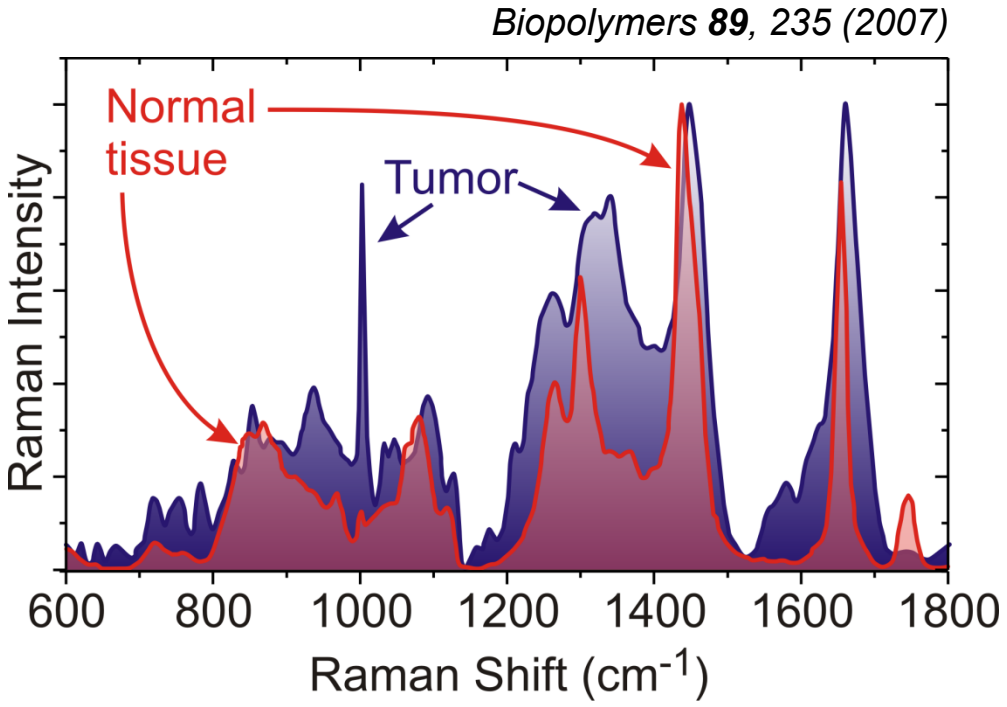
Raman Scattering

Tissues display characteristic vibrational spectra

→ "fingerprint" - "**chemical signature**"!

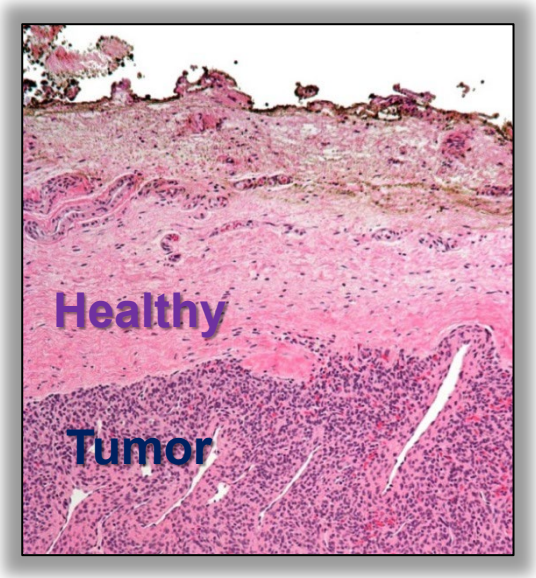
Non-invasive (no staining, no labelling)





Motivation: tumour identification

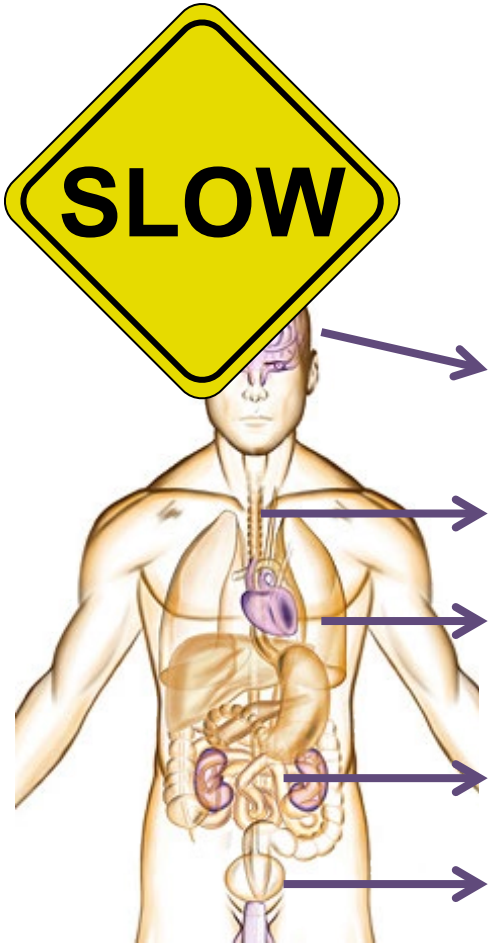
Biopsies stained with dyes (H&E)



Opinion of a histo-pathologist: not objective!

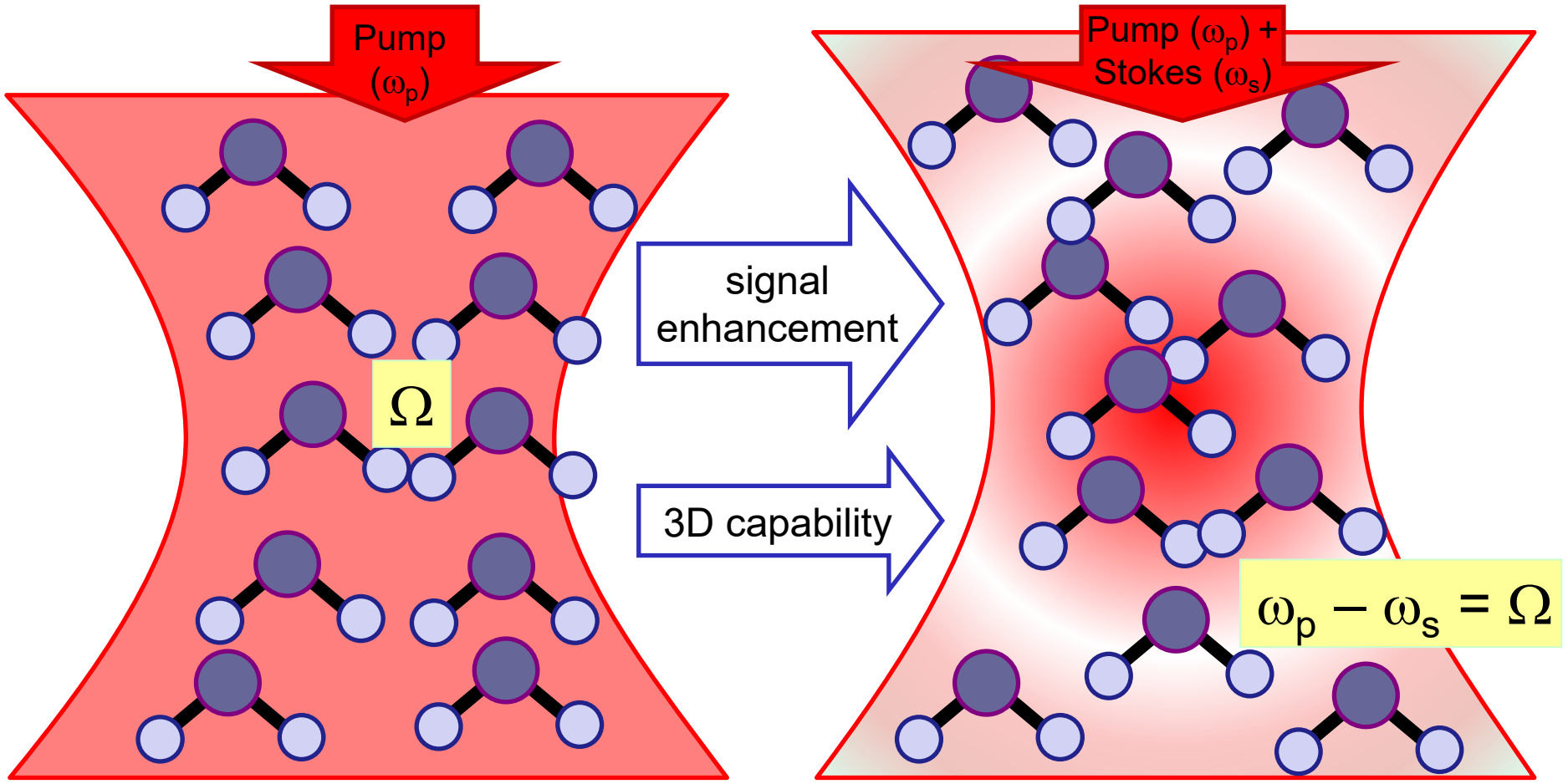
- Low sensitivity (60-90%)
  → many **false negatives**!
- Time consuming
- Destructive
- No *in-vivo* imaging

Spontaneous Raman for tumour identification



Tumor location	Specificity	Sensitivity	Ref.
Brain metastases	97-100%	96-99%	Chemometr. Intell. Lab. **117**, 224 (2012)
Esophagus	87%	88%	Phys. Med. Biol. **54**, 7077 (2009)
Breast in vitro	100%	92%	Analyst **135**, 3042 (2010)
Polyps in the colon	95% ex vivo 89% in vivo	91% ex vivo 100% in vivo	Gastrointest. Endosc. **57**, 396 (2003)
Bladder	96-99%	78-98%	J. Raman Spectrosc. **33**, 564 (2002)

From spontaneous to coherent Raman



Spontaneous Raman:
incoherent thermal vibrations

Coherent Raman: **coherent** molecular vibrations (driven by Pump+Stokes)

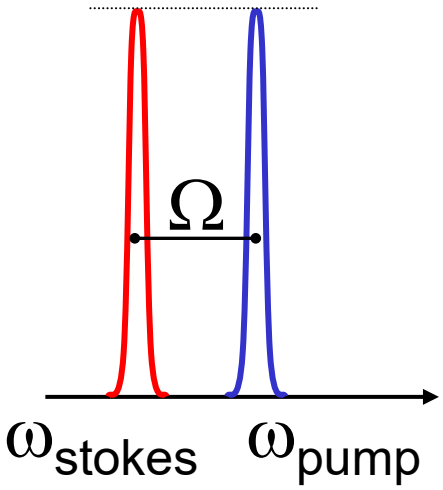
Coherent anti-Stokes Raman Scattering (CARS)

Requires two synchronized narrowband pulses, the pump (ω_p) and Stokes (ω_s).

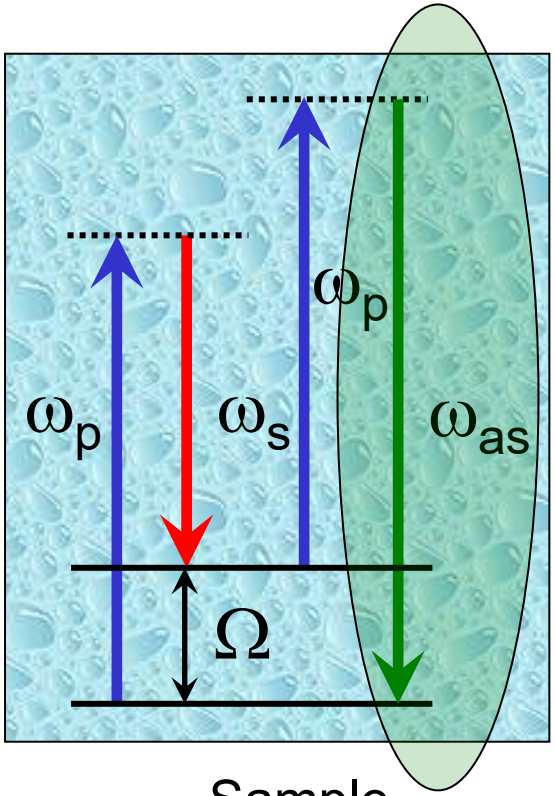
Resonance condition: $\omega_p - \omega_s = \Omega$

Background free

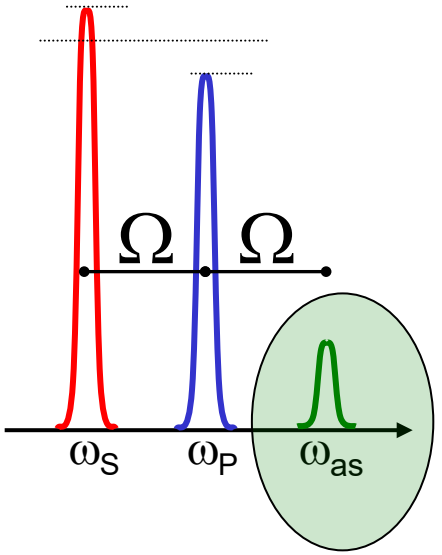
$\omega_{aS} = 2\omega_p - \omega_S = \omega_p + \Omega$



Input spectra



Sample

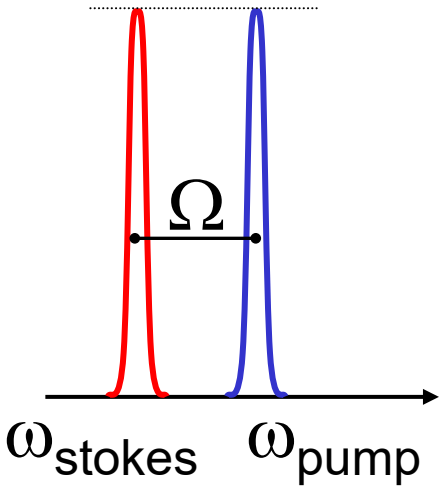


Anti-Stokes

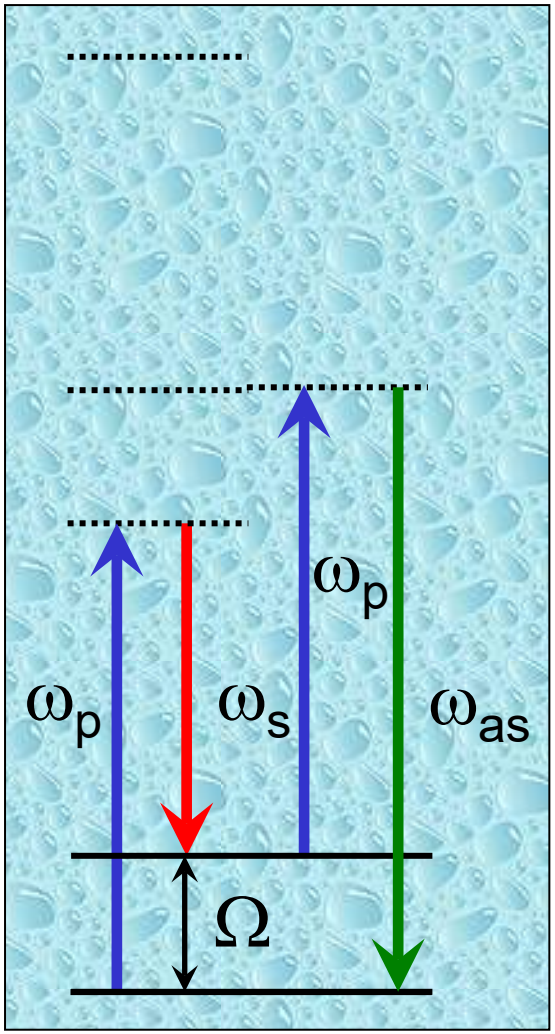
Output spectra

Non-resonant background (NRB) in CARS

Four-wave-mixing process!

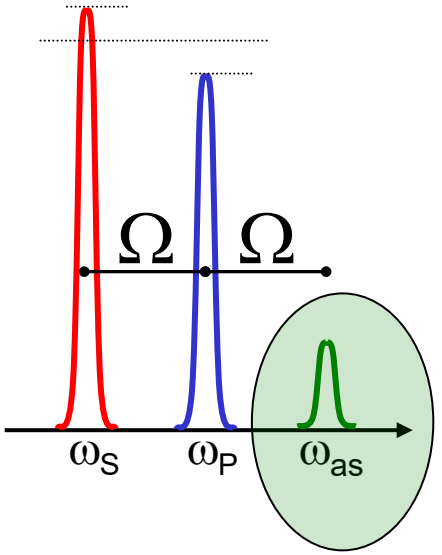


Input spectra



Sample

Anti-Stokes emission outside resonance $\omega_{aS} = 2\omega_p - \omega_S$ and from third molecules



Anti-Stokes

Output spectra

NRB insight (I)

The CARS signal is given by:

$$E_{CARS} = -i\chi^{(3)} E_{pu}^2 E_S * L$$

$$\chi^{(3)} = \chi_R^{(3)} + \chi_{NR}^{(3)}$$

Complex resonant response

Real non-resonant background (NRB)

We detect:

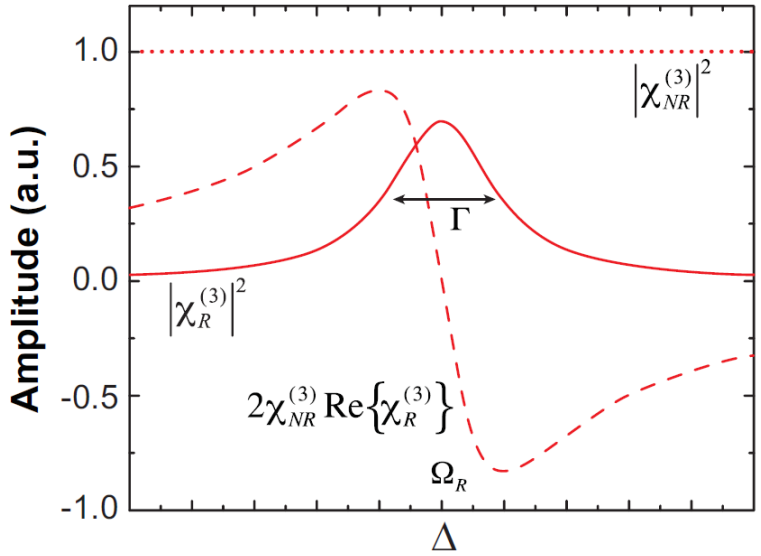
$$I_{CARS} \propto \left|\chi^{(3)}\right|^2 I_p^2 I_S L^2$$

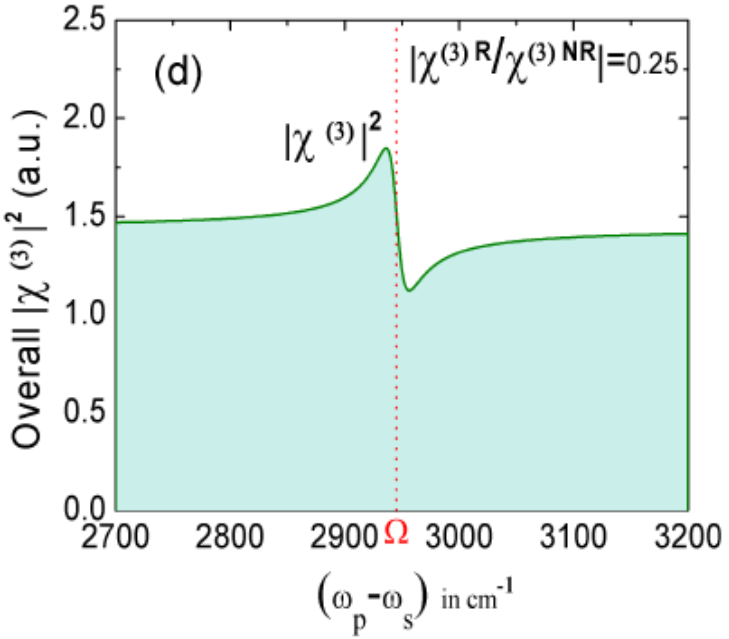
The CARS signal depends on:

- the square of the pump intensity
- the Stokes intensity
- the square of the propagation length L
- the square modulus of third-order susceptibility

NRB insight (II)

$$I_{CARS} \propto \left|E_{CARS}\right|^2 \propto \left|\chi_R^{(3)} + \chi_{NR}^{(3)}\right|^2 = \left|\chi_R^{(3)}\right|^2 + \left|\chi_{NR}^{(3)}\right|^2 + 2\chi_{NR}^{(3)}\,\mathrm{Re}\left(\chi_R^{(3)}\right)$$ NRB





Drawbacks of CARS:

- The NRB can distort and even overwhelm the resonant signal of interest;
- The CARS signal scales **quadratically** with sample **concentration**.

Stimulated Raman Scattering (SRS)

Stimulated decay from a virtual level

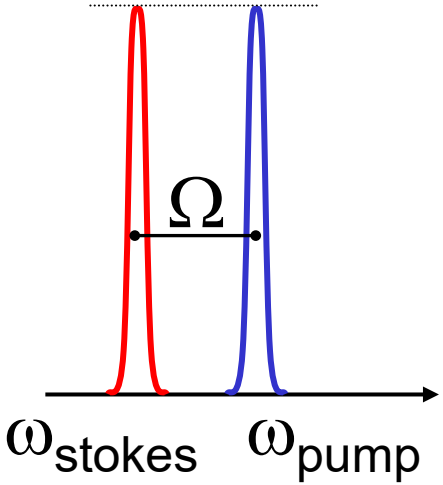
Resonance condition: $\omega_p - \omega_s = \Omega$

Free from non-resonant signals

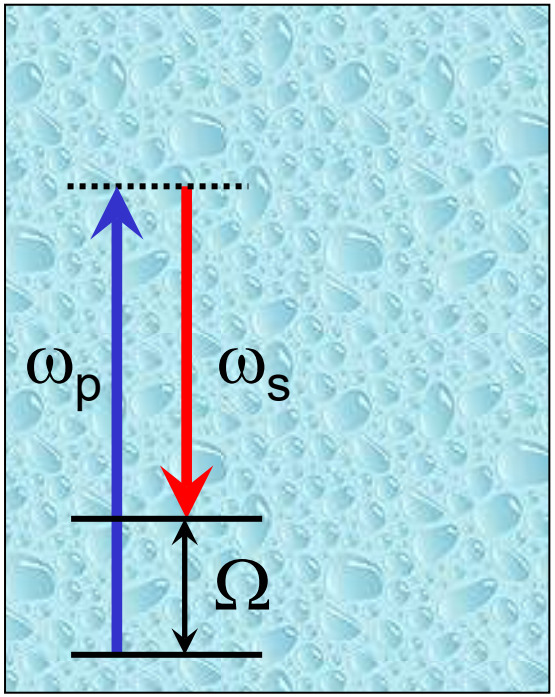
Detect small changes ($<10^{-4}$) $\Rightarrow$ demodulation

SRG: Stimulated Raman Gain

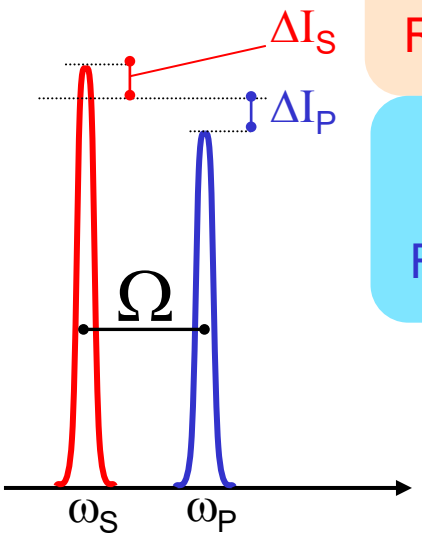
SRL: Stimulated Raman Loss



Input spectra



Sample



Output spectra

Stimulated Raman Scattering (SRS)

The Stokes field amplification amounts to:

$$\Delta E_S \propto -i\chi^{(3)}\left|E_{pu}\right|^2 E_S$$

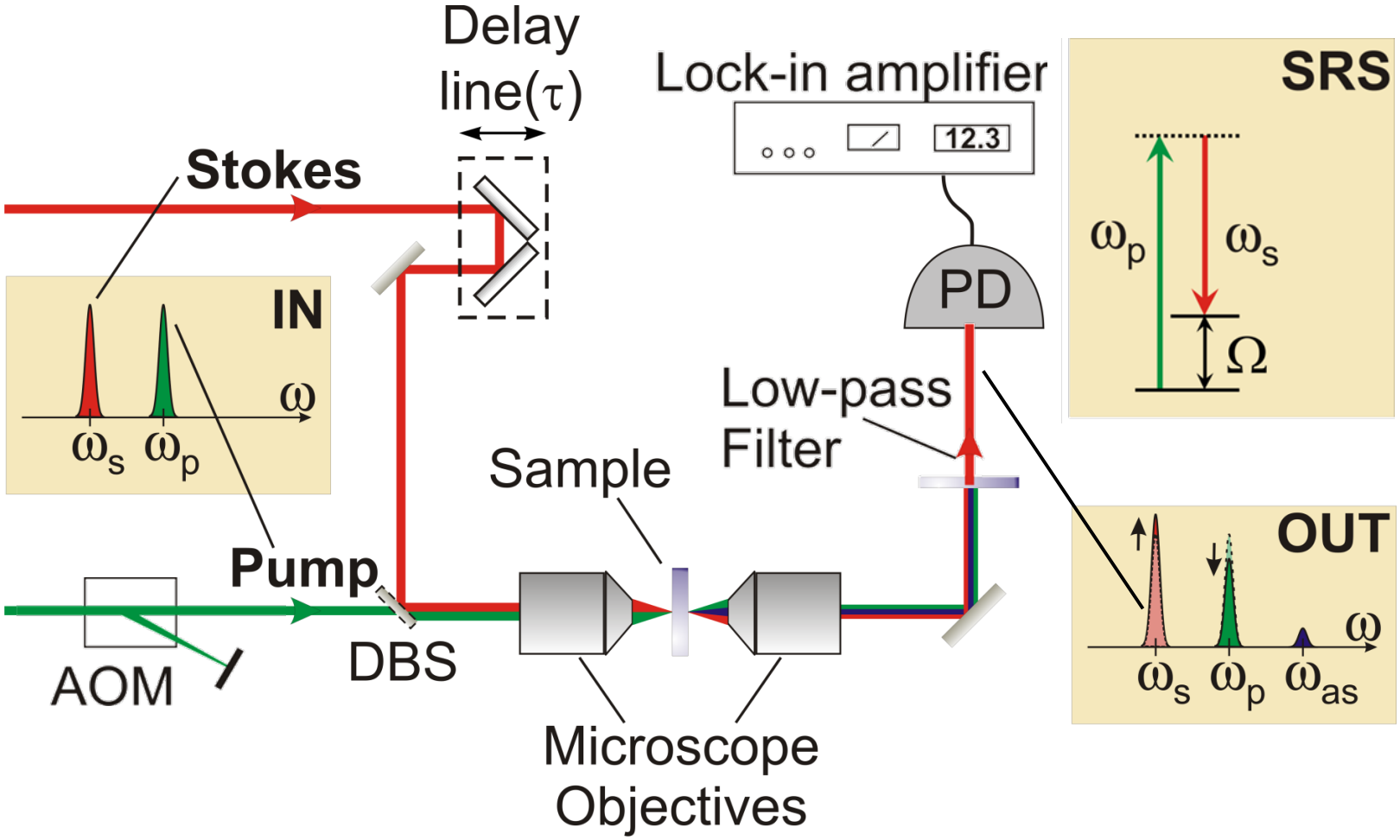
and sits on the linear background given by the Stokes field E_S.
The SRS signal is given by:

$$\Delta I_{SRS} = \left|E_S + \Delta E_S\right|^2 - \left|E_S\right|^2 \cong 2\,\mathrm{Re}\left(E_S^* \Delta E_S\right) \propto \mathrm{Im}\left(\chi_R^{(3)}\right) I_{pu} I_S$$

Advantages of SRS:

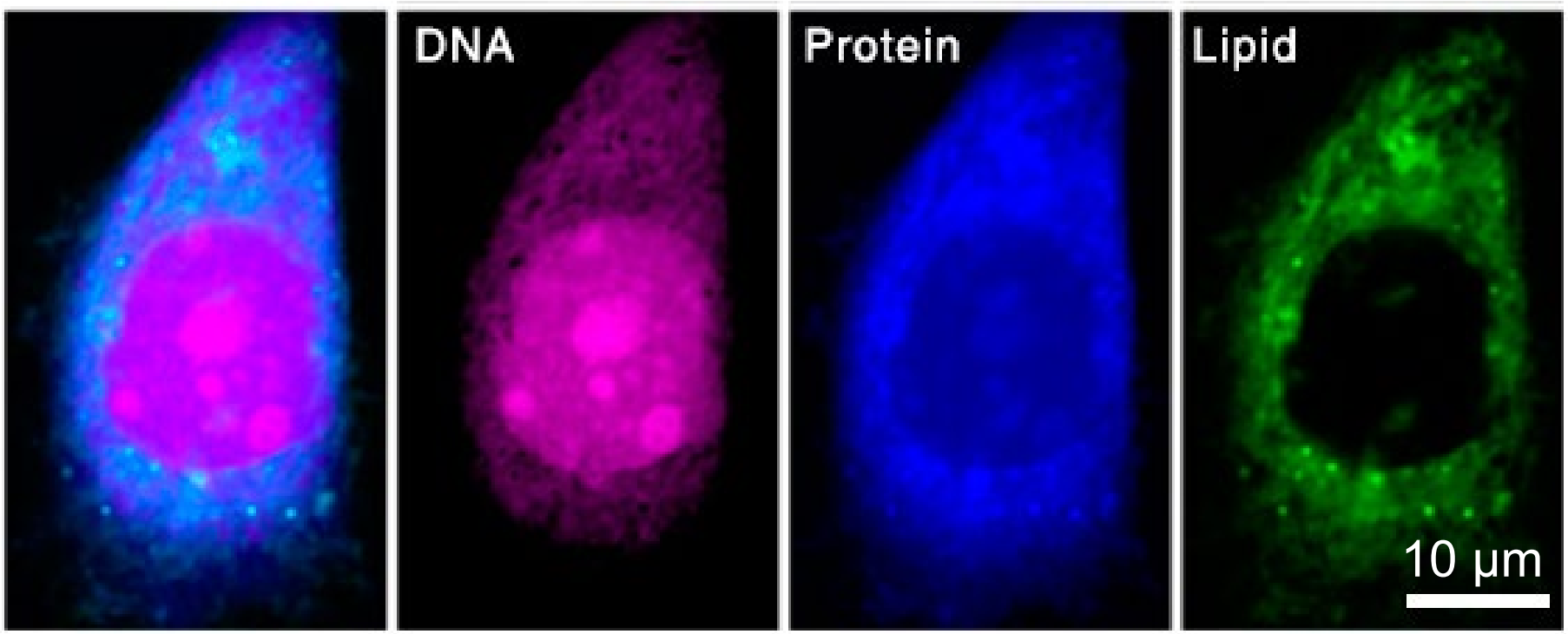
- The signal is proportional to the imaginary part of $\chi^{(3)}$ and **the real NRB is suppressed**;
- The SRS signal scales **linearly** with sample **concentration**;
- The small nonlinear signal ΔE_S is multiplied by the phase-locked Stokes field $E_S \Rightarrow$ **self-heterodyne amplification**.

Single-frequency SRS: basic layout



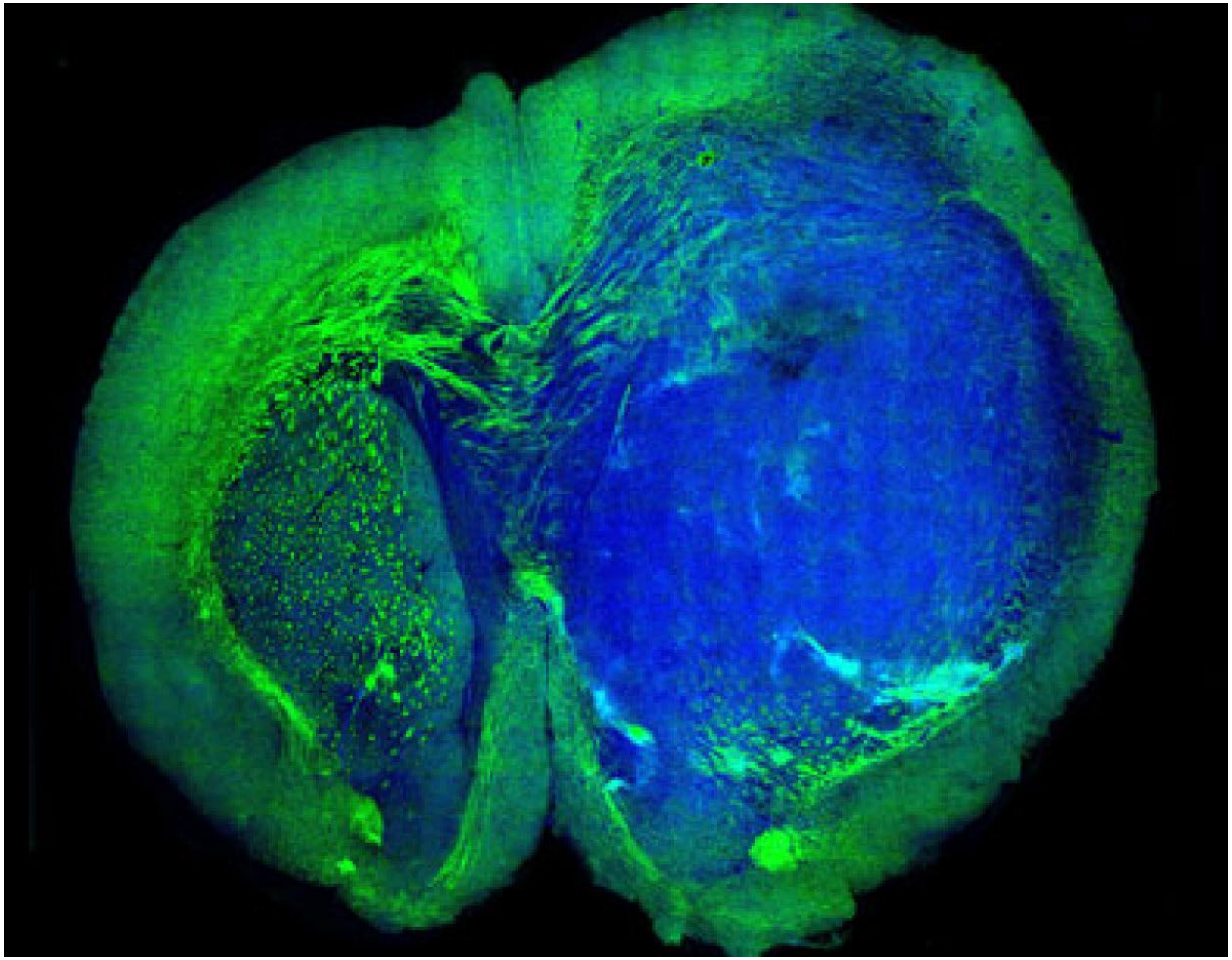
- High frequency (multi-MHz) modulation and lock-in detection → high speed (up to video rate)

Label-free SRS imaging of cells



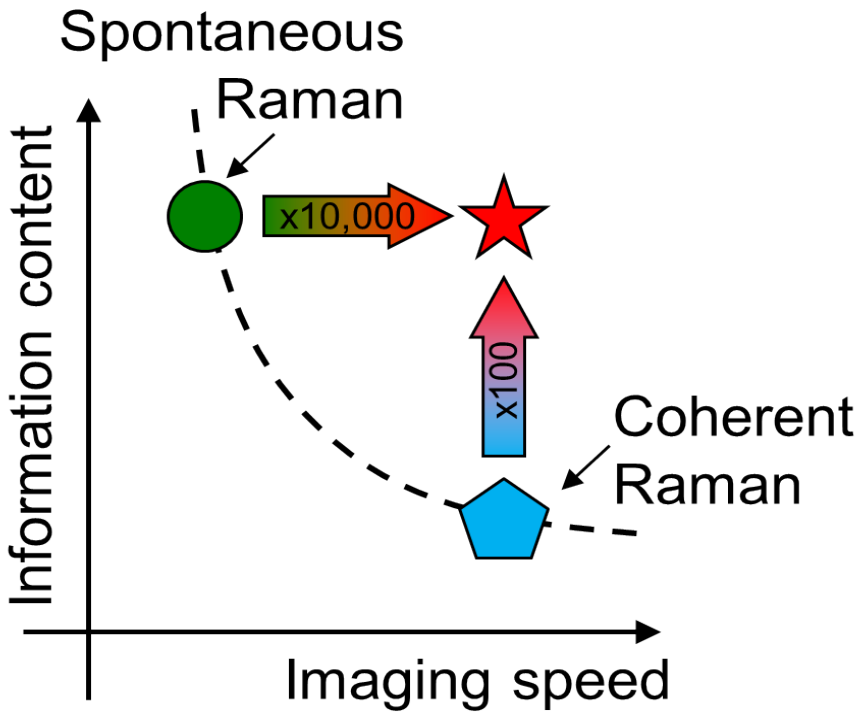
PNAS **112**, 11624 (2015)

Label-free SRS imaging of brain tumors



Science Translational Medicine **5**, 201ra119 (2013)

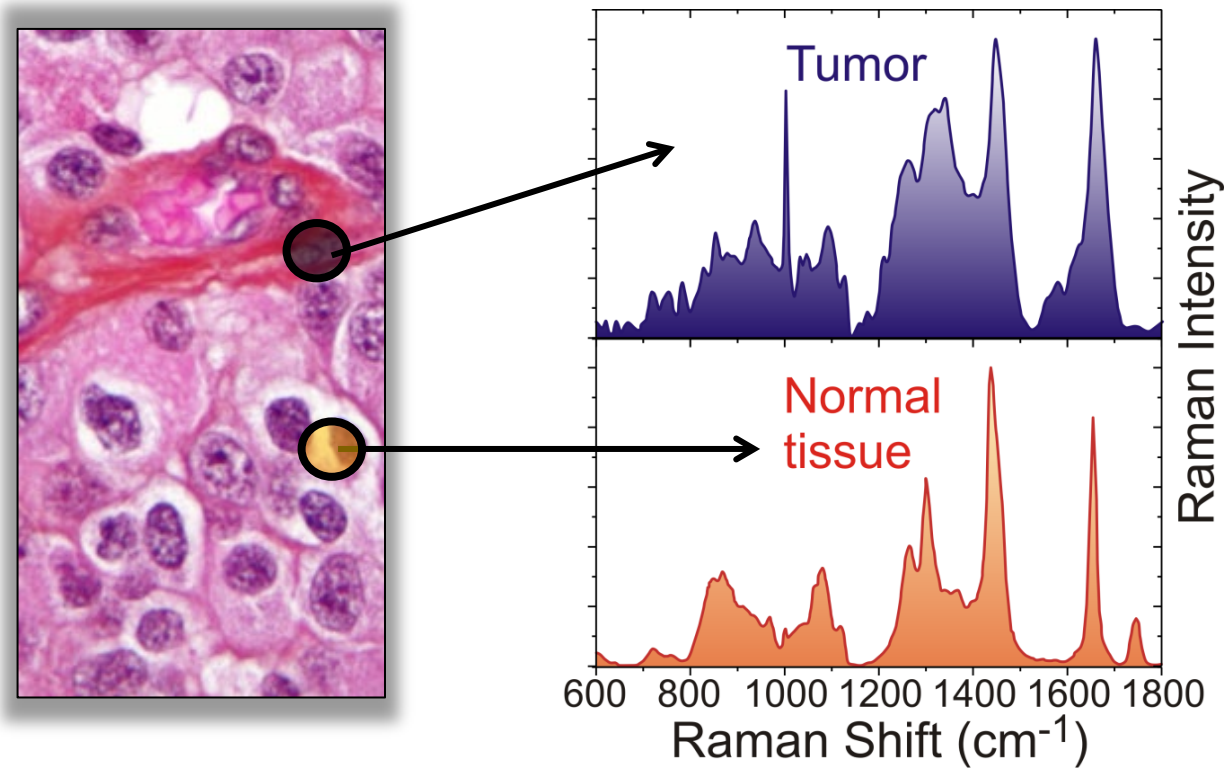
GOAL: fast and broadband SRS microscopy



Combine the best of two worlds: the information content of **spontaneous Raman** and the imaging speed of **coherent Raman**.

GOAL: fast and broadband SRS microscopy

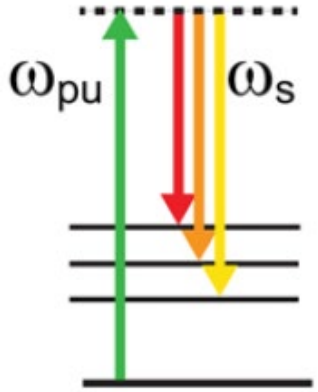
Broadband high-speed coherent Raman Microscope

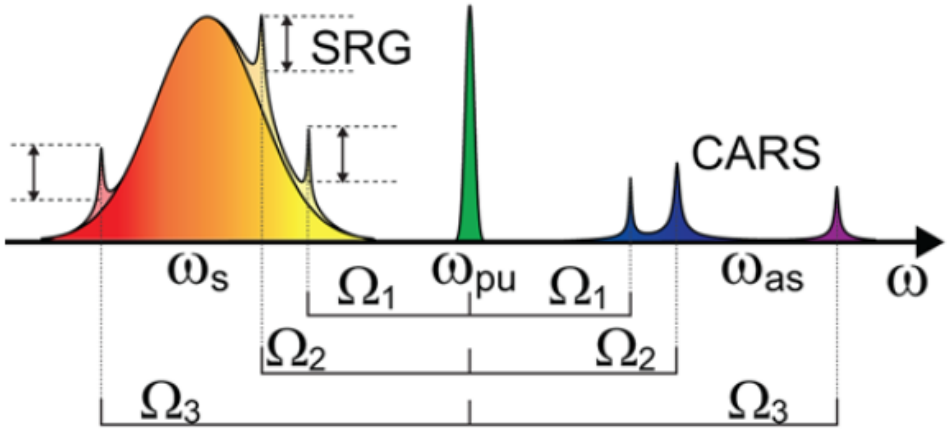


Real-time **classification** of cells and tissues

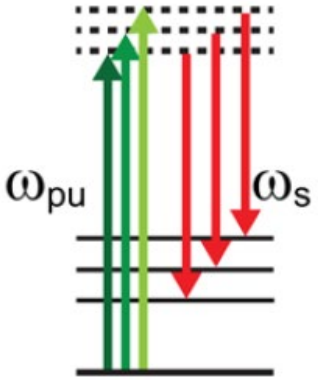
Broadband stimulated Raman scattering

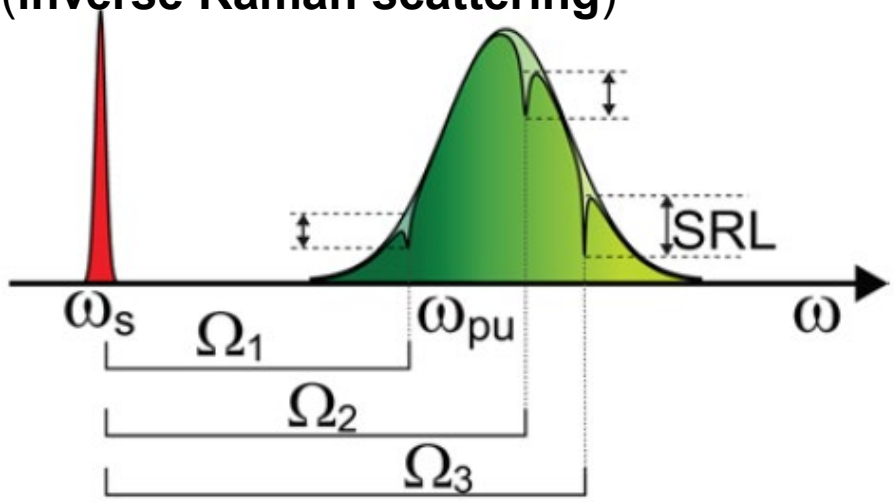
Combination of narrowband pump and broadband Stokes, measuring the stimulated Raman gain of the Stokes



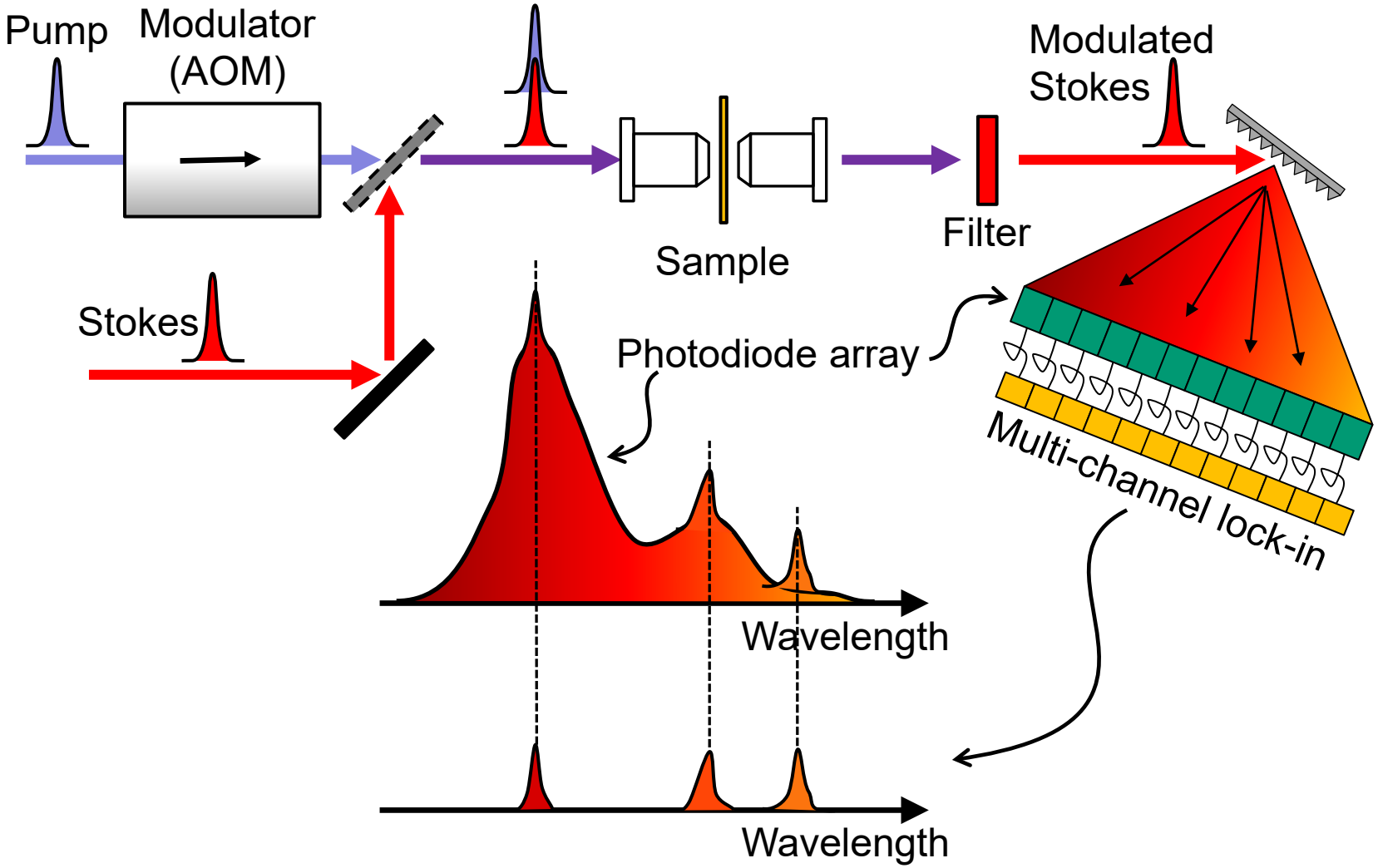


*A*lternative mechanism: broadband pump and narrowband Stokes, measuring the stimulated Raman loss of the pump (**inverse Raman scattering**)

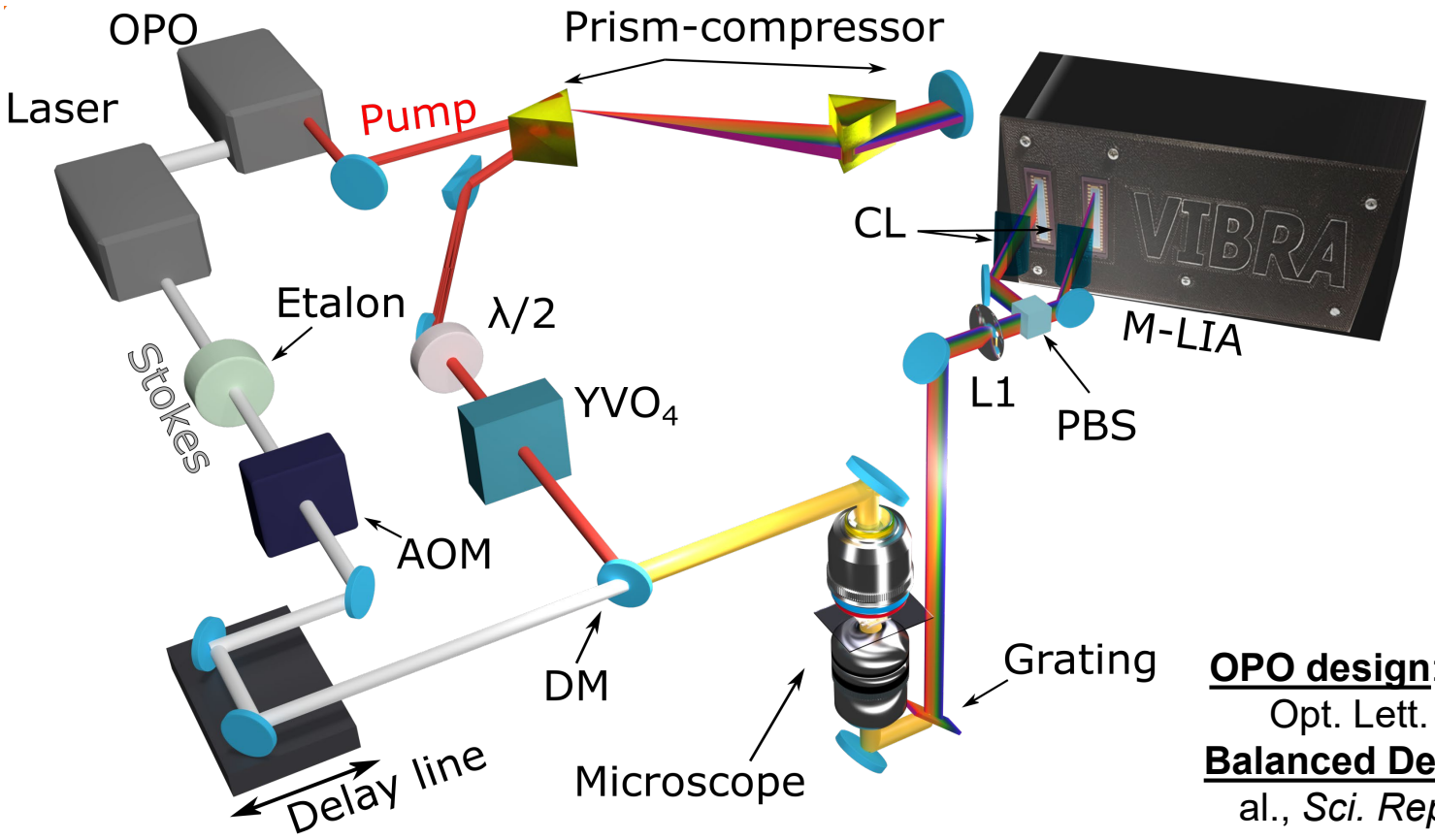




Multi-channel lock-in SRS



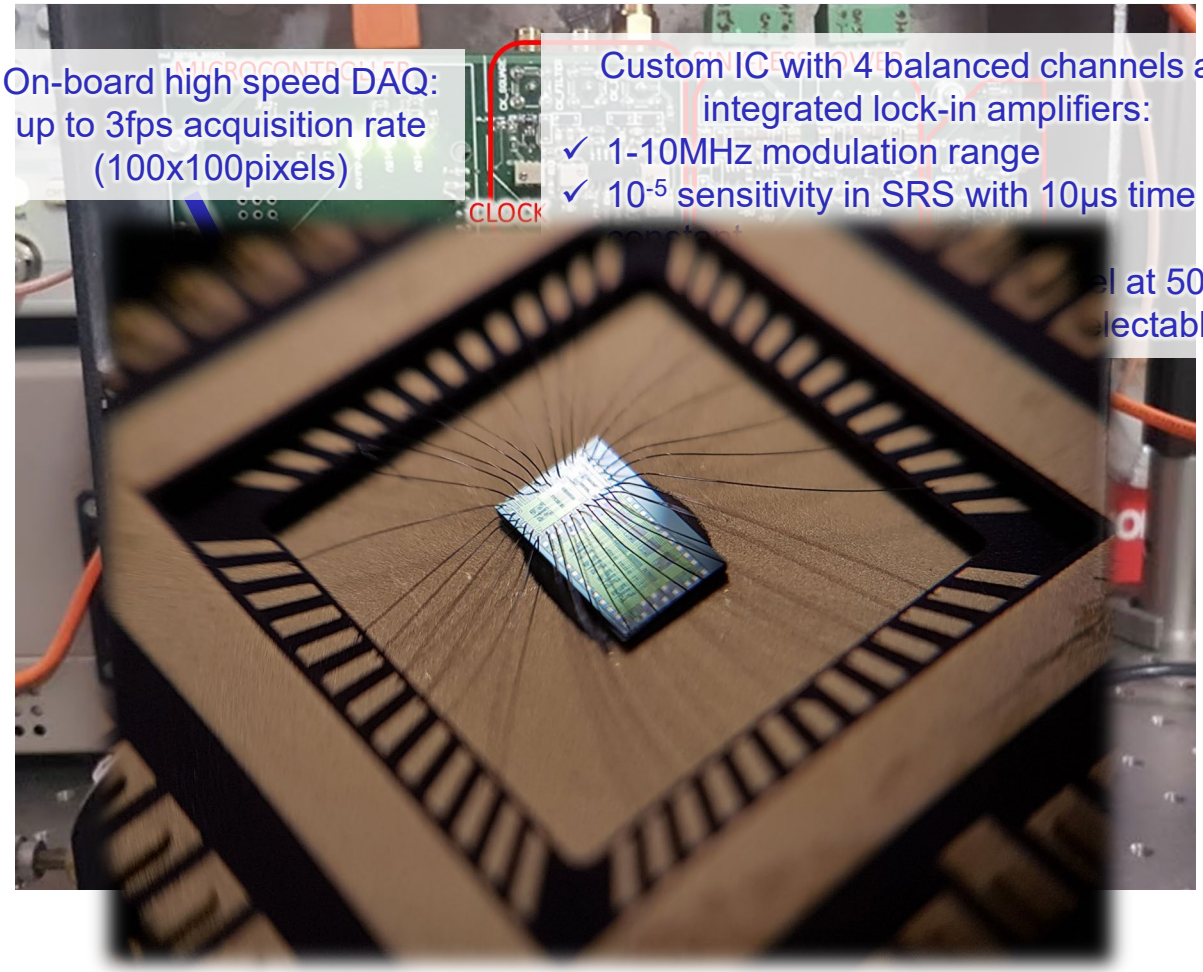
Multi-channel lock-in SRS



A. De La Cadena

OPO design: N. Coluccelli *et al.*, Opt. Lett. **42**, 4545 (2017)
Balanced Detection: F. Crisafi et al., *Sci. Rep.* **7**, 10745 (2017)

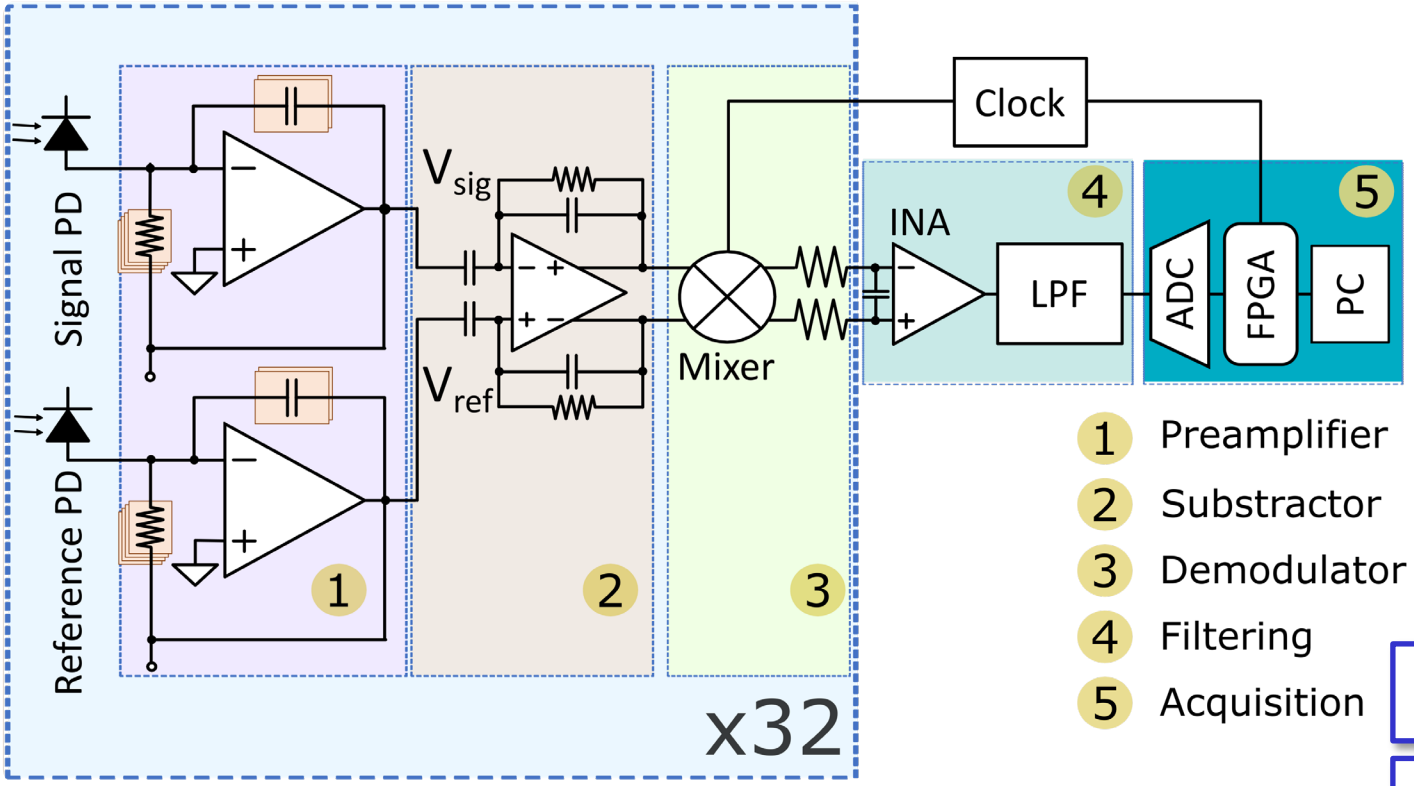
Multi-channel Lock-In Amplifier



G. Sciortino *et al.*, IEEE J. Solid State Circuits (2021)

A. Ragni *et al.*, Integration, the VLSI Journal **67**, 44 (2019)

Multi-channel Lock-In Amplifier



1. Preamplifier
2. Substractor
3. Demodulator
4. Filtering
5. Acquisition

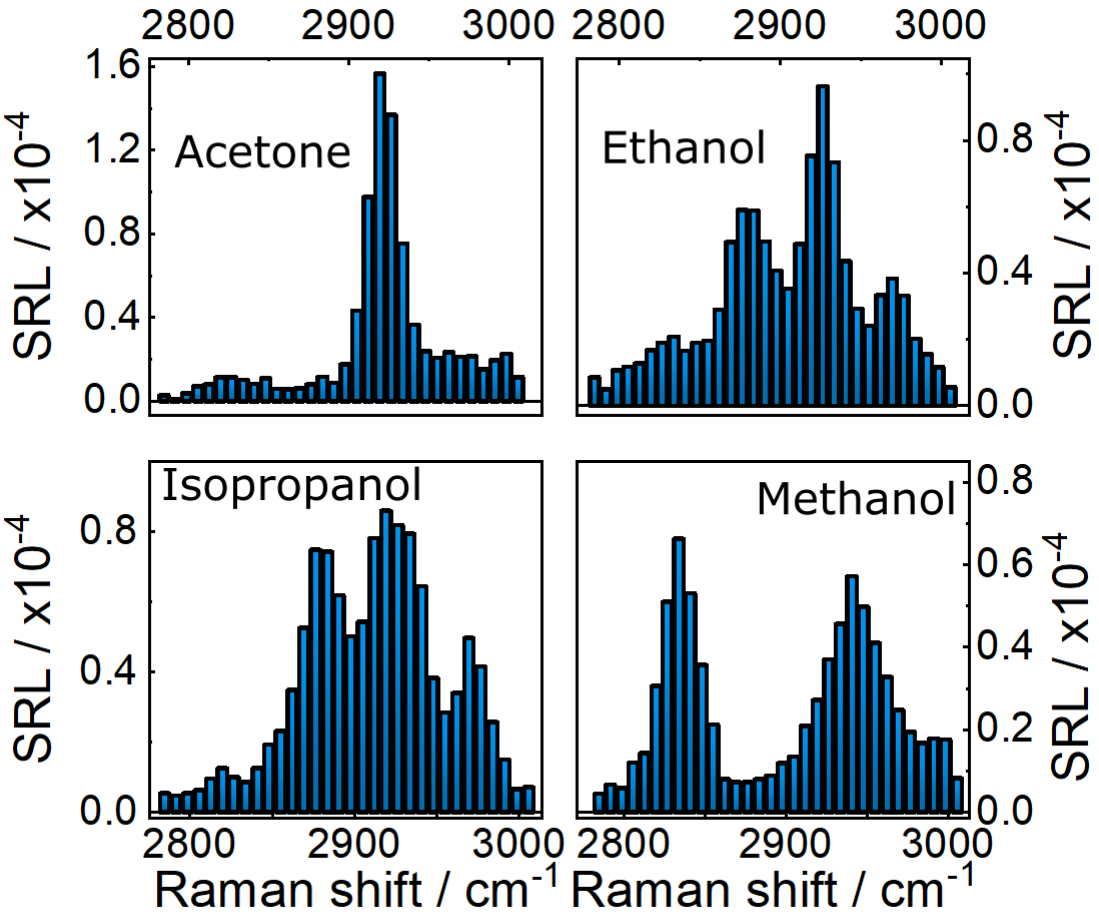
A. Ragni

G. Sciortino

G. Sciortino *et al.*, IEEE J. Solid State Circuits (2021)

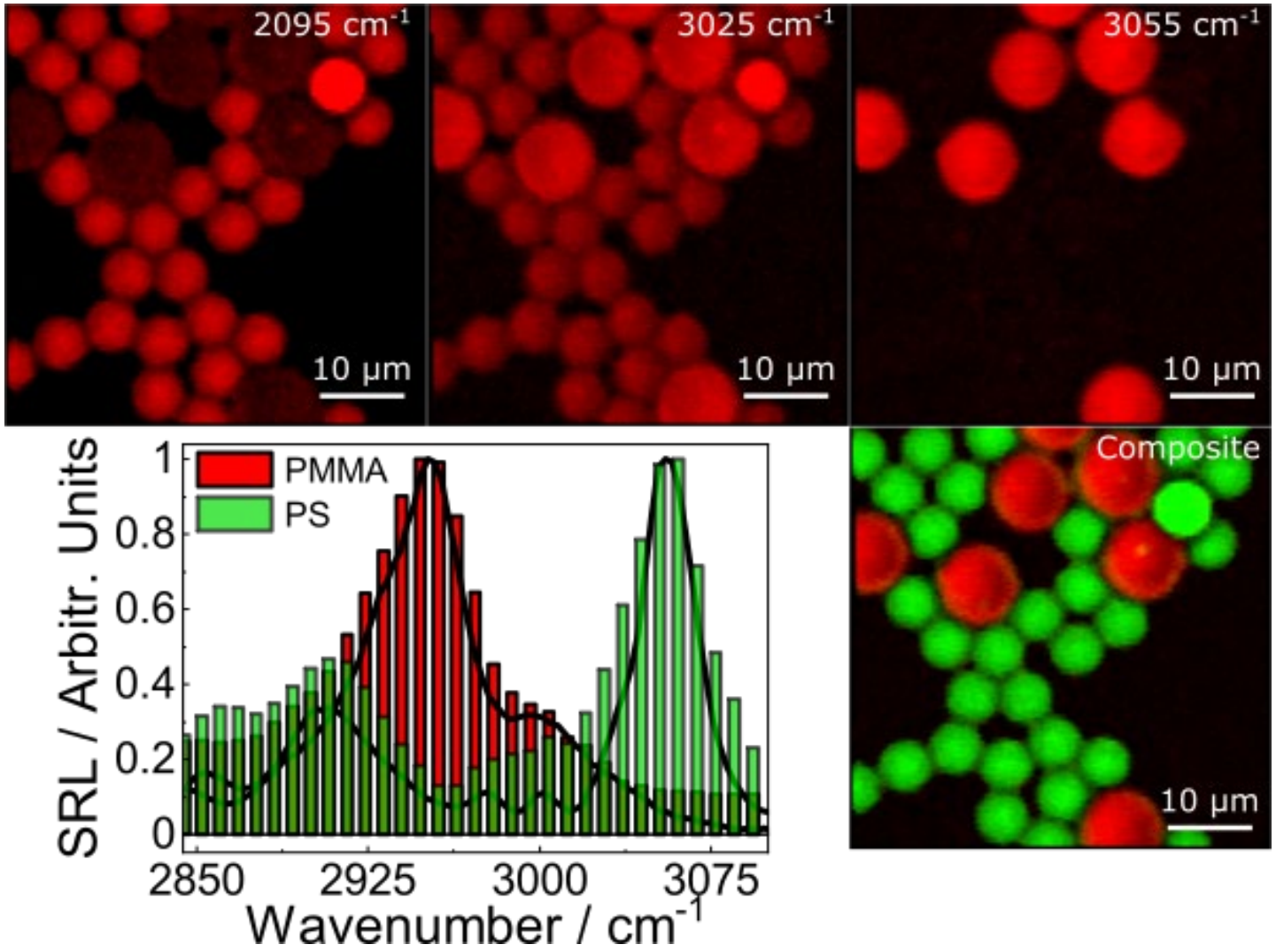
A. Ragni *et al.*, Integration, the VLSI Journal **67**, 44 (2019)

Broadband SRS spectra of solvents

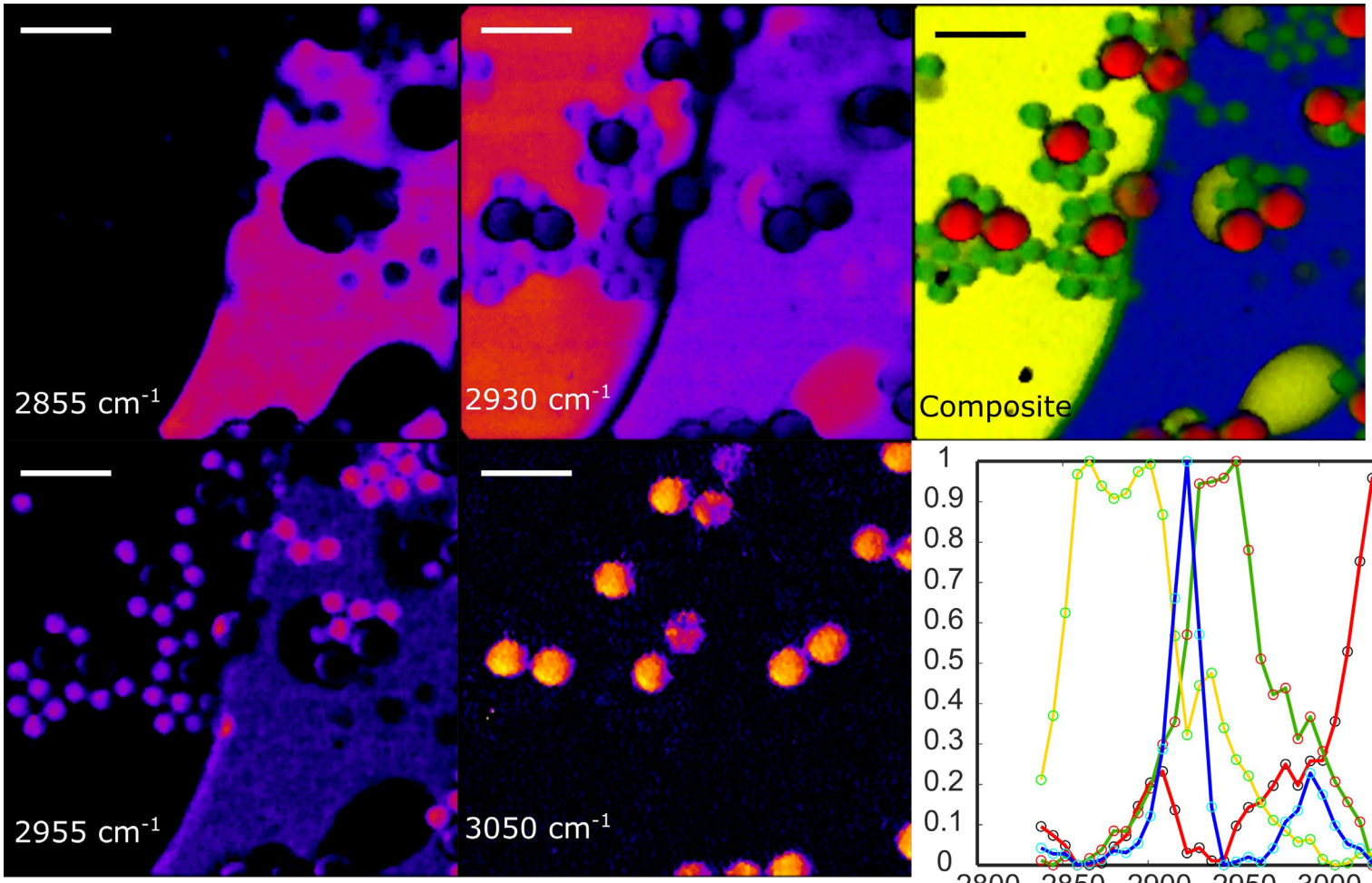


- Very good agreement with spontaneous Raman spectra

Broadband SRS imaging of PMMA/PS beads

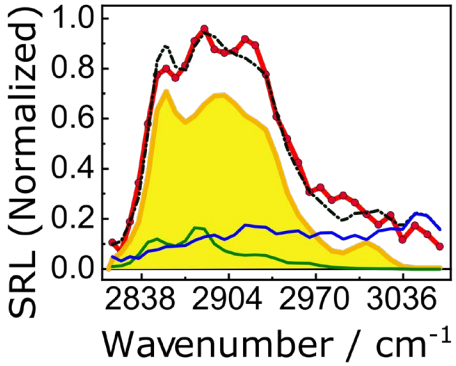


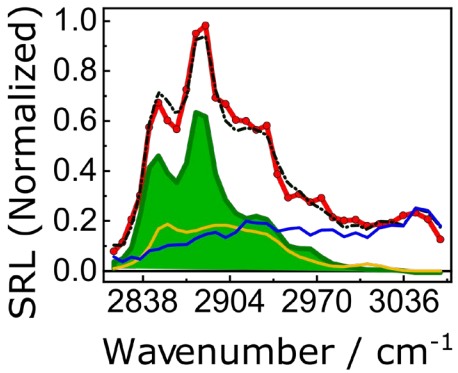
Distinguishing four separate chemical species

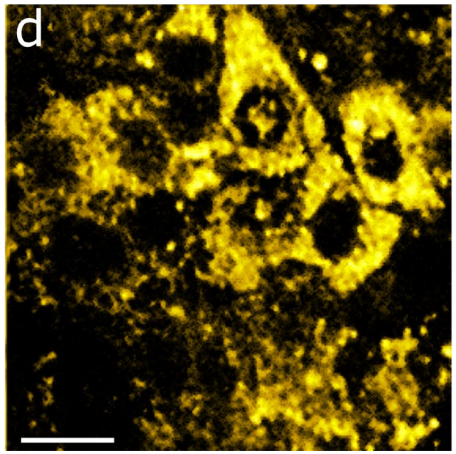


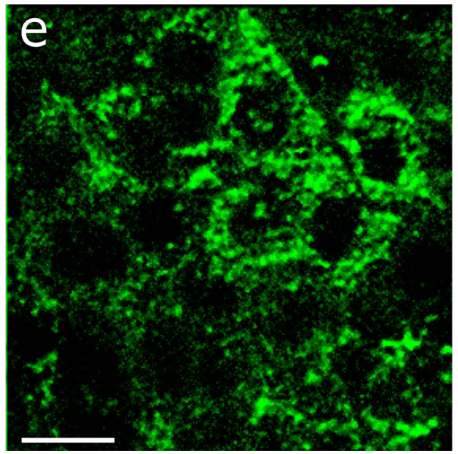
Distinguishing fatty acid uptake in hepatic cells

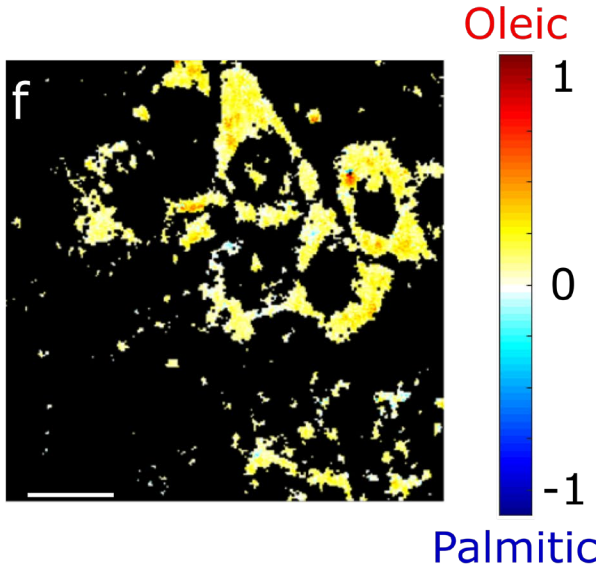
Broadband SRS-imaging of HepaRG cells grown in a culture media containing oleic and palmitic fatty acids







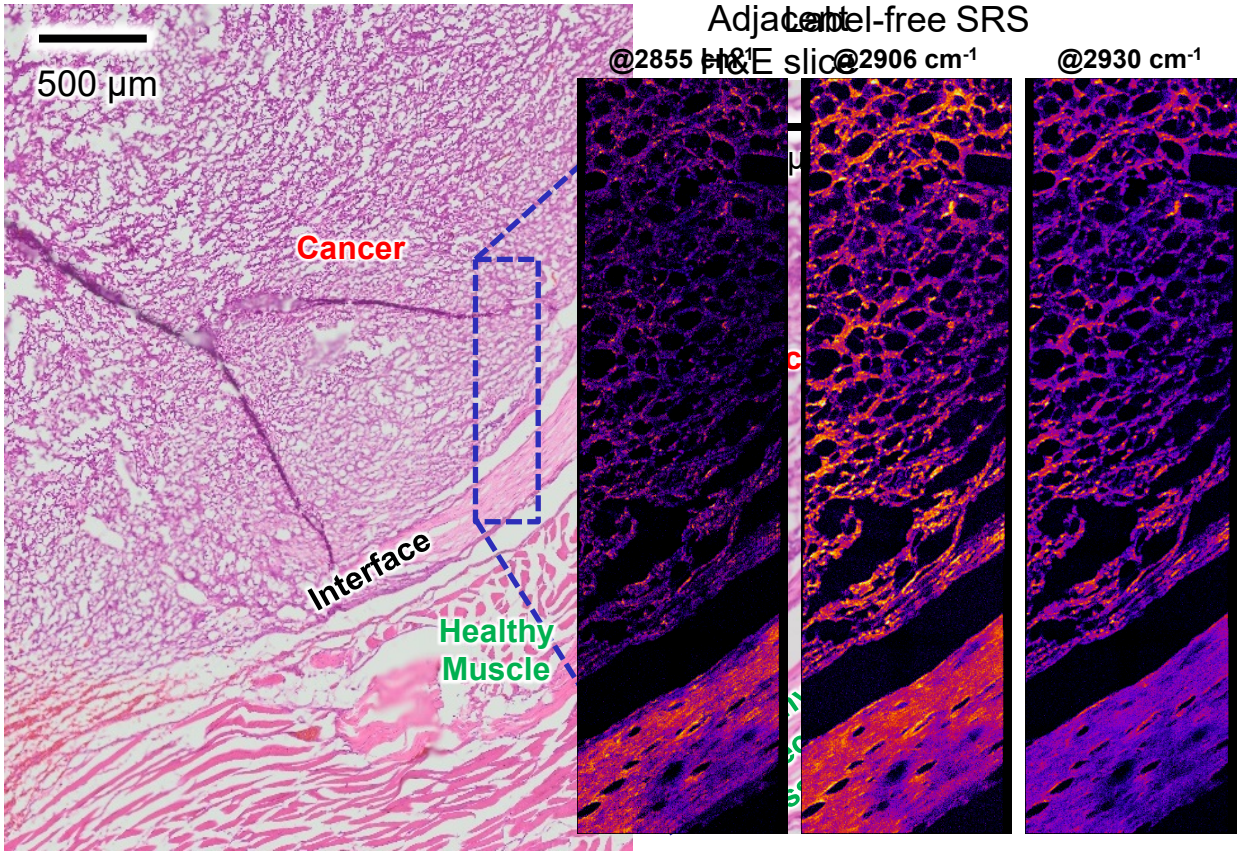




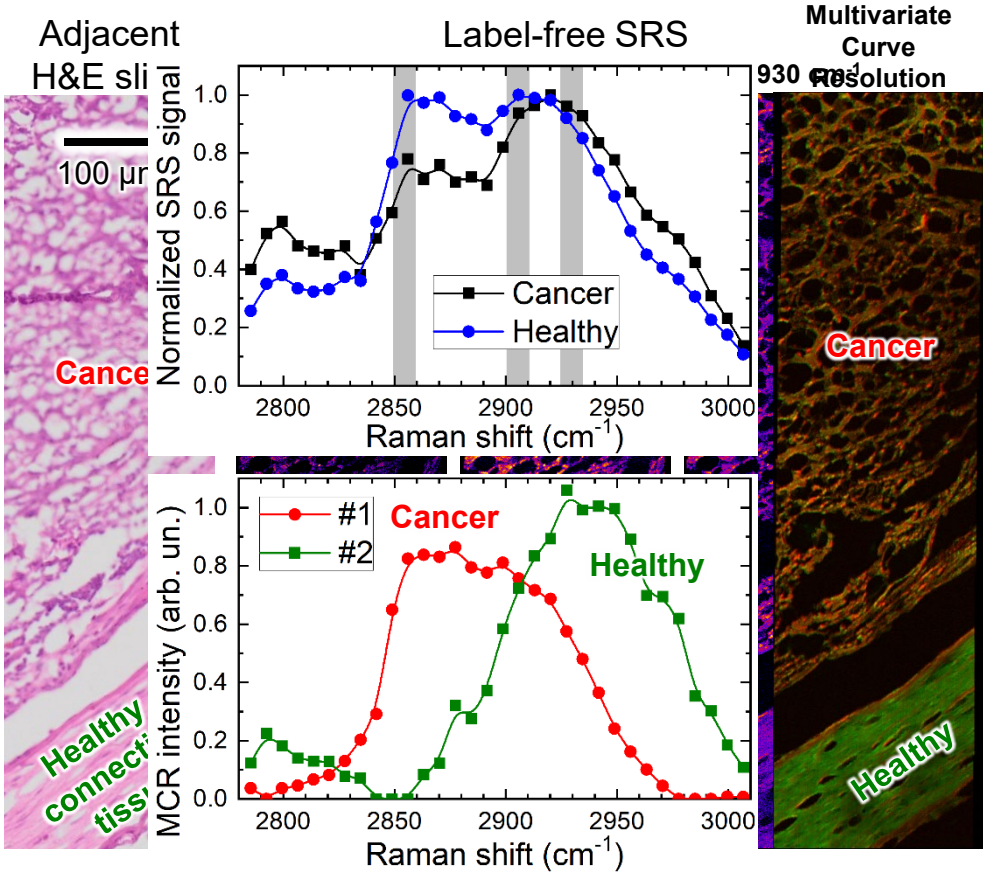
oleic acid

palmitic acid

Tumor model – Mouse fibrosarcoma



Tumor model – Mouse fibrosarcoma



Conclusions

- SRS is a powerful approach for fast label-free imaging of biomolecules

- Broadband SRS combines acquisition speed with information content

- Broadband SRS enables high-speed label-free tissue imaging for virtual histopathology

CRI
chemometric imaging

https://www.cambridgeramanimaging.com/