Chalmers University of Technology

**Annicka Enejder**  
Associate Professor, group leader of Molecular Microscopy  
Develops microscopy techniques (CARS, SRS, SHG, and Raman) where inherent molecular vibrations are probed to 3D images

**Juris Kiskis**  
PhD student in the Group of Molecular microscopy  
Juris works on combining non-linear optical microscopy with scanning probe microscopy to achieve molecular specific nano-scale imaging. The goal is to study the formation of beta-amyloid aggregates and the role of lipids in neurodegenerative disorders without the need for staining and at spatial resolutions significantly higher than that achieved by optical microscopy alone.
Non-linear Raman scattering microscopy techniques

Jūris Kiškis

Group of Molecular Microscopy
Department of Chemical and Biological Engineering
Chalmers University of Technology
Göteborg
Imaging lipid metabolism in **live** Caenorhabditis elegans using fingerprint vibrations
Imaging lipid metabolism in *live* Caenorhabditis elegans using *fingerprint vibrations*
Imaging using fingerprint vibrations
Imaging using **fingerprint** vibrations

When I look at *c. elegans*, I see this
Objectives of the lecture

After the lecture I hope that you will be able to:

• explain how different light-molecule interactions can be used in microscopy and what information about molecule can be extracted using energy diagram of the molecule.

• Make a sketch of spontaneous, stimulated and coherent anti-Stokes Raman scattering using energy diagram of the molecule and explain how Raman spectra are measured.

• Compare labeling microscopy with coherent Raman scattering microscopy in terms of chemical specificity, resolution and invasiveness of the method.
Unstained and Stained Specimens in Brightfield Illumination

Figure 2
Basophilic and acidophilic staining
Basophilic and acidophilic staining

- Cationic or basic components
  - Cytoplasmic proteins

- Anionic or acidic components
  - Nucleic acids
Immunohistochemistry
Immunohistochemistry

Targeting antigens with specific antibodies tagged with a visible label
Fluorescent protein labeling
Fluorescent protein labeling

Genetically modifying original protein to include a sequence of fluorescent protein.
<table>
<thead>
<tr>
<th>Labeling</th>
<th>Selectivity</th>
<th>Sample image</th>
</tr>
</thead>
<tbody>
<tr>
<td>No labeling</td>
<td>No selectivity/Natural pigments</td>
<td></td>
</tr>
<tr>
<td>Basophilic and acidophilic staining</td>
<td>Acidic/Basic components</td>
<td></td>
</tr>
<tr>
<td>Immuno-histochemistry</td>
<td>What antibodies bind</td>
<td></td>
</tr>
<tr>
<td>Fluorescent protein labeling</td>
<td>Expressed genetically modified proteins</td>
<td></td>
</tr>
</tbody>
</table>
Imaging using molecular vibrations
A graph showing the Raman shift (cm$^{-1}$) against $\Delta I/I (x10^5)$ for various substances:

- Oxidized lipids
- Fat droplets
- LROs
- Protein

There is a peak at 1680 cm$^{-1}$. A close-up image below the graph shows a microscopic view with different colors representing each substance.
Vibrations of a methylene group (-CH₂-)
Vibrations of a methylene group (-CH$_2$-)
Energy levels of the molecule

First excited electronic state ($E_1$)

Vibrational levels of $E_1$

Ground electronic state ($E_0$)

Vibrational levels of $E_0$
Absorption (VIS)
Fluorescence

Absorption

Emission

Energy

V4 -> V1 -> E1
V3 -> V2 -> V1
V2 -> V1
V1

E0
Absorption (mid-IR)

Absorption

Energy

\( E_0 \)

\( V_1 \)

\( V_2 \)

\( V_3 \)

\( V_4 \)
Absorption (mid-IR)

$E_0 \rightarrow v_1 \rightarrow v_2 \rightarrow v_3 \rightarrow v_4$

Energy

Absorption
Energy levels of the molecule
Energy levels of the molecule
Energy levels of the molecule

- O-H stretch
- C-H stretch
- C-C stretch
- P-O stretch
- C-O stretch
Absorption spectrum of liquid water
Absorption spectrum of liquid water

IR vibrational spectroscopy
1000-4000 cm$^{-1}$
Raman scattering

Rayleigh

Energy

$E_1$

$E_0$

$v_1$

$v_2$

$v_3$

$v_4$
Fluorescence background in Raman

Stokes
Raman scattering

$E_1$
$v_1$
$v_2$
$v_3$
$v_4$
$E_0$

Energy
Fluorescence background in Raman

Stokes
Raman scattering
Fluorescence?

E₁

V₄
V₃
V₂
V₁

E₀

Energy
Fluorescence background in Raman

![Fluorescence background in Raman diagram](image-url)
Non-linear Raman scattering techniques
Stimulated Raman scattering (SRS)

$E_0$

$v_1$

$v_2$

$v_3$

$v_4$
Stimulated Raman scattering (SRS)

Photon of the same energy “stimulates” emission of identical photon

\[ E_0, v_1, v_2, v_3, v_4 \]
Stimulated Raman scattering (SRS)

Photon of the same energy "stimulates" emission of identical photon

\[ v_1, v_2, v_3, v_4 \]

Energy
Stimulated Raman scattering (SRS)
Coherent anti-Stokes Raman scattering (CARS)

\[ \omega_p, \omega_s \]

\[ v_1, v_2, v_3, v_4 \]

\[ \omega_p - \omega_s = \Omega \]

\[ E_0 \]

\[ \Omega \]
Coherent anti-Stokes Raman scattering (CARS)

Energy levels:

\[ \omega_p, \omega_s, \omega_1, \omega_2, \omega_3, \omega_4 \]

Pump:

\[ \omega_p - \omega_s = \Omega \]

\[ Y_p = Y_s \]
Coherent anti-Stokes Raman scattering (CARS)

\[ \omega_{as} = \omega_p + \Omega \]

\[ \omega_p - \omega_s = \Omega \]

\[ E_0 \]

\[ \omega_p \quad \omega_s \quad \omega_{as} \]

CARS

Pump

Stokes

Energy

\[ v_1 \quad v_2 \quad v_3 \quad v_4 \]
Coherent anti-Stokes Raman scattering (CARS)

\[ \omega_p, \omega_s, \omega_{as} \]

\[ v_1, v_2, v_3, v_4 \]

\[ \Omega \]

\[ \omega_p - \omega_s = \Omega \]

\[ \omega_{as} = \omega_p + \Omega \]
Nonlinear optics
Single photon vs two photon excitation fluorescence
Non-linear processes
Multimodal non-linear microscope

- SU - scanning mirror
- FG - function generator
- OM - optical modulator
Applications
RNAi screening for fat regulatory genes with SRS microscopy

Label-free Chemically Specific Imaging in Planta with Stimulated Raman Scattering Microscopy

Commercially available fungicides azoxystrobin and chlorothalonil

Label-free Chemically Specific Imaging in Planta with Stimulated Raman Scattering Microscopy

(C) Chlorothalonil applied to a maize leaf blue = SRL at 2234 cm\(^{-1}\) from the CN bond, green = SRL at 2930 cm\(^{-1}\) from the CH\(_3\) vibrations).

(D) Azoxystrobin applied to a maize leaf red = SRL at 2225 cm\(^{-1}\) from the CN bond, green = SRL at 2930 cm\(^{-1}\) from the CH\(_3\) vibrations)

Label-free Chemically Specific Imaging in Planta with Stimulated Raman Scattering Microscopy

Many agrochemicals do not contain Raman vibrations within the silent region. To aid chemically specific imaging of these compounds deuterium labeling was investigated.

Label-free Chemically Specific Imaging in Planta with Stimulated Raman Scattering Microscopy


Deuterated Glyphosate crystal in formulation.

Deuterated Glyphosate on maize leaf.
Chemical imaging of lignocellulosic biomass by CARS microscopy

Green - cellulose
Red – xylan
Blue - lignin

Cellulose alignment

Label-Free, Real-Time Monitoring of Biomass Processing with Stimulated Raman Scattering Microscopy

Label-Free, Real-Time Monitoring of Biomass Processing with Stimulated Raman Scattering Microscopy

<table>
<thead>
<tr>
<th>Method</th>
<th>Selectivity</th>
<th>Sample image</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basophilic and acidophilic staining</td>
<td>Acidic/Basic components</td>
<td><img src="image1" alt="Sample image" /></td>
</tr>
<tr>
<td>Immuno-histochemistry</td>
<td>What antibodies bind</td>
<td><img src="image2" alt="Sample image" /></td>
</tr>
<tr>
<td>Fluorescent protein labeling</td>
<td>Expressed genetically modified proteins</td>
<td><img src="image3" alt="Sample image" /></td>
</tr>
<tr>
<td>Raman scattering</td>
<td>Molecular vibrations</td>
<td><img src="image4" alt="Sample image" /></td>
</tr>
</tbody>
</table>
Objectives of the lecture

After the lecture I hope that you are able to:

• Using energy diagram of the molecule explain how different light-molecule interactions can be used in microscopy and what information about molecule can be extracted.

• Make a sketch of spontaneous, stimulated and coherent anti-Stokes Raman scattering using energy diagram of the molecule and explain how Raman spectra are measured.

• Compare fluorescence labeling microscopy with coherent Raman scattering microscopy in terms of chemical specificity, resolution and invasiveness of the method.
Nonlinear Raman – staining/labeling

Raman  Staining/labeling

• Specific to what?
  Think in terms of your research

• Invasive? How?

• Resolution. What are limiting factors?

• What else might be of interest to compare?
Nonlinear Raman – staining/labeling

Raman
• Probes vibrations. Specific only if substances have different spectral components
• High powers, in case of intrinsic pigments can lead to photodamage
• NIR light + nonlinearity: resolution comparable to VIS. Advantage – intrinsic confocality.

Staining/labeling
• Specificity depends on the label. In case of antibodies – as specific as antibodies can be.
• Staining/labeling modifies the object of study. Care should be taken about the level of interference.
• Can be used with multi-photon excitation techniques
Do you have questions?
Do you have questions?

• What is Raman scattering?
• Molecular vibrations – what is it?
• Why nonlinear microscopy is intrinsically “confocal”?
• There are many nonlinear optical processes? Why should I care? 😊
Thanks!

If you still have questions, feel free to contact me at juris@chalmers.se