

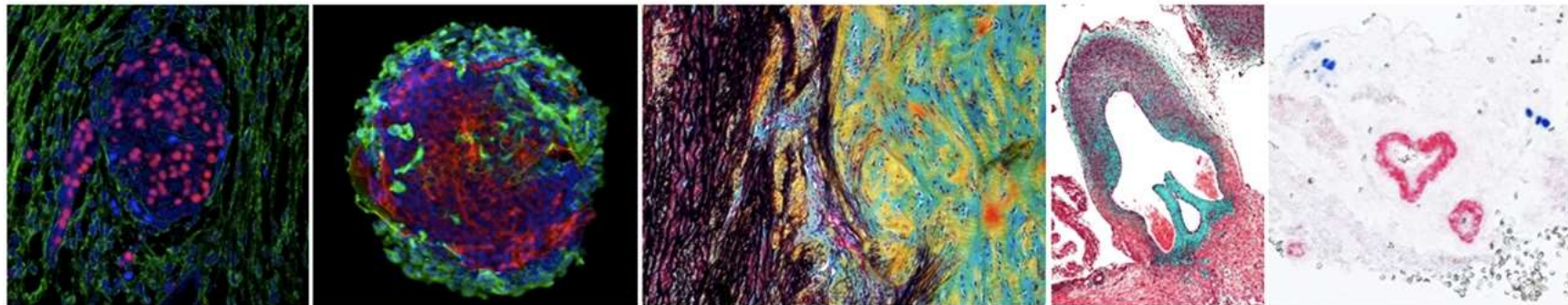
Non-invasive imaging and biomedical applications

Eva Brauchle

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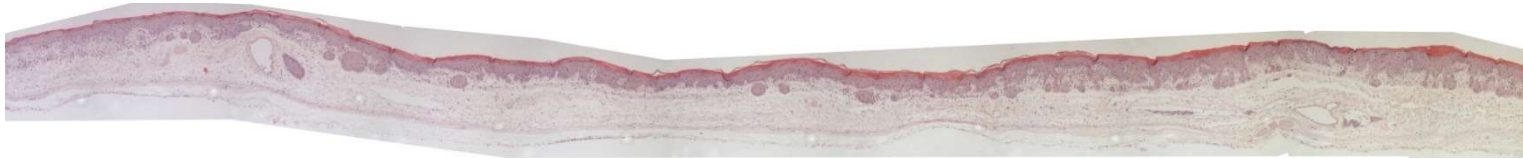


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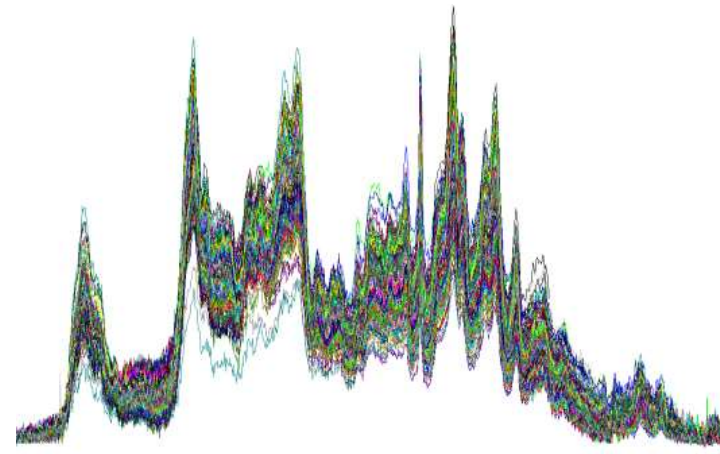
EBERHARD KARLS
UNIVERSITÄT
TÜBINGEN



Non-invasive imaging and biomedical applications

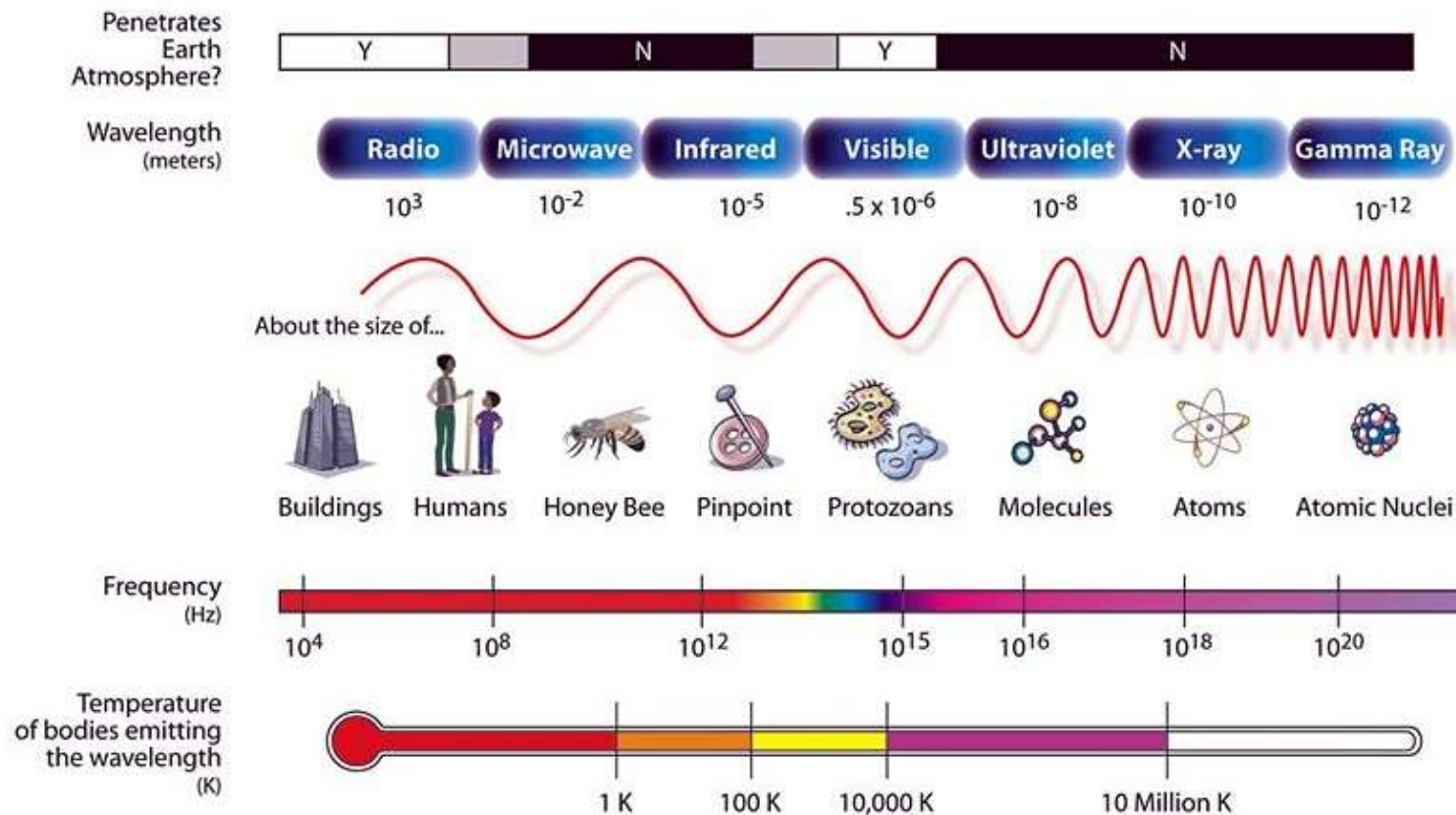


- Introduction: Basics of Microscopy
- Raman microscopy
 - Basic principle
 - Cell analysis
 - Tissue (ECM) analysis
- Multiphoton Imaging
 - SHG
 - TPEF
 - Applications



Introduction

Light - an electromagnetic wave

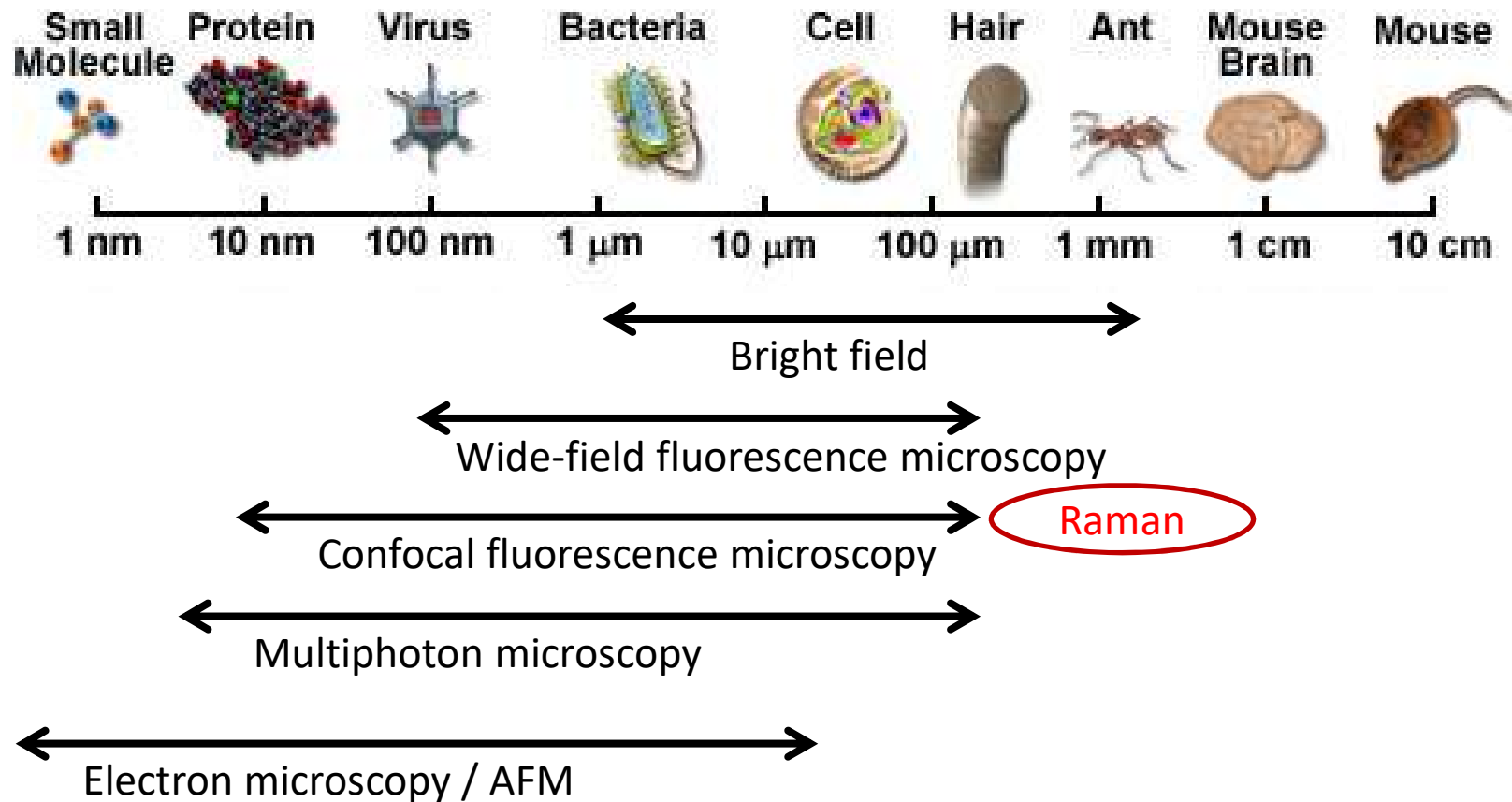


<http://www.scienceblogs.de/frischer-wind/2009/04/es-grunt-so-rot-ledbeleuchtung-im-gewachshaus.php>

Eva Brauchle

Introduction

Spatial Resolution of Biological Imaging Techniques



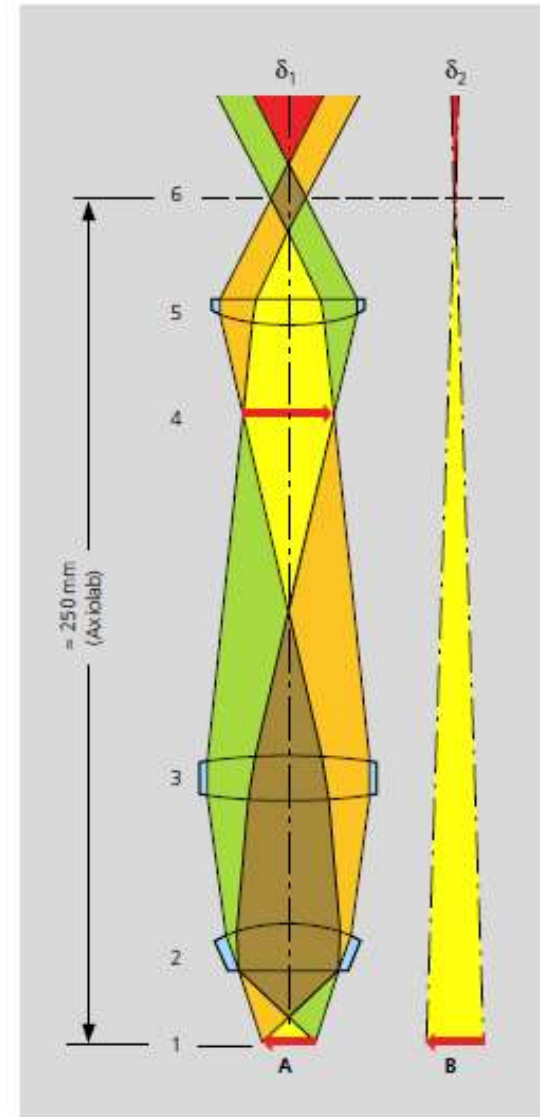
<http://zeiss-campus.magnet.fsu.edu/articles/superresolution/introduction.html>

Basic light pathway

1. Object
2. Objective
3. Tube lens
4. Magnified intermediate image
5. Eyepiece (ocular)
6. Eye

Magnification:

$$M_{\text{microscope}} = M_{\text{objective}} \times M_{\text{ocular}}$$

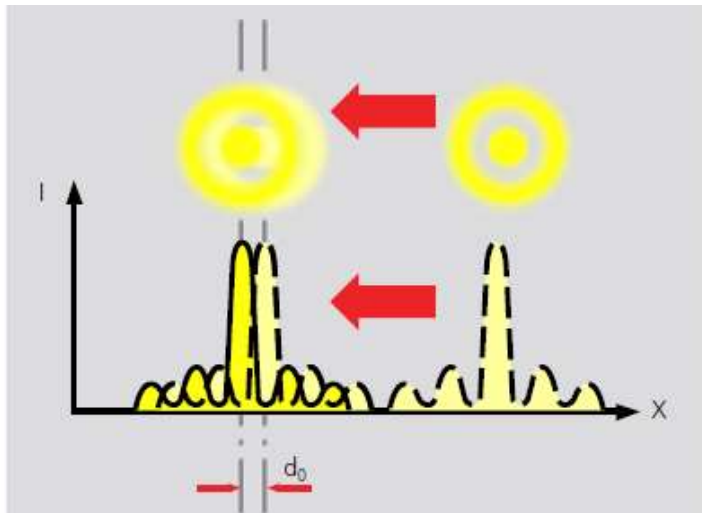


Resolution

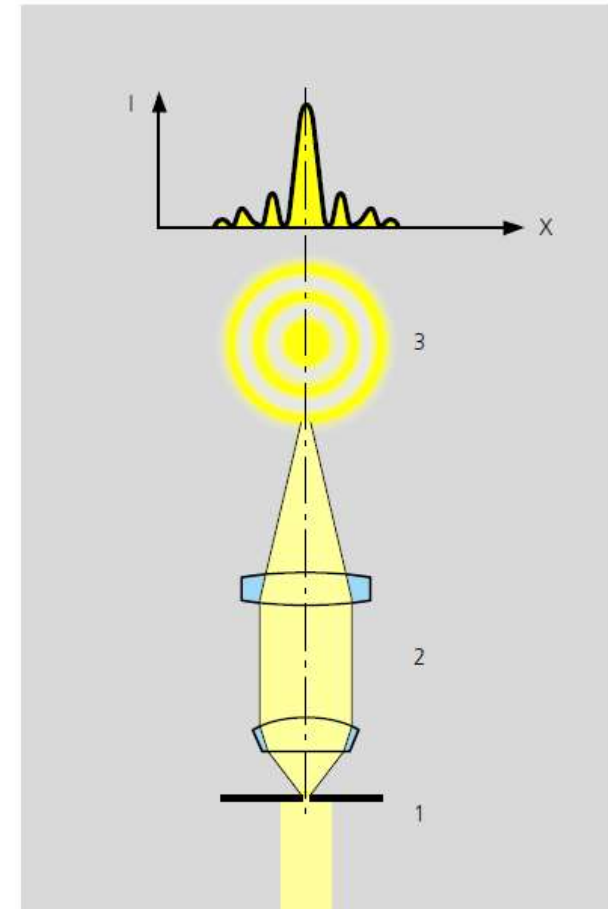
- d_0 = distance at which two points are seen separable

$$d_0 = \frac{1.22\lambda}{N.A_{\text{obj.}} + N.A_{\text{Cond}}} \quad \text{or more simply } d_0 = \frac{\lambda}{2N.A}$$

λ = wavelength of light, e.g. 550 nm (green)



Zeiss: Microscopy from the very beginning

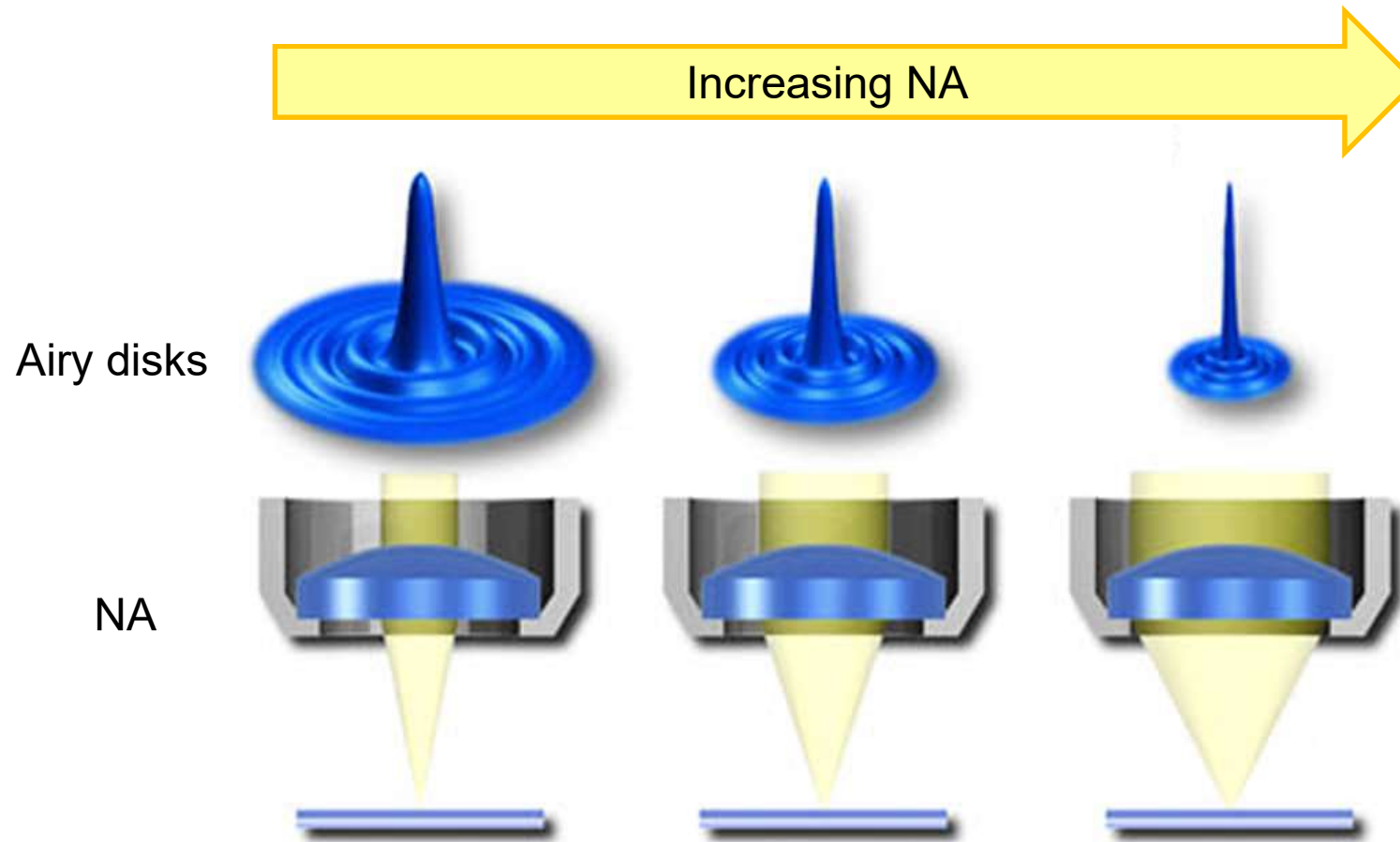


Zeiss: Microscopy from the very beginning

- 1 Object
- 2 Objective and tube lens
- 3 Diffraction rings = "airy disk"

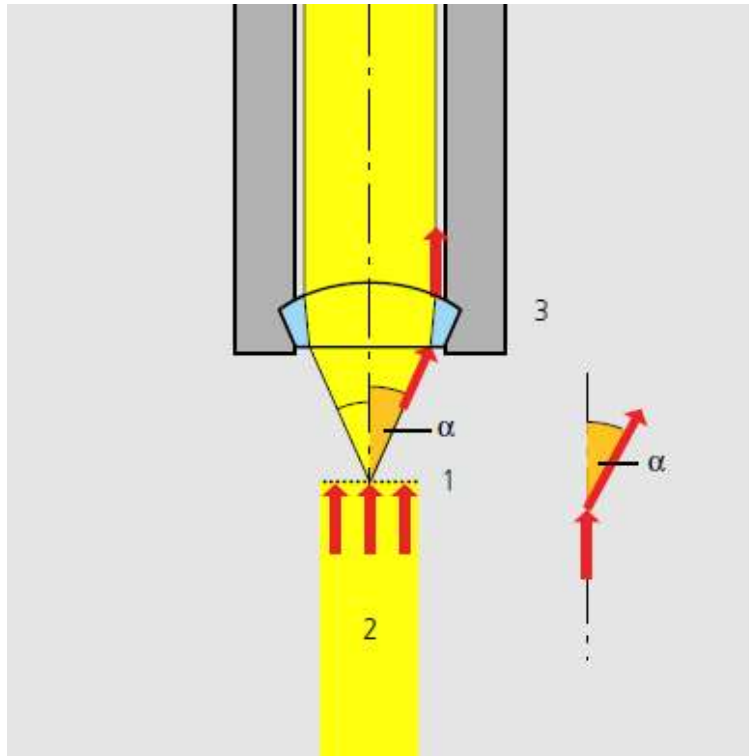
=> The resolution is dependent on the N.A.

Resolution



<http://www.olympusmicro.com/primer/anatomy/numaperture.html>

Numerical Aperture (N.A.)



Zeiss: Microscopy from the very beginning

- 1 Object
- 2 Incident light
- 3 Objective

- N.A. = “opening” of an objective
- The smaller the object the more light is diffracted
- To collect sharp images from small objects, the objective needs to collect as much diffracted light as possible

$$\text{Numerical Aperture} = \text{N.A.} = n \cdot \sin \alpha$$

α is half the opening angle of the objective.

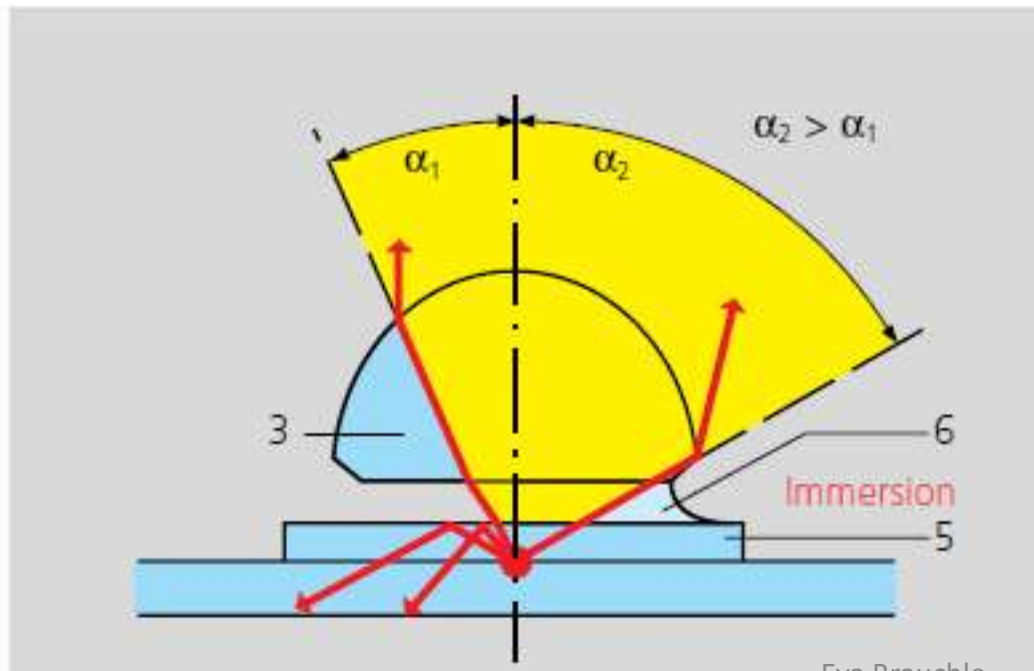
n is the refractive index of the immersion medium used between the objective and the object.

($n = 1$ for air; $n = 1.51$ for oil or glass)

Increasing the resolution

- N.A. is dependent on the refractive index n
- Increasing n due to reduction of the reflections on the microscopic devices

=> more light reaches the objective

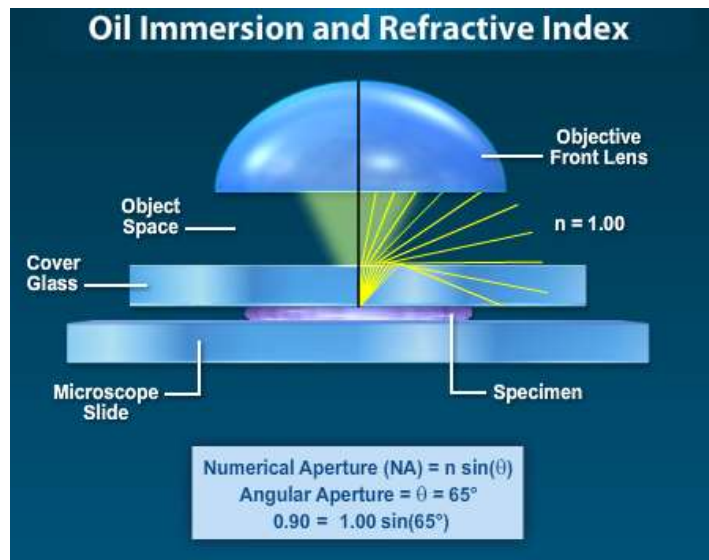


3 Objective
5 Cover slip
6 Immersion liquid

Refractive index

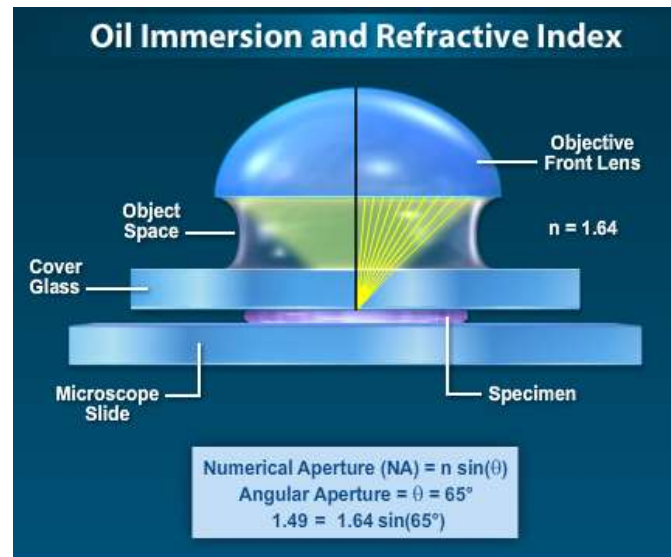
Refractive index n is a value that describes the speed of light in a second medium compared to light speed in vacuum

Low refractive index



<http://zeiss-campus.magnet.fsu.edu/tutorials/basics/oilimmersionrefractiveindex/index.html>

High refractive index



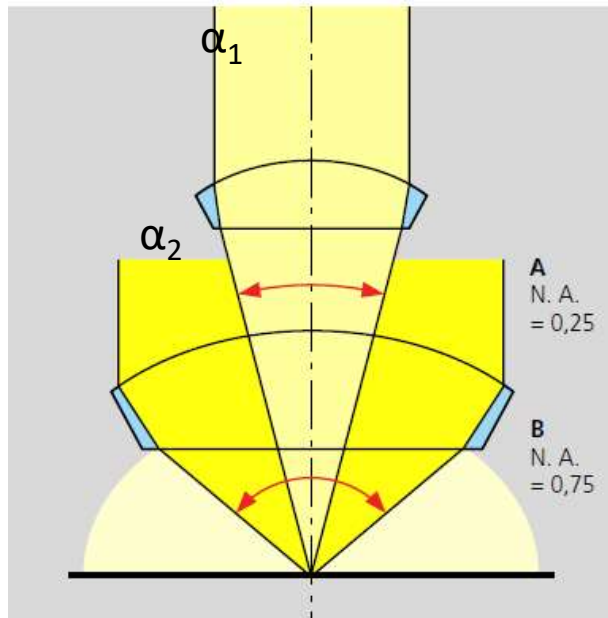
Refractive indices n of immersion liquids

Air	= 1.00
Water	= 1.33
Glycerol	= 1.47
Oil	= 1.57

Increasing the resolution

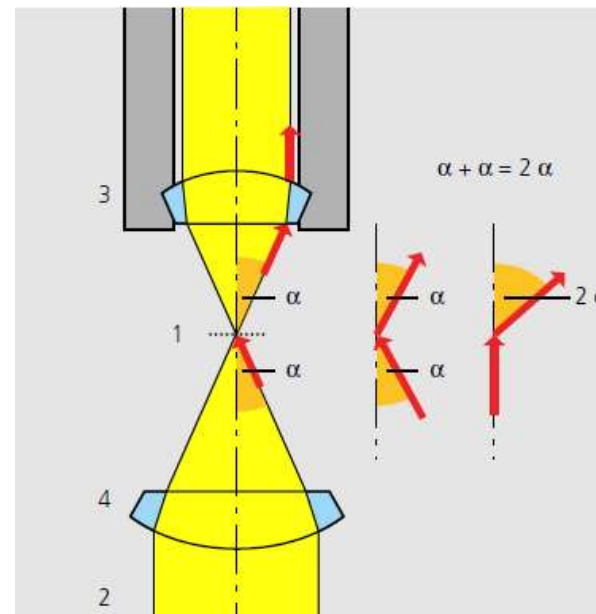
$$d_0 = \frac{\lambda}{2 \text{N.A.}}$$

Increase N.A. of the objective



Zeiss: Microscopy from the very beginning

Condenser lens (4) with the same N.A. as the objective



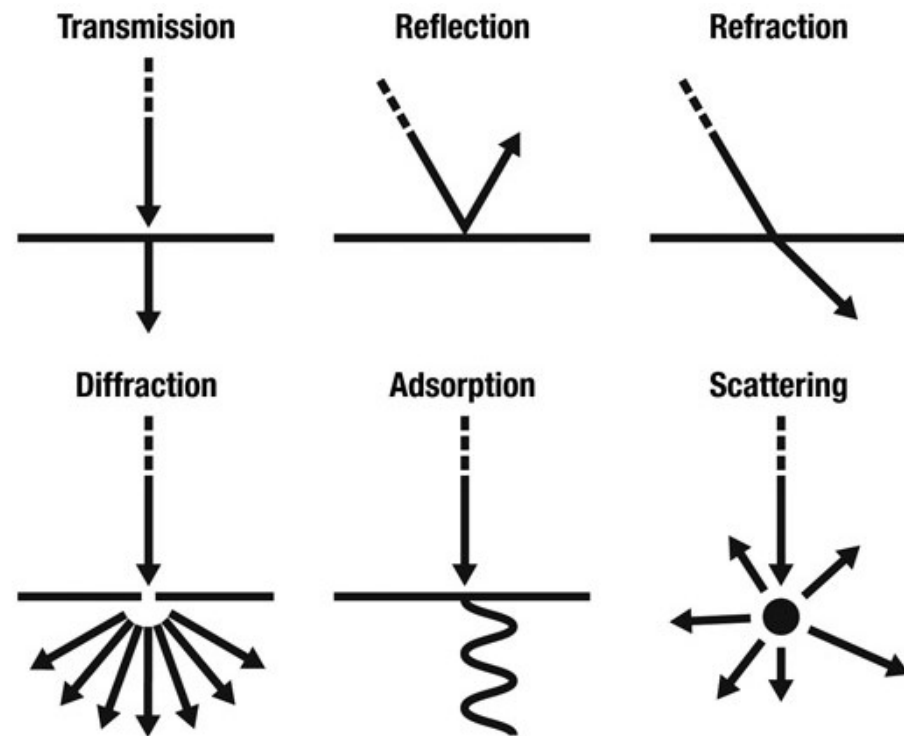
Zeiss: Microscopy from the very beginning

Advantages of optical technologies

- No sample preparation
- Non-invasive and marker-free (no dyes or antibodies needed)
- Suitable for all kind of samples (chemicals, food, biologicals)
- For in vitro and in vivo studies
- Molecular information

Basics of optical spectroscopy

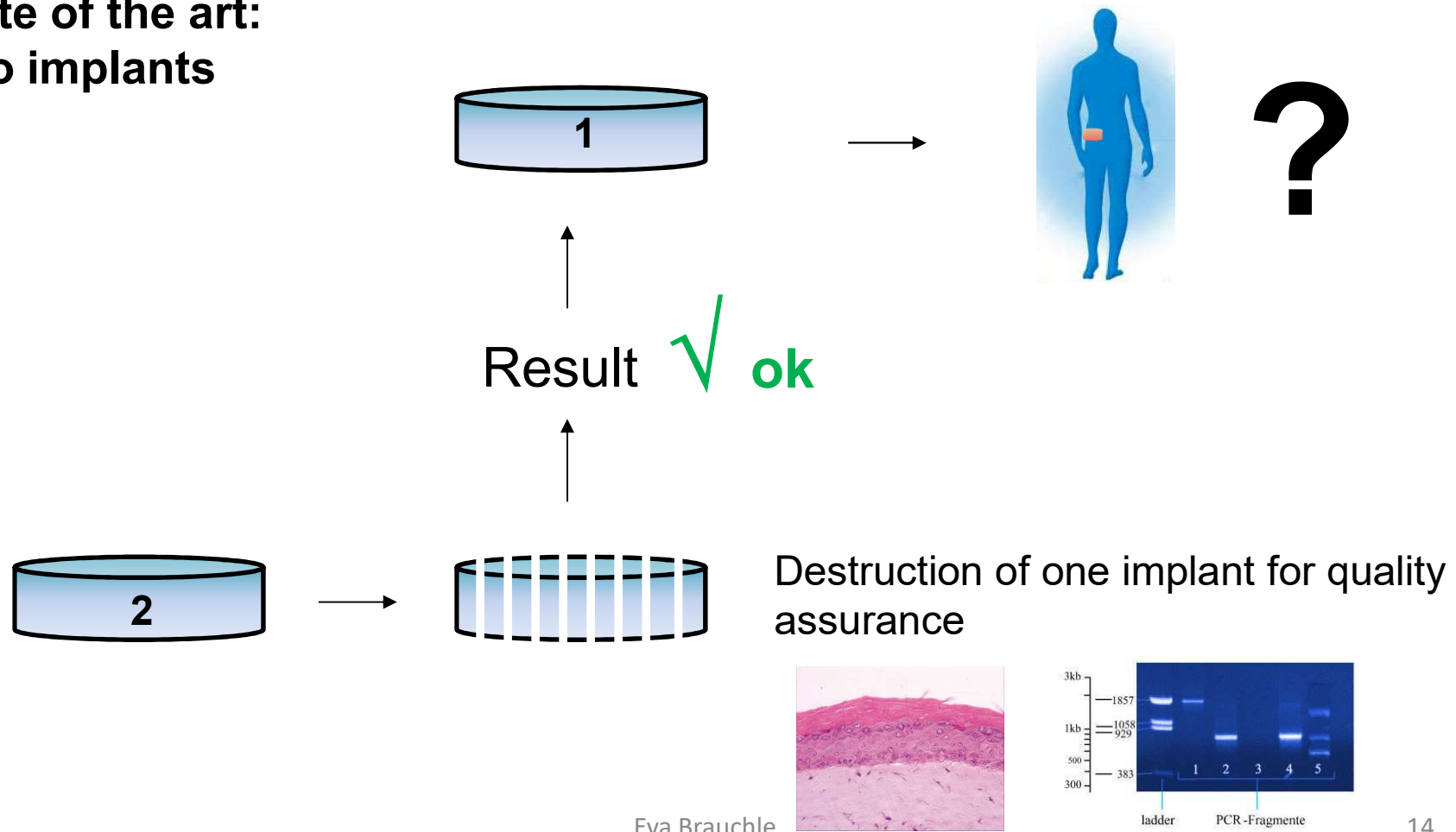
- Spectroscopy: interaction of light and matter



<http://weeklsciencequiz.blogspot.de/2011/09/when-light-meets-matter.html>

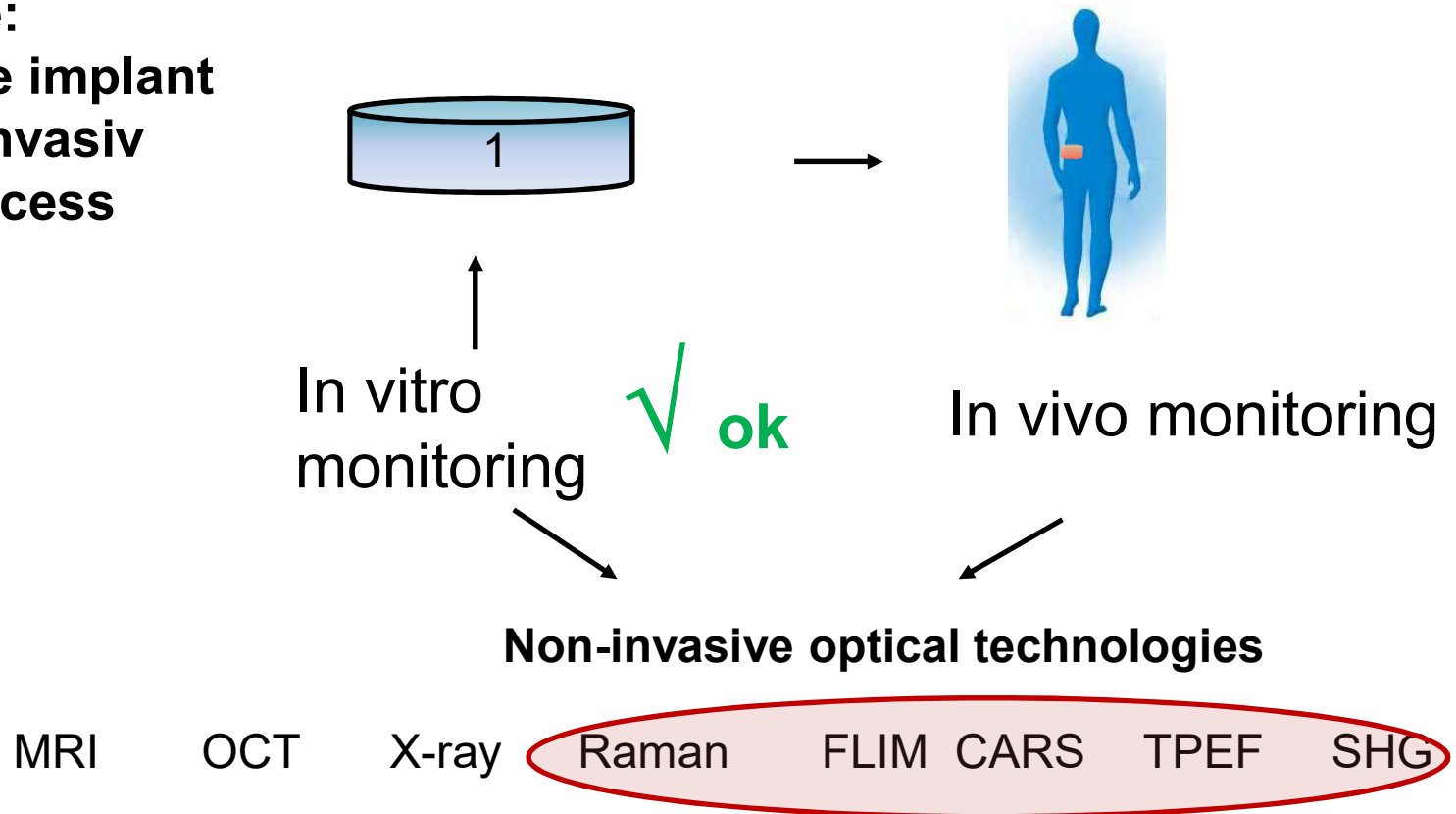
Advantages of optical technologies in tissue engineering

State of the art:
Two implants



Advantages of optical technologies in tissue engineering

**The future:
One single implant
and non-invasive
online process
control**



Theory of Raman spectroscopy



Chandra. Venkata Raman
(* 1887, † 1970)

A New Type of Secondary Radiation.

IF we assume that the X-ray scattering of the 'unmodified' type observed by Prof. Compton corresponds to the normal or average state of the atoms and molecules, while the 'modified' scattering of altered wave-length corresponds to their fluctuations from that state, it would follow that we should expect also in the case of ordinary light two types of scattering, one determined by the normal optical properties of the atoms or molecules, and another representing the effect of their fluctuations from their normal state. It accordingly becomes necessary to test whether this is actually the case. The experiments we have made have confirmed this anticipation, and shown that in every case in which light is scattered by the molecules in dust-free liquids or gases, the diffuse radiation of the ordinary kind, having the same wave-length as the incident beam, is accompanied by a modified scattered radiation of degraded frequency.

⋮

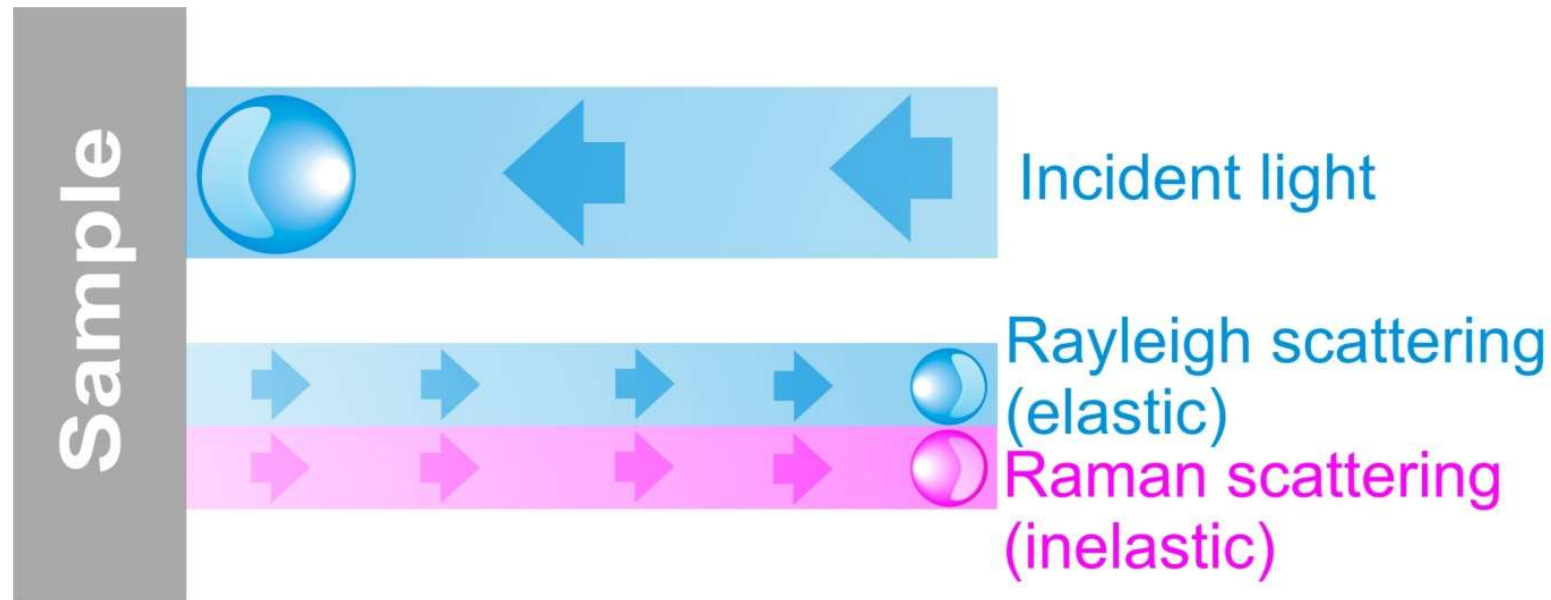
Raman and Krishnan in Letters to Nature, 1928

Theory of Raman spectroscopy

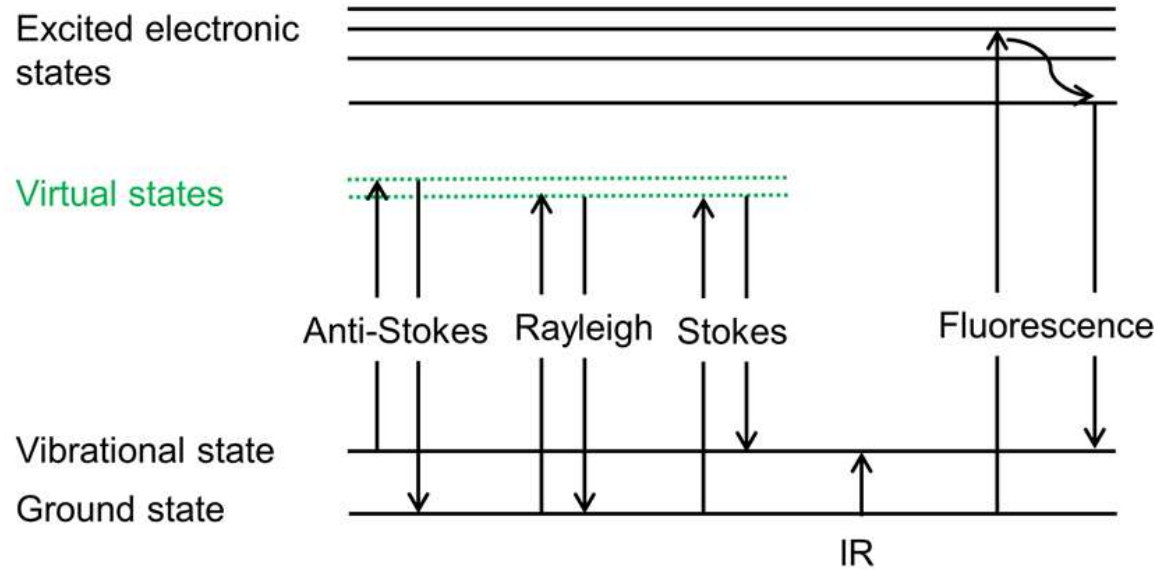


Chandra. Venkata Raman
(* 1887, † 1970)

- Vibrational spectroscopy
- Detection of inelastic light scattering due to molecular vibrations



Theory of Raman spectroscopy



Rayleigh scattering



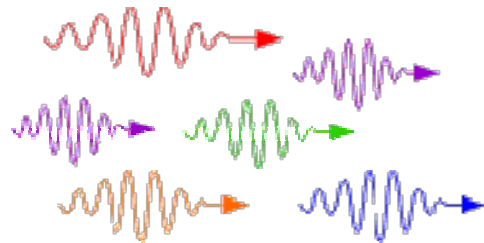
Light

- Light waves consist of single photons
- Energy E of light is proportional to the frequency ν

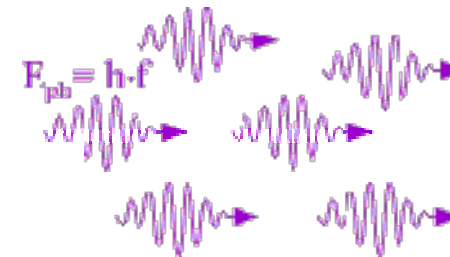
$$E = h \cdot \nu$$

- Light intensity (given in mW) means density of photons

**Photons of a light spectrum
(different energies)**



**Photons of monochromatic
light (equal energies)**



Photons

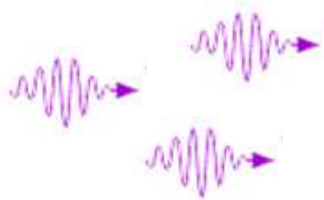
How many photons per second are emitted by a laserpointer with laser wavelength 500 nm and the power of 5 mW.

$$E_{\text{Photon}} = h \cdot \nu = 0,4 \cdot 10^{-18} \text{ Joule}$$

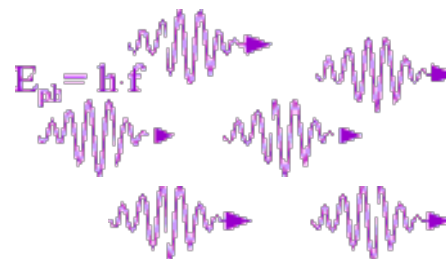
$$5 \text{ mW} = 0,005 \text{ J/s}$$

$$\Rightarrow 13 \times 10^{15} \text{ photons per second}$$

Low energy



High energy



Eva Brauchle

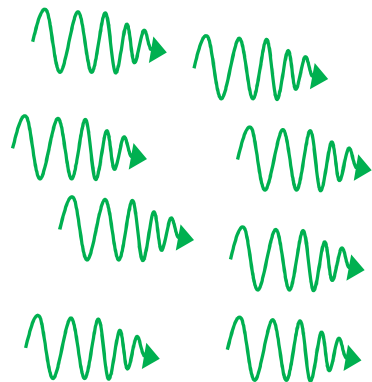


Theory of Raman spectroscopy

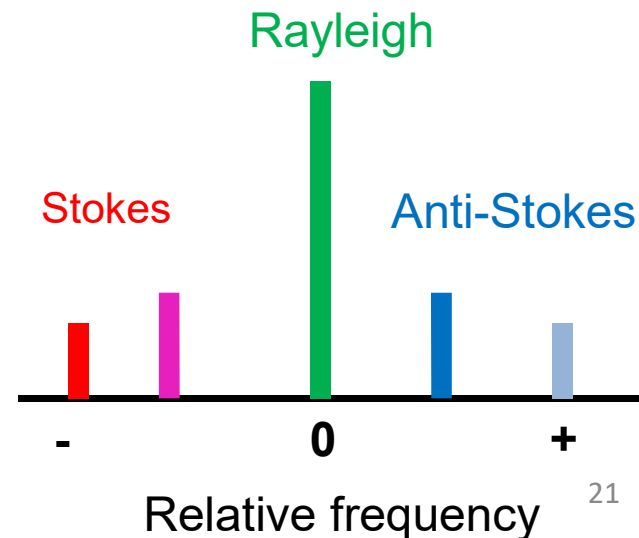
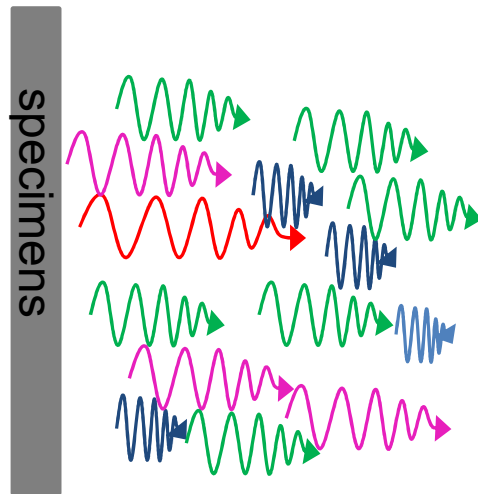
Light scattering is a two-photon process:

- One photon is absorbed another one is emitted at the same time
- Most frequently emitted photons have the same energy as the incident light (elastic scattering)
- Only few photons are shifted in their frequency (inelastic scattering)
- Stokes scattering refers to photons shifted towards red (lower energy)
- Photons with an increased energy correspond to Anti-Stokes scattering

Incident photons

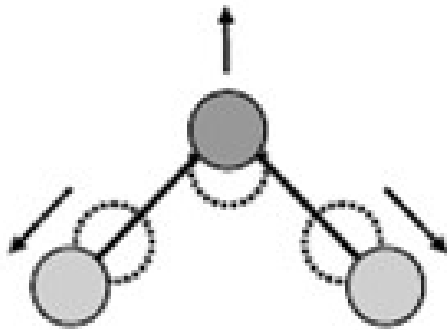


Scattered photons

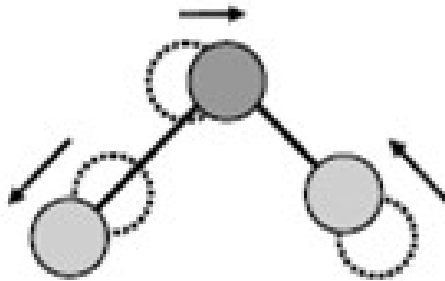


Theory of Raman spectroscopy

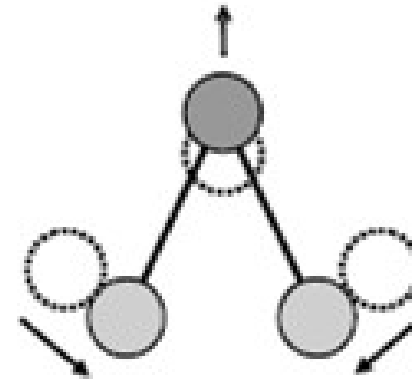
Examples of molecular vibrational modes



Symmetric vibrations



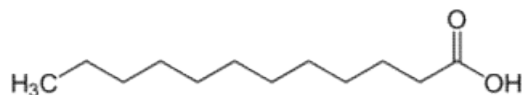
Assymetric vibrations



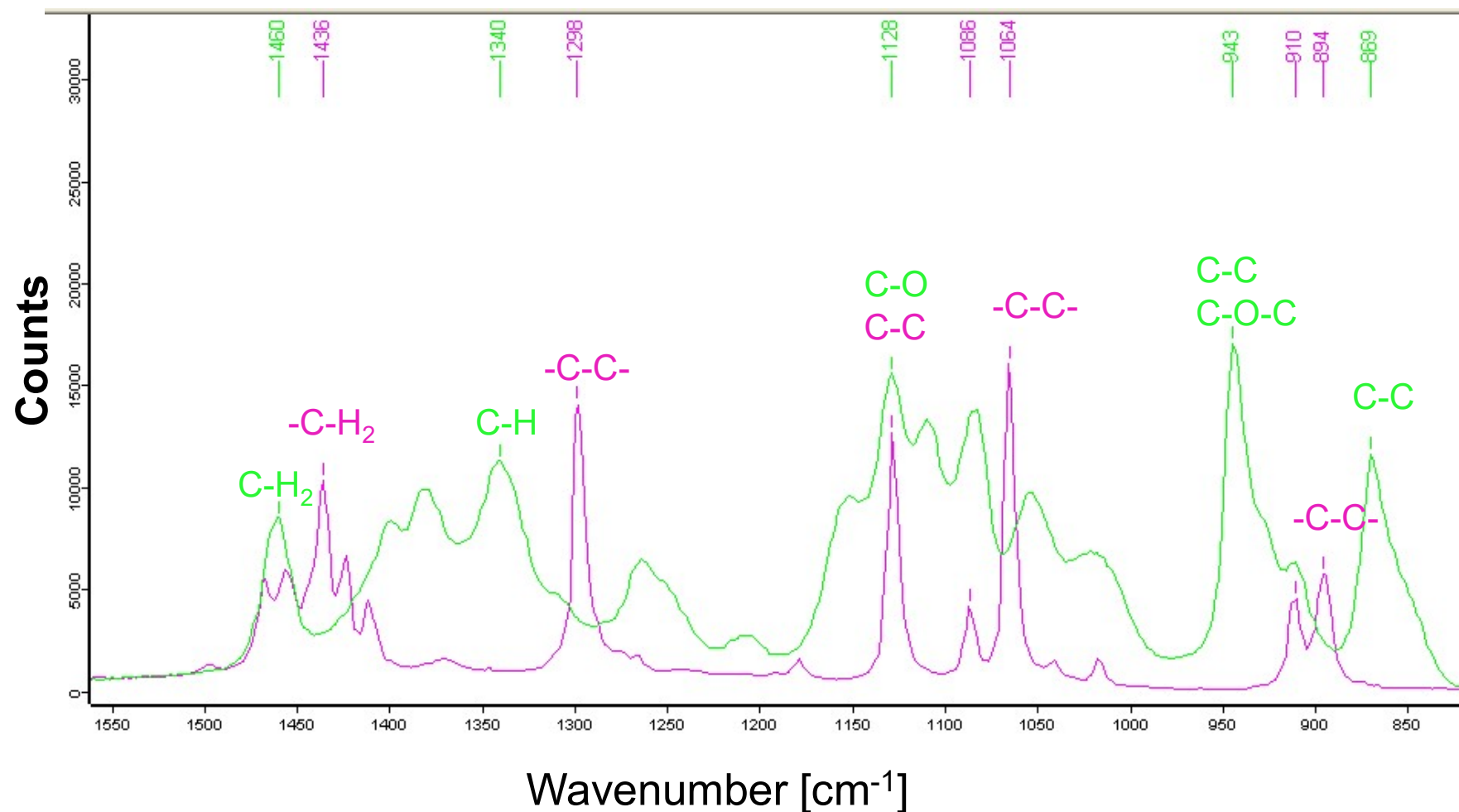
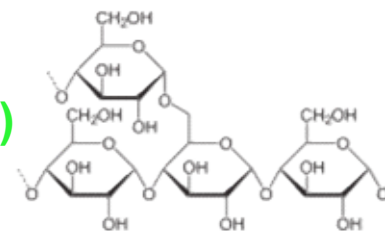
Deformation

Assignments to vibrational modes

Fatty acid

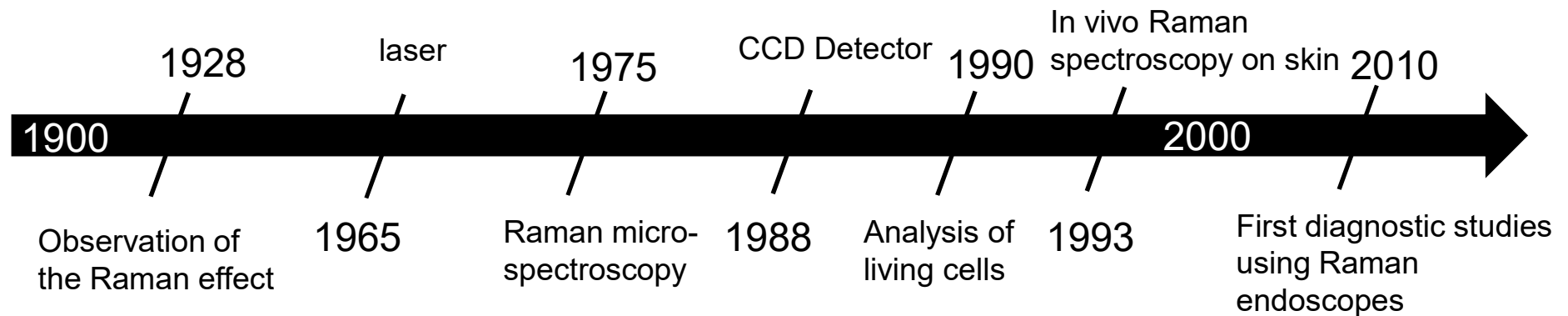


Starch
(carbohydrate)

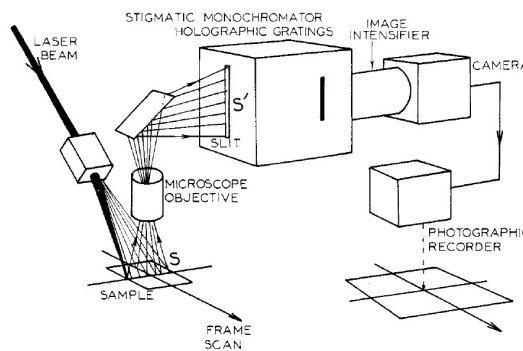


Raman spectroscopic systems

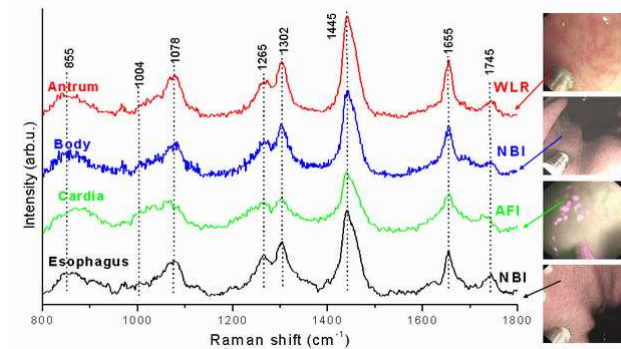
History



Adar et al. 2007



Delhaye et al. 1975



Dr Huang's Research Group, Singapore

Raman spectroscopic systems

Today



<http://www.riverd.com/aboutspectra.html>



Raman micro-spectroscopy



<http://www.thermoscientific.de/product/dxr-smartraman-spectrometer.html>

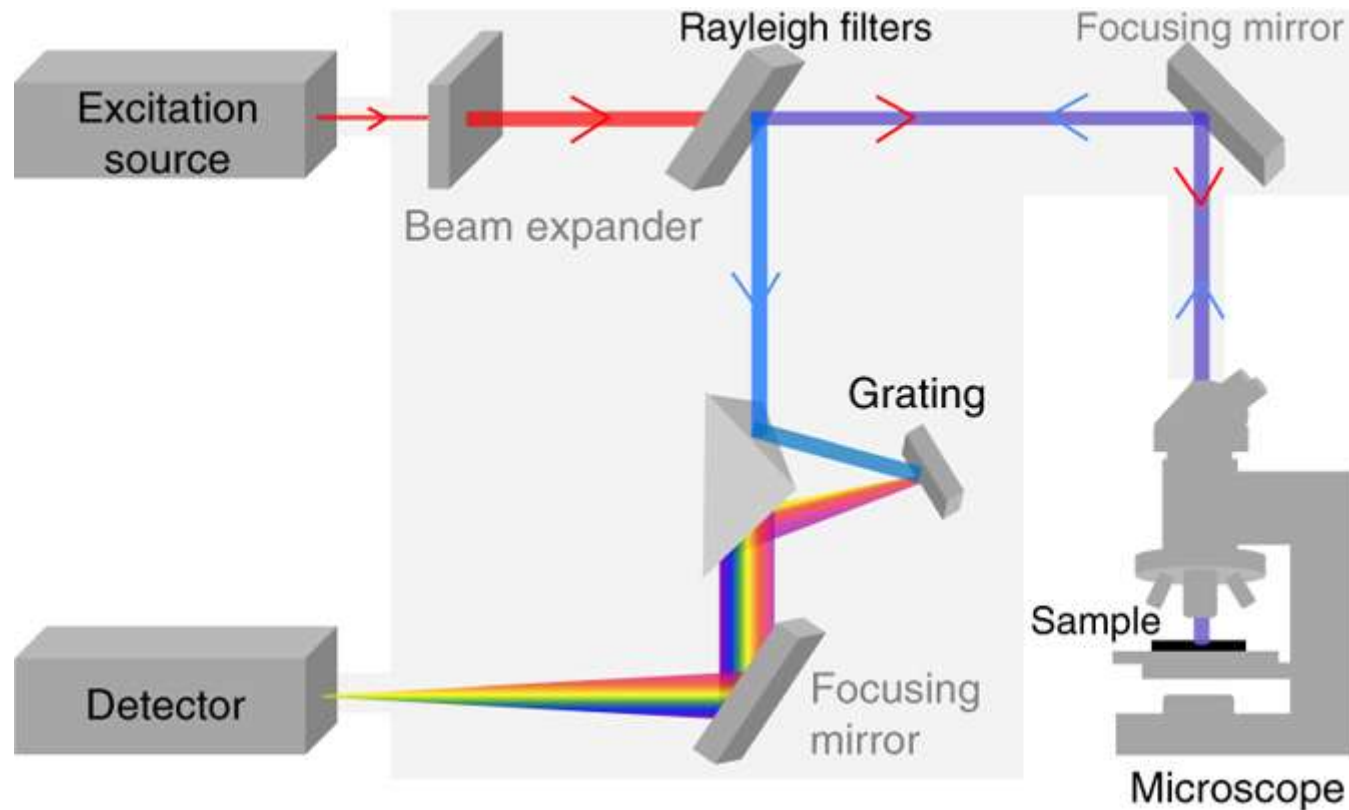


<http://www.laboratoryequipment.com/product-releases/2013/03/raman-spectrometer-system-process-control>



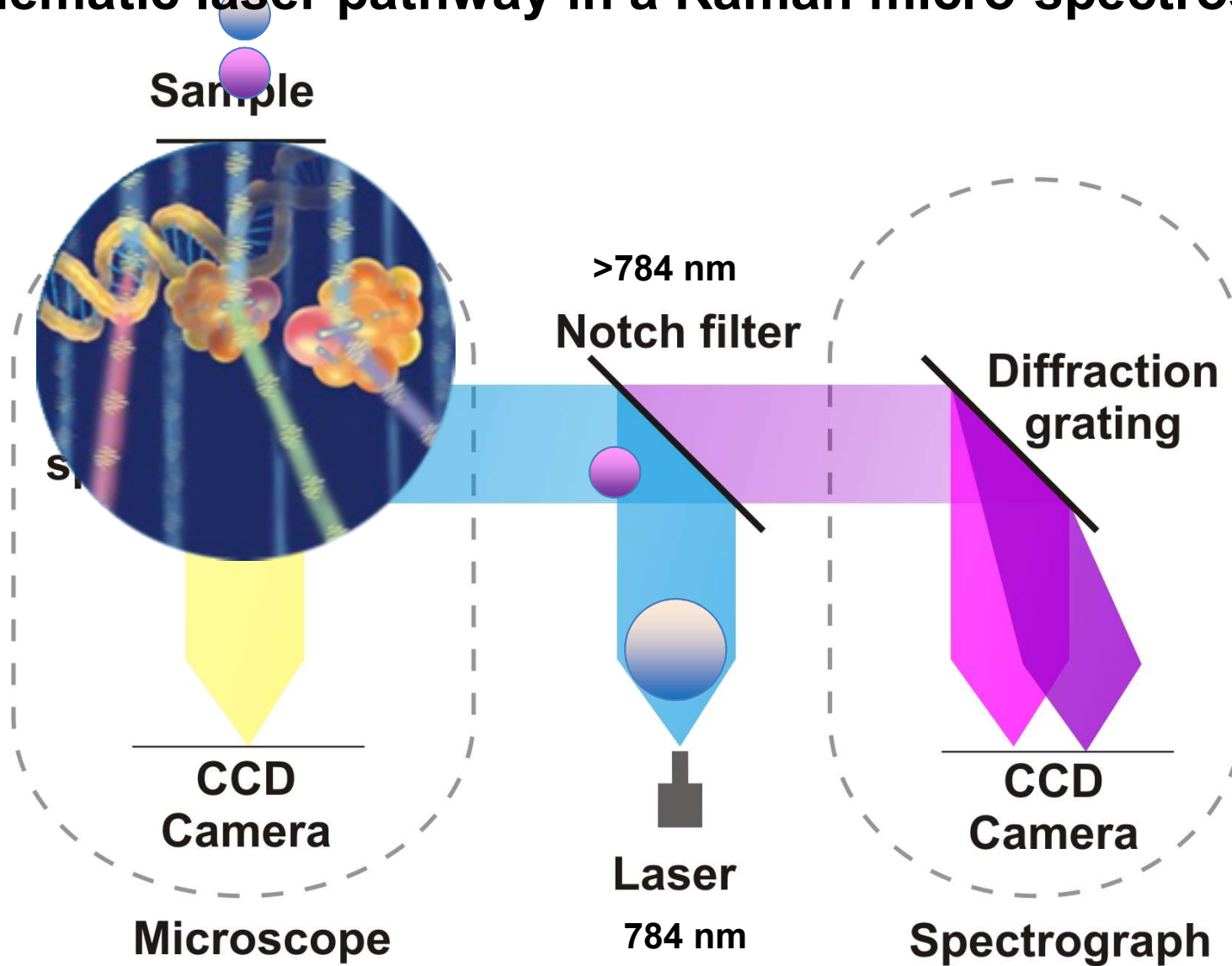
http://1.1.1.1/bmi/www.htds.fr/en/files/2012/07/instrumentation-analytique_spectroscopie-moleculaire_spectroscopie-raman-Truscan1.jpg

Basic Set-up of a Raman microscope



<http://way2science.com/raman-spectroscopy-2/>

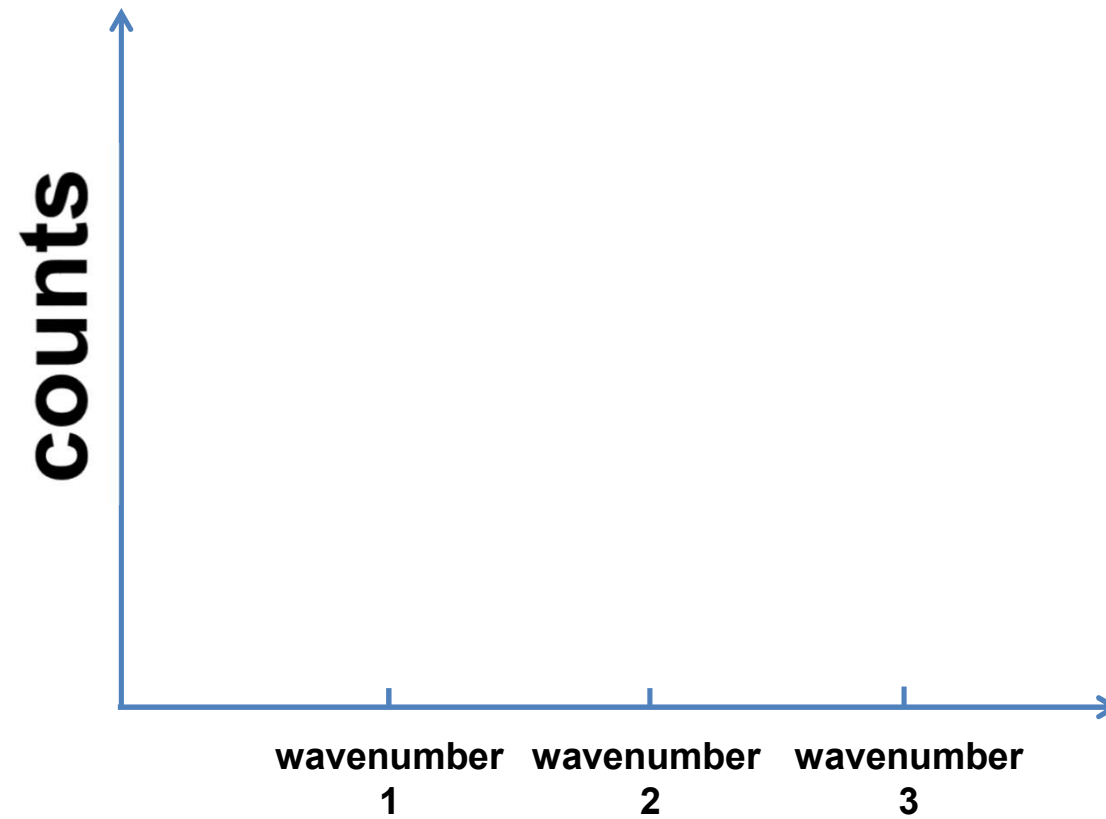
Schematic laser pathway in a Raman micro-spectroscope



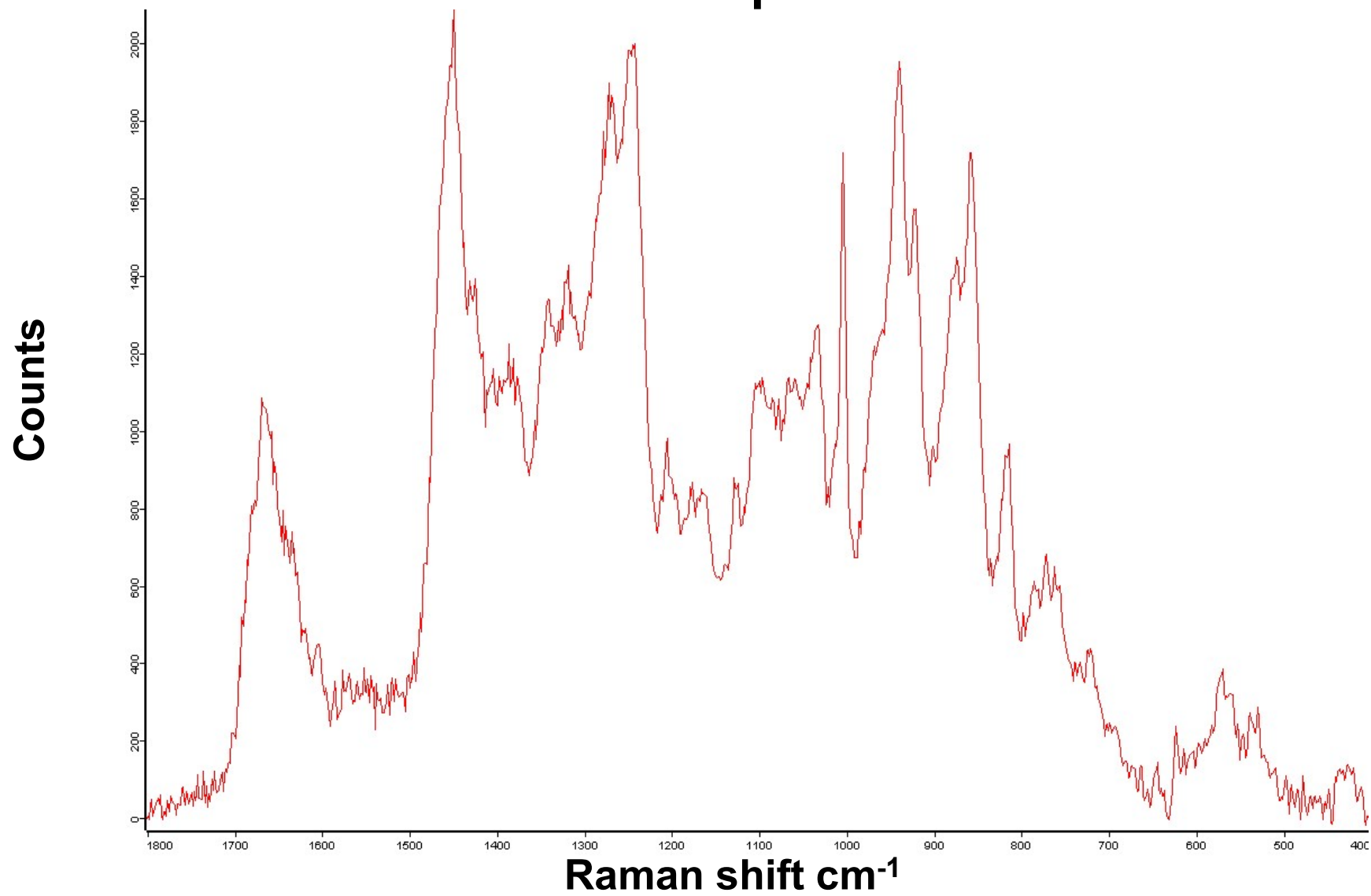
Raman spectroscopy



Spectral grating

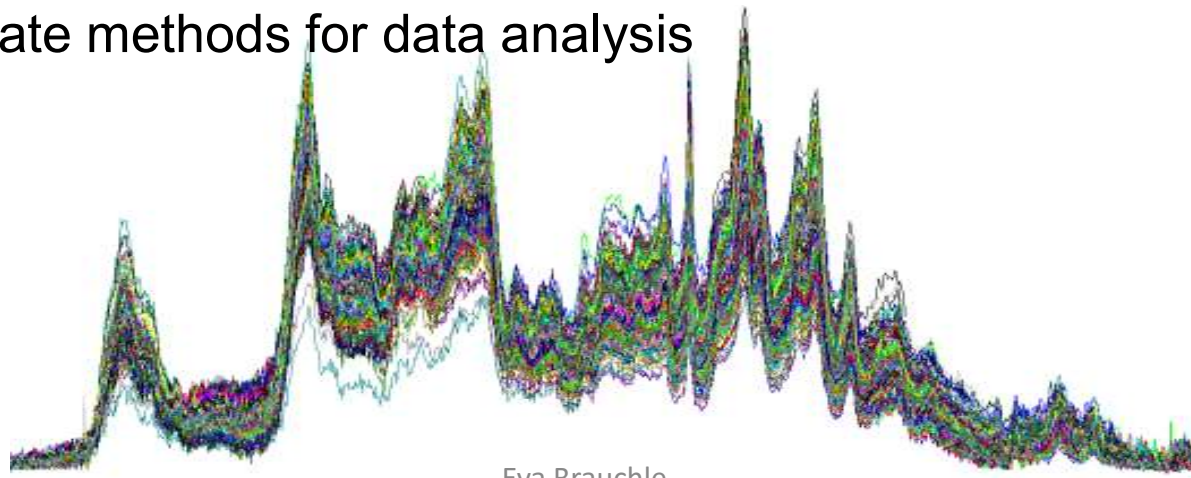


Raman spectrum

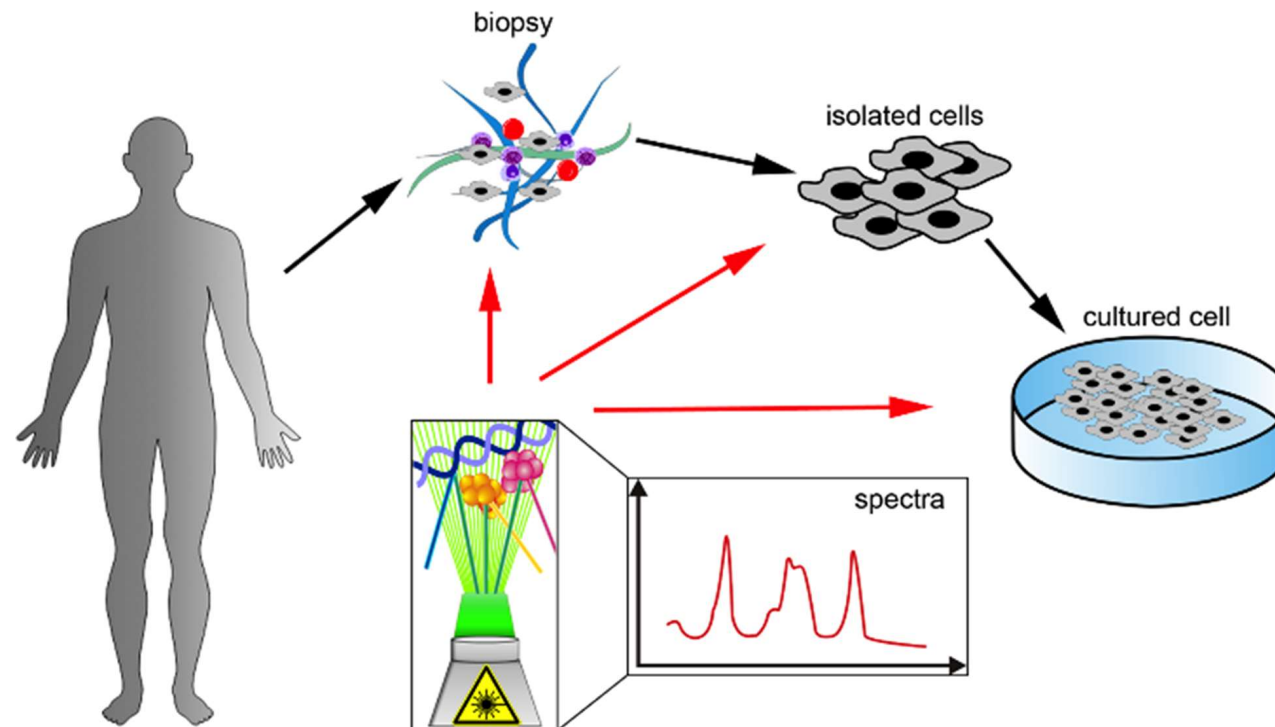


Raman micro-spectroscopy in biomedical applications

- Visible or NIR-lasers (532 or 784 nm)
- Non-invasive and label-free measurements on tissues or cultured cells
- Under physiological conditions (minimal H₂O signal)
- Molecular fingerprints from samples
- Small sample volumes
- Monitoring of changes over time or identification
- Strongly overlapping signals (proteins, nucleic acids, lipids and carbohydrates)
- Multivariate methods for data analysis



Potential Applications of Raman microscopy in Tissue Engineering



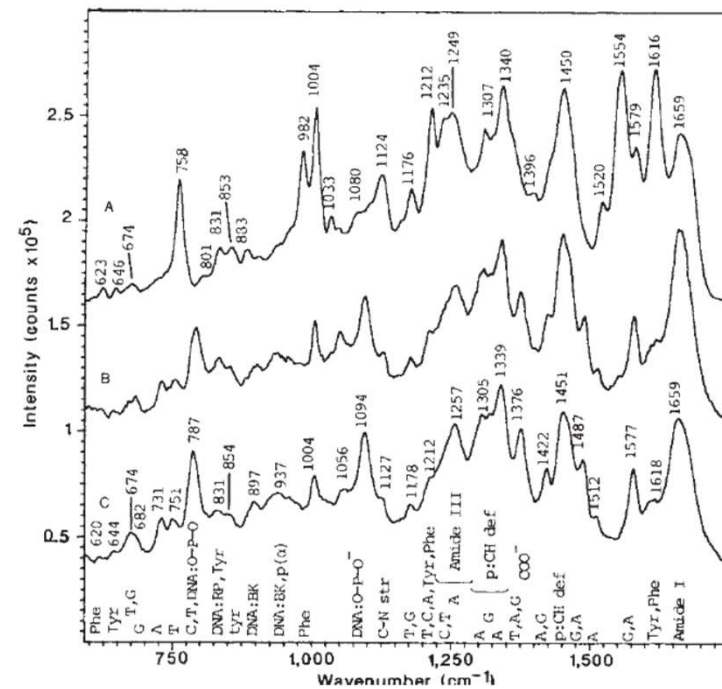
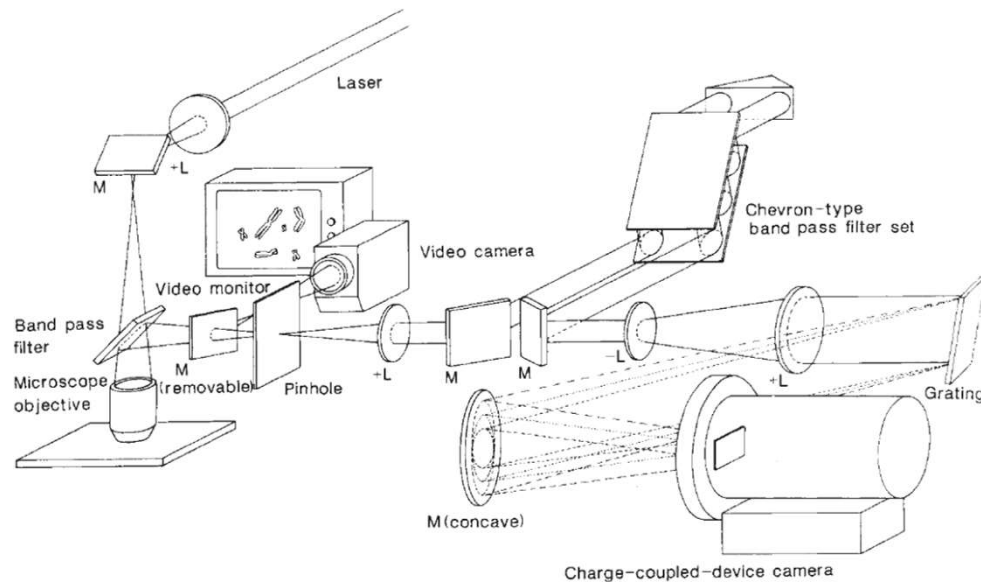
Raman spectroscopy

Studying single living cells and chromosomes by confocal Raman microspectroscopy

G. J. Puppels*, **F. F. M. de Mul***, **C. Otto***, **J. Greve***,
M. Robert-Nicoud†, **D. J. Arndt-Jovin†** & **T. M. Jovin†**

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† Max Planck Institute for Biophysical Chemistry,
Department of Molecular Biology, PF 2841 D-3400 Göttingen, FRG



Raman spectra of cells

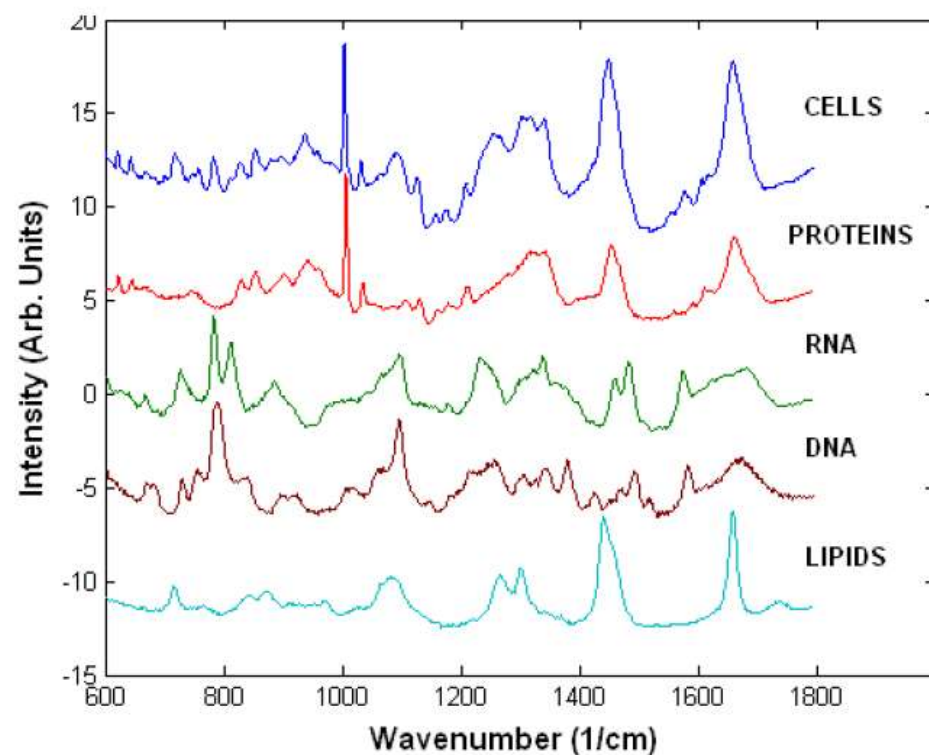
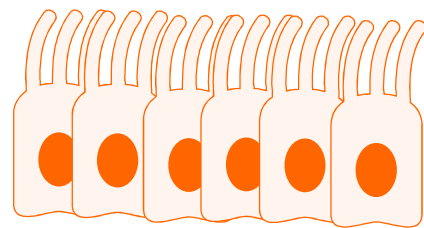


Table 1. Peak assignment for Raman spectra of living cells [23].

Peak (cm ⁻¹)	Assignment			
	Nucleic Acids	Proteins	Lipids	Carbohydrates
1736			C=O ester	
1680-1655		Amide I	C=C str.	
1617		C=C Tyr, Trp		
1607		C=C Phe, Tyr		
1578	G, A			
1480-1420	G, A, CH def	C-H	CH def	CH def
1342	A, G	C-H		CH def
1320	G	C-H		
1301			CH ₂ twist	
1284-1220	T, A	Amide III	=CH bend	
1209		C-C ₆ H ₅ , Phe, Trp		
1176		C-H bend Tyr		
1158		C-C/C-N str.		
1128		C-N str.		C-O str
1095-1060	PO ₂ ⁻ str.		Chain C-C str.	C-O, C-C str
1033		C-H in-plane Phe		
1005		Sym. Ring br Phe		
980		C-C BK str. β-sheet	=CH bend	
937		C-C BK str. α-helix		C-O-C glycos.
877			C-C-N ⁺ sym str	C-O-C ring
854		Ring br Tyr		
828	O-P-O asym.str.	Ring br. Tyr		
811	O-P-O str. RNA			
788	O-P-O str. DNA			
782	U,C,T ring br			
760		Ring breath Trp		
729	A			
717			CN ⁺ (CH ₃) ₃ str	
667	T, G			
645		C-C twist Tyr		
621		C-C twist Phe		

Notinger, Sensors, 2007

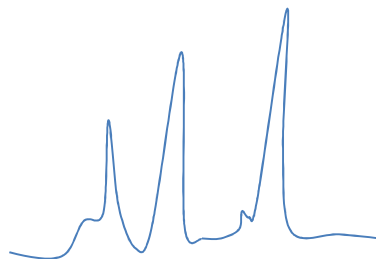
Analysis of Raman spectra



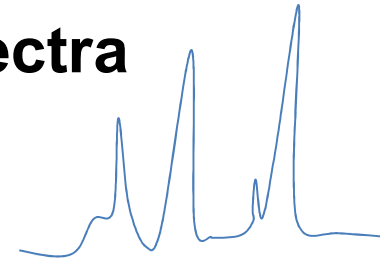
Specimens



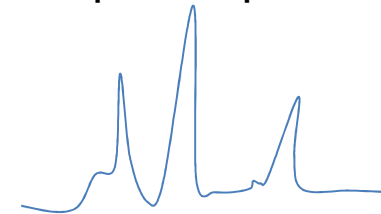
Factor



Spectrum



Intensity increase of a specific peak



Intensity decrease of a specific peak

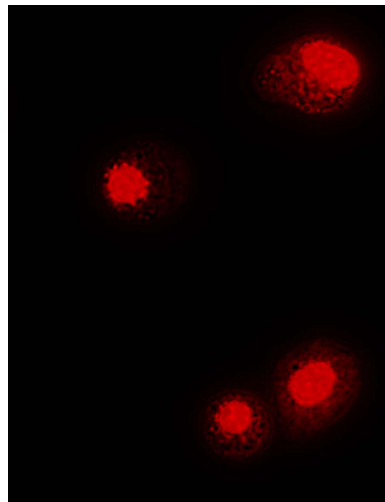


Change of the overall spectrum

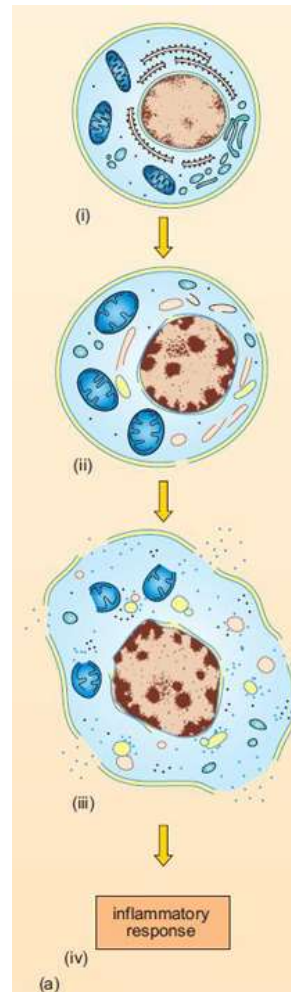
Differentiation of viable and dead cells

Propidium-Iodid

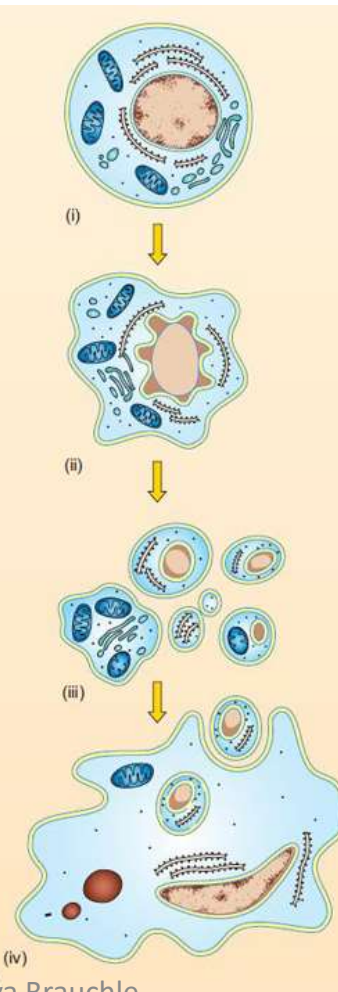
Passes the
membrane of
dead cells



necrosis



apoptosis

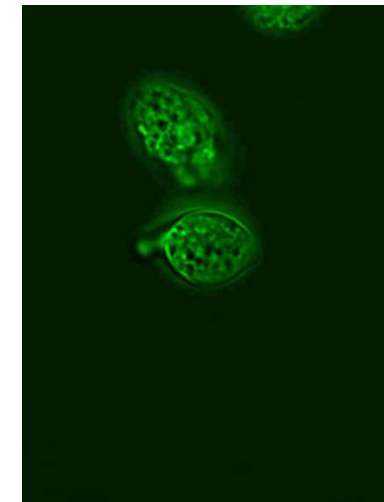


Annexin V

Specific marker
for apoptotic cells



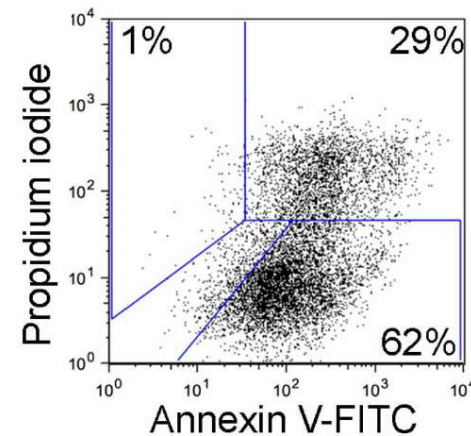
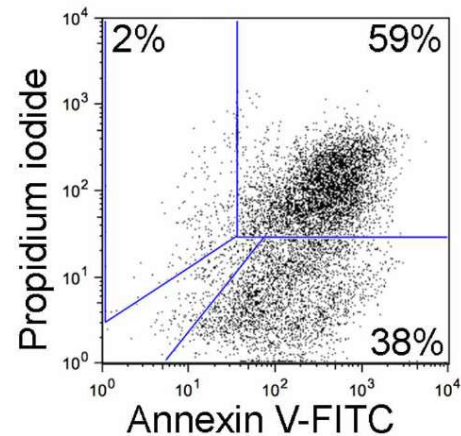
Annexin V-FITC



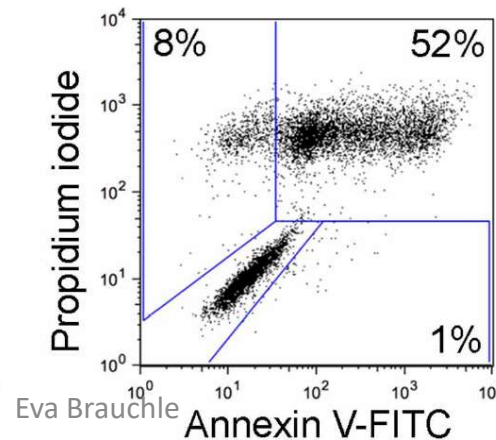
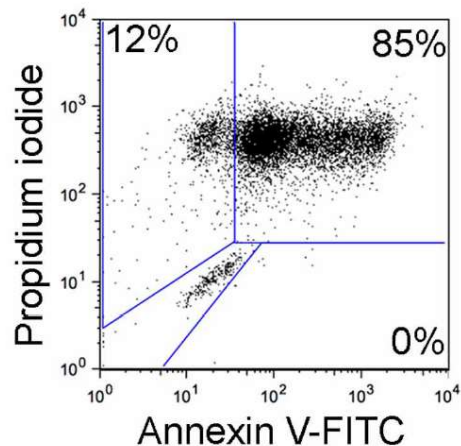
Differentiation of viable and dead cells

Classical FACS analysis

Apoptosis



Necrosis

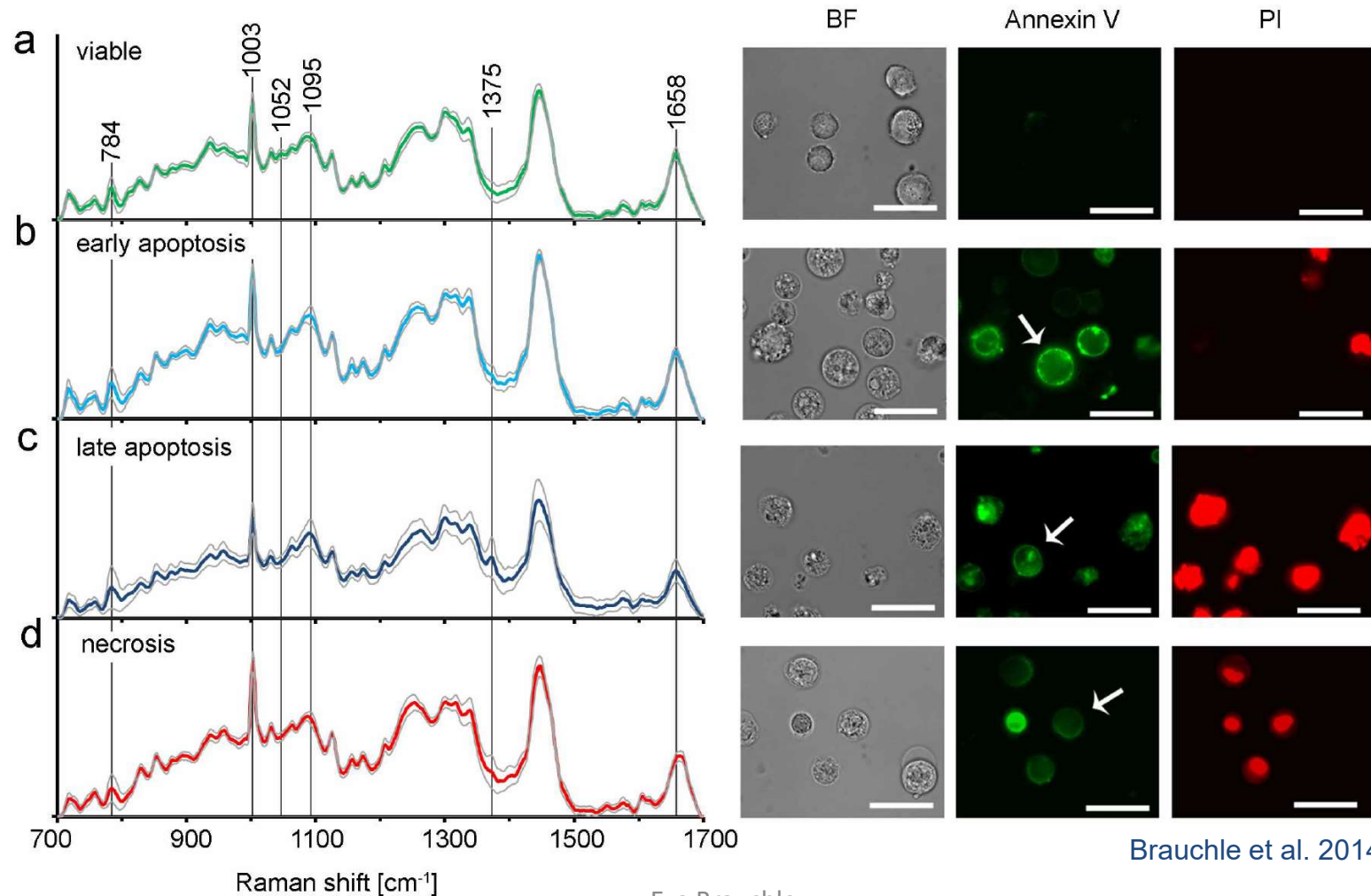


Brauchle et al. 2014

Eva Brauchle

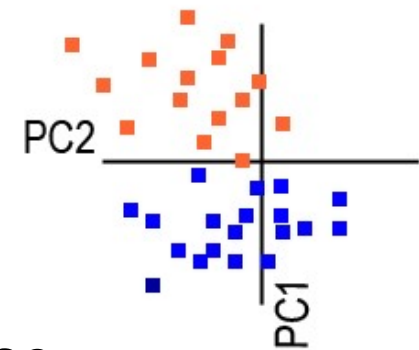
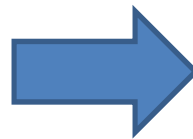
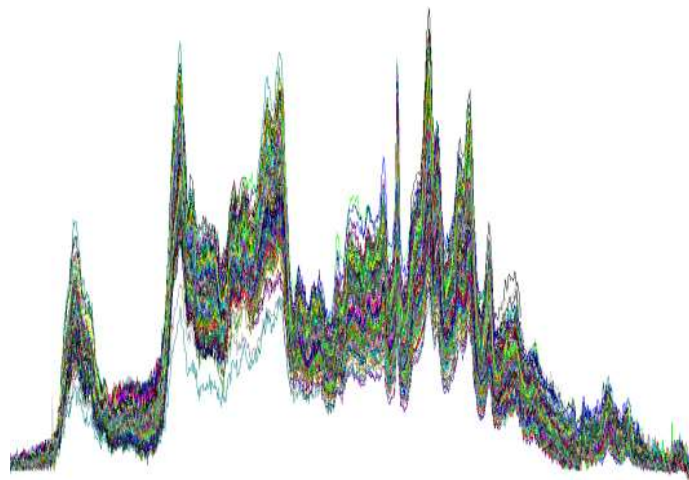
Differentiation of viable and dead cells

Raman micro-spectroscopy



Principal components analysis (PCA)

- Analysis of spectral data
- Reduction of variables:
1000 data points per spectrum -> 7 score values per spectrum
- Fast analysis of the predominant variances within the data set and visualization (unsupervised method)
- Use of PCA model for qualitative prediction



Principal components analysis (PCA)

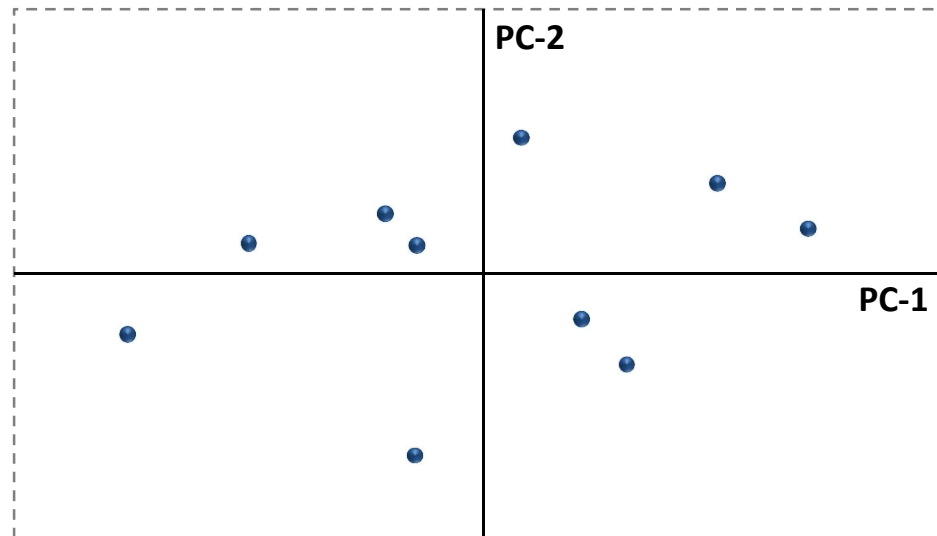
Mathematical view

The diagram illustrates the mathematical equation for Principal Component Analysis (PCA): $X = TP^T + E$. Each matrix is represented by a rounded rectangle with its dimensions indicated by letters: X is $N \times M$, T is $N \times A$, P^T is $A \times M$, and E is $N \times M$. The equation shows the decomposition of the data matrix X into the product of the scores matrix T and the loadings matrix P^T , plus the residues matrix E .

X	Data matrix
T	Scores matrix
P^T	Loadings matrix
E	Residues matrix

Principal components analysis (PCA)

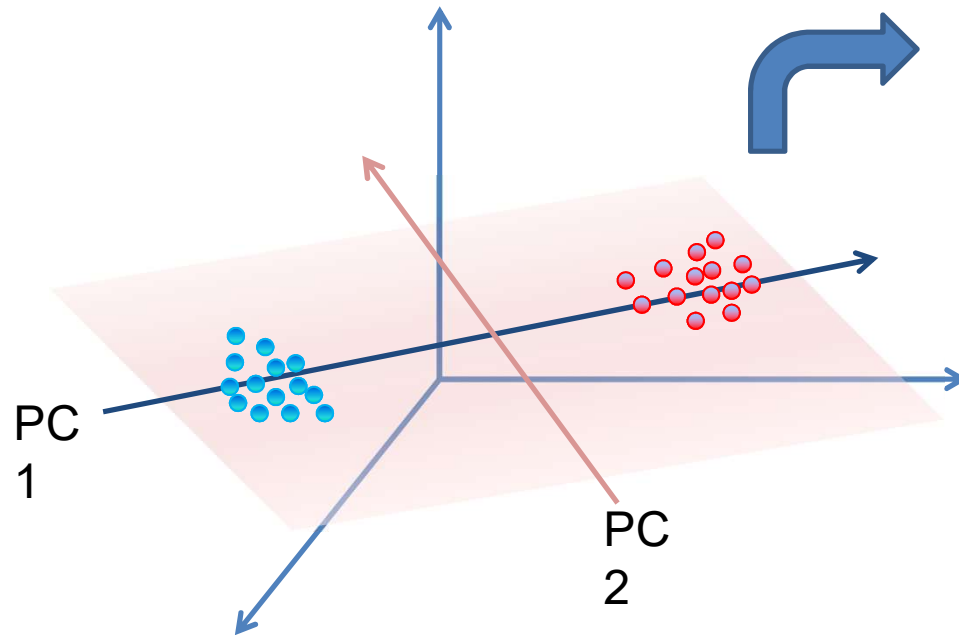
Graphical Explanation



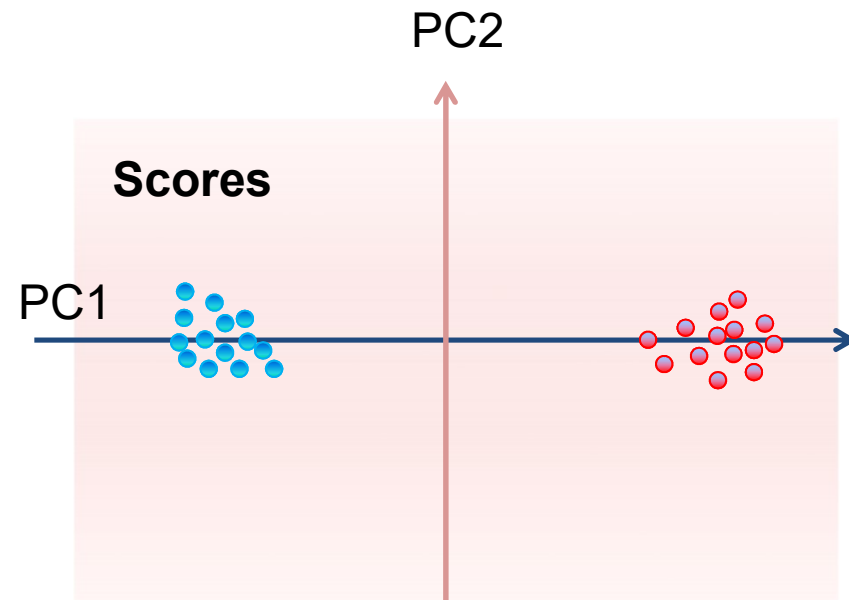
Principal components analysis (PCA)

Simplified description with only 3 variables

Probe	Raman shift 1	Raman shift 2	Raman shift 3
A	Counts 1	Counts 2	Counts 3
B	Counts 1	Counts 2	Counts 3



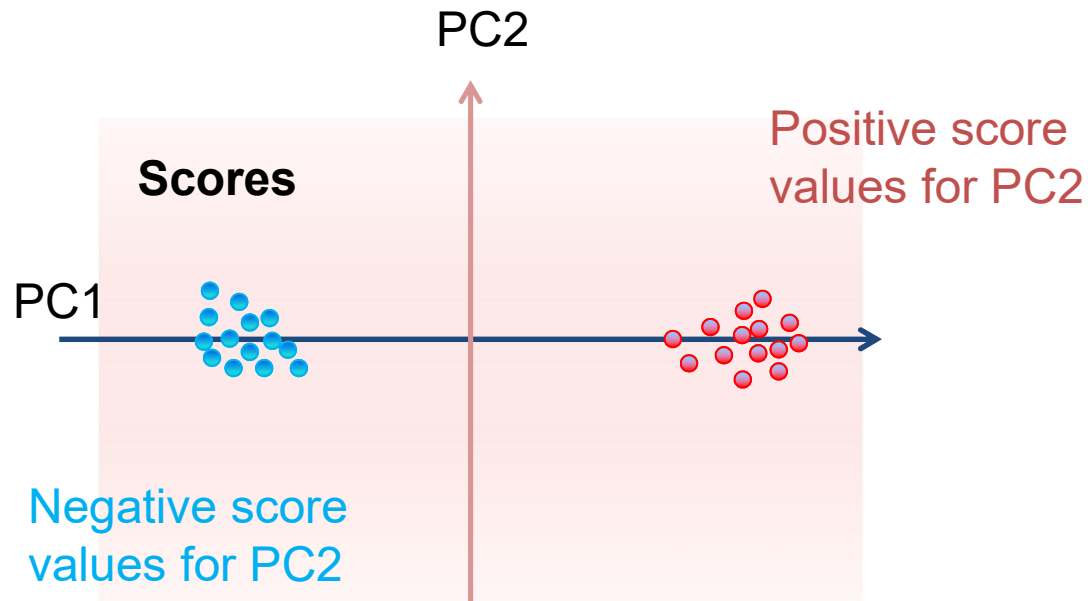
Projection of the data into a 3-dimensional space



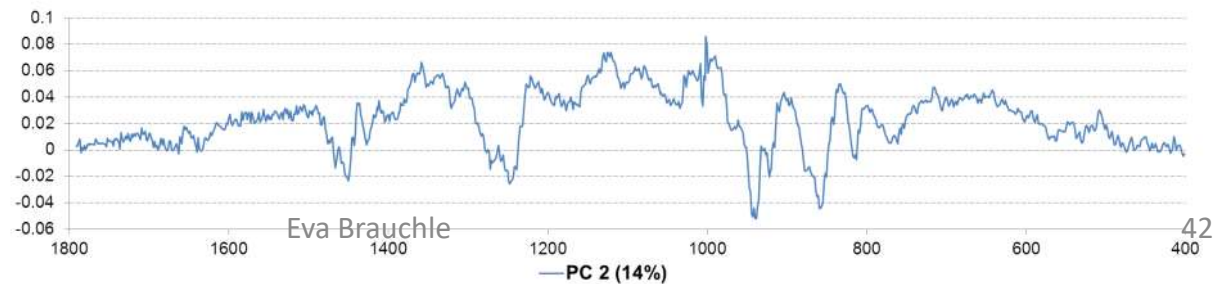
- PC Loadings describe the new axes
- Scores are the new variables of spectra

Principal components analysis (PCA)

Simplified description with only 3 variables



Loadings have spectral dimensions



Principal components analysis (PCA)

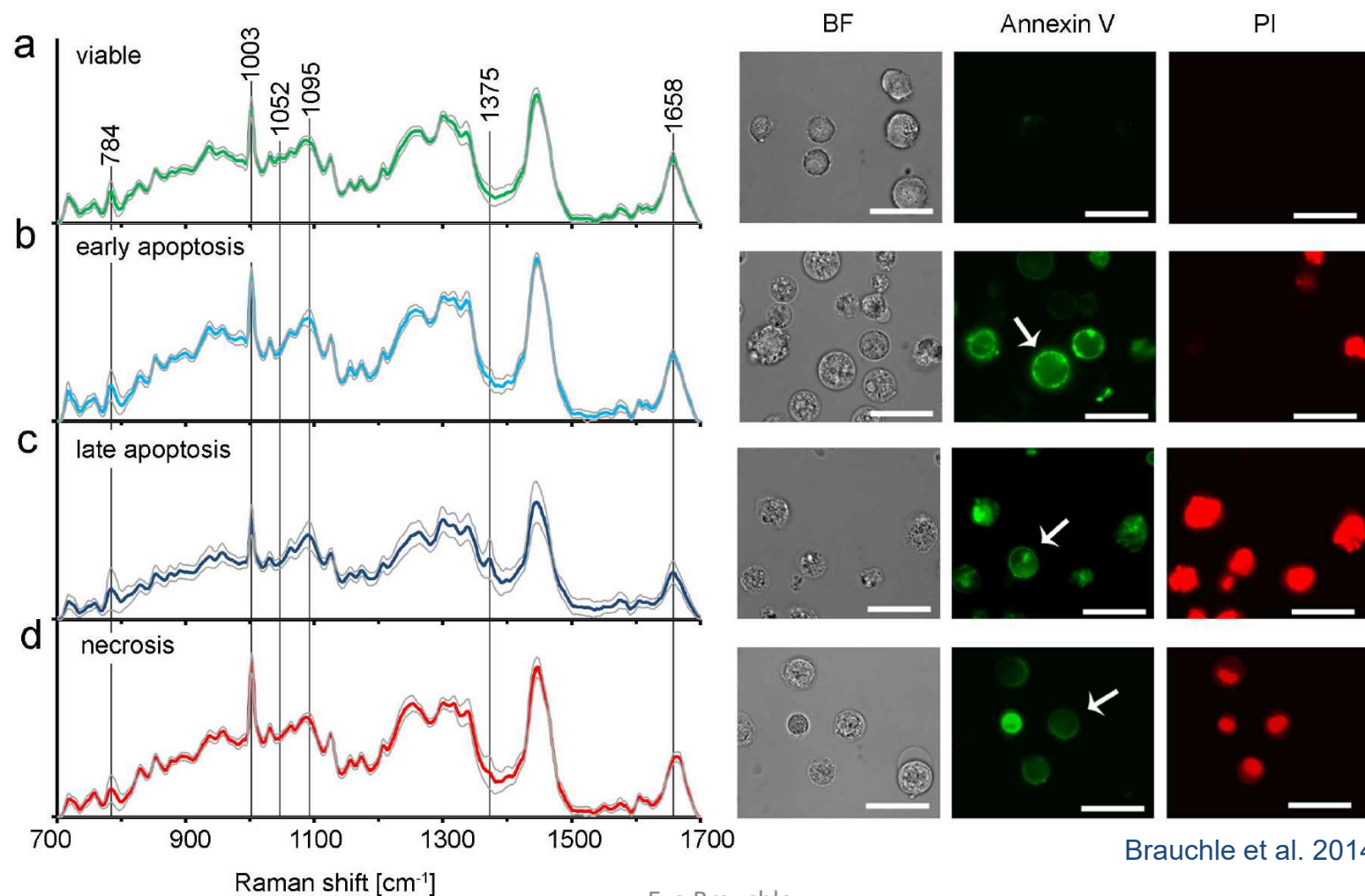
- Loadings and scores are calculated from the original data
- Impact of variances within the overall data set are described by loadings
- Variables within the dataset are reduced to few score values

⇒ Fast analysis of huge datasets

⇒ Identification of correlating peaks

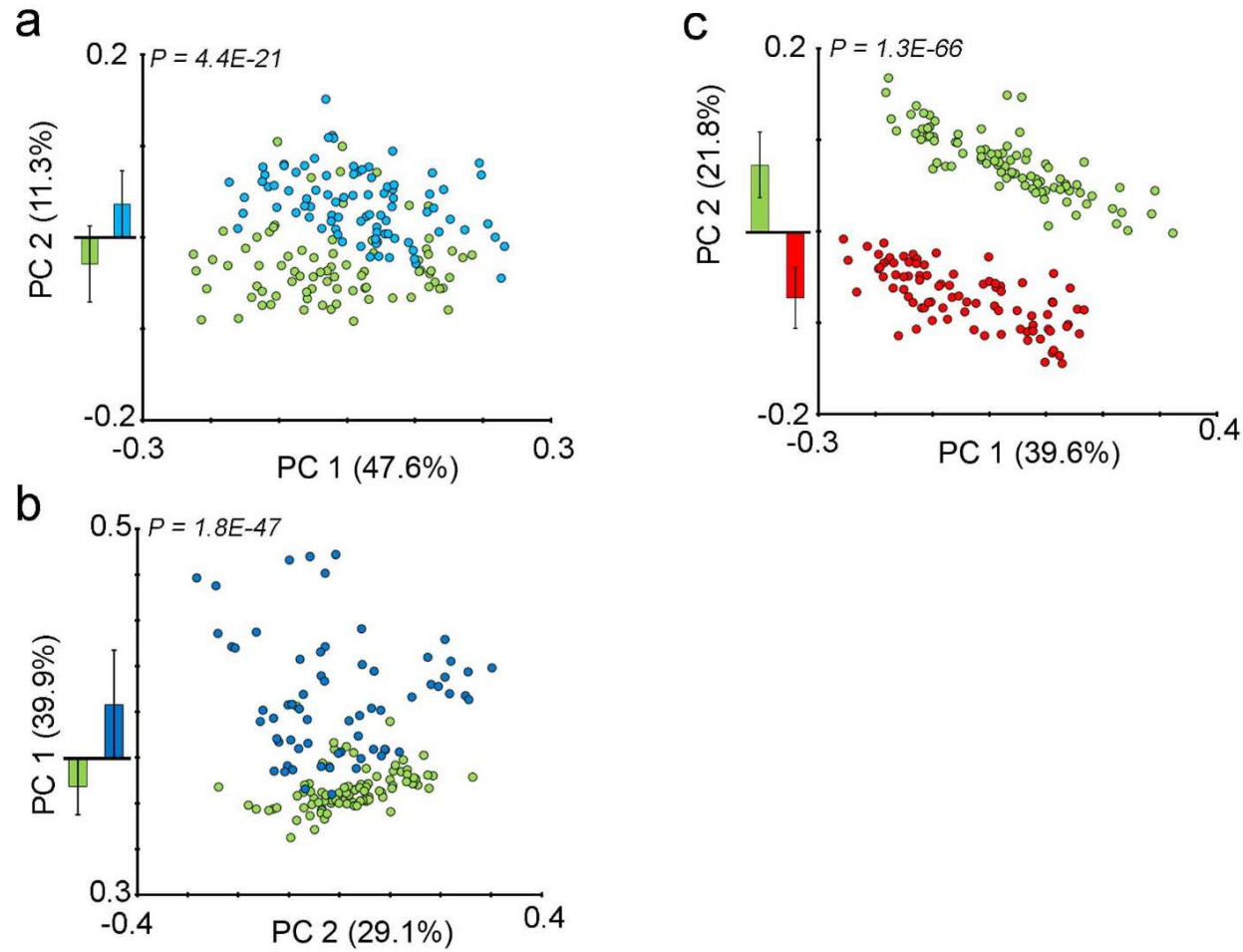
Differentiation of viable and dead cells

Raman micro-spectroscopy



Differentiation of viable and dead cells

PCA

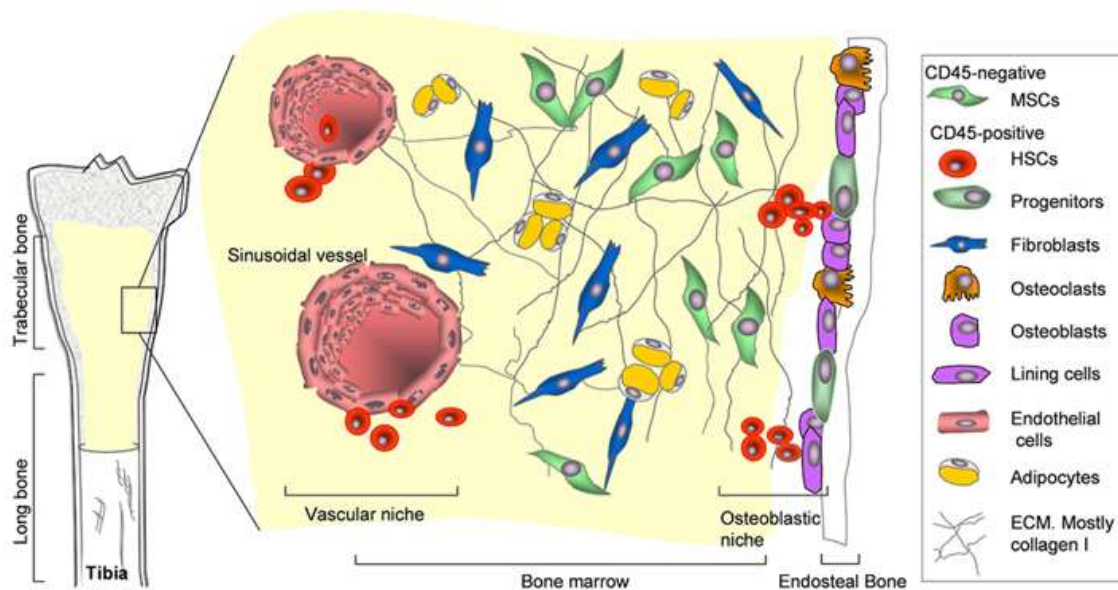


Brauchle et al. 2014

Differentiation of cell phenotypes

Background:

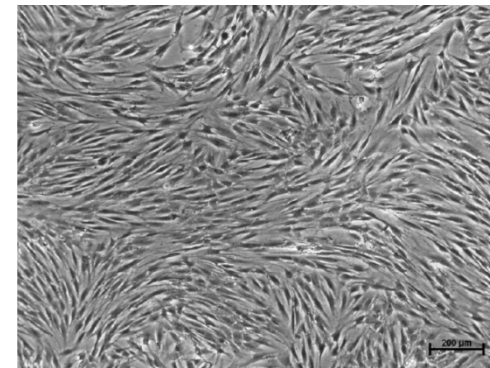
- Mesenchymal stem cells (MSCs) can be isolated from bone marrow
- During the isolation procedure fibroblasts can contaminate MSCs



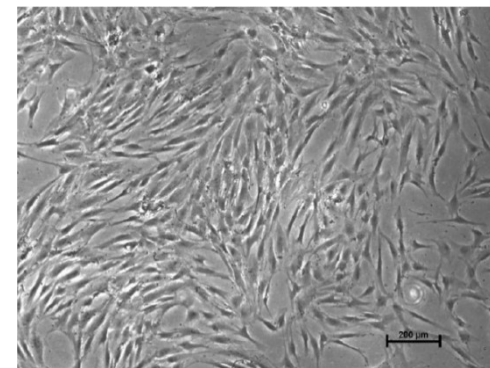
<http://www.bioscience.org/2007/v12/af/2440/figures.htm>

Eva Brauchle

Fibroblasts



MSCs

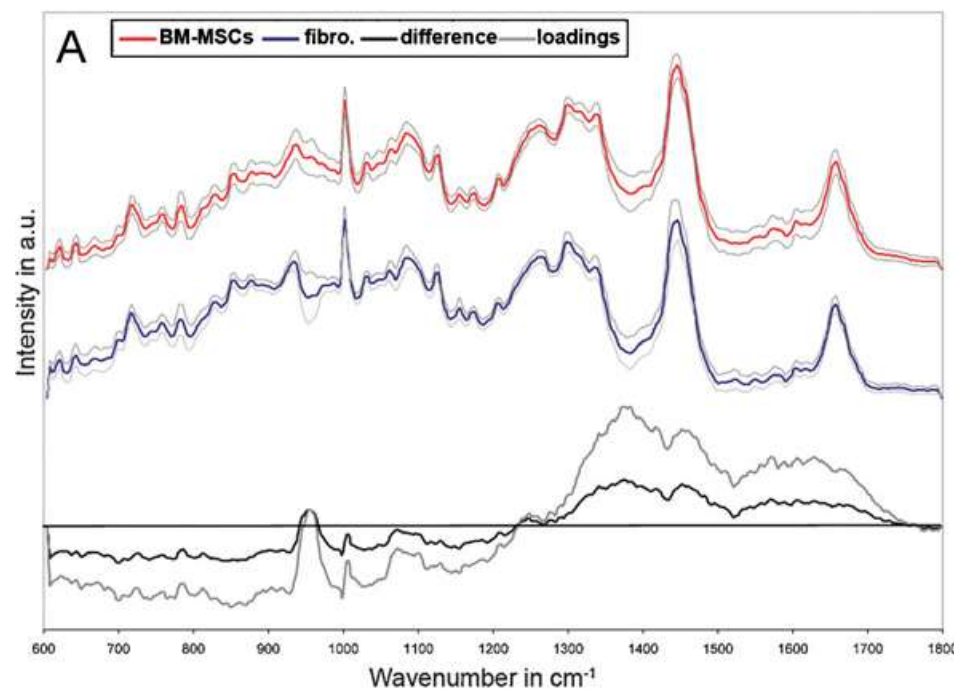


M. Pudlas et al., Medical Laser
Application, 2011

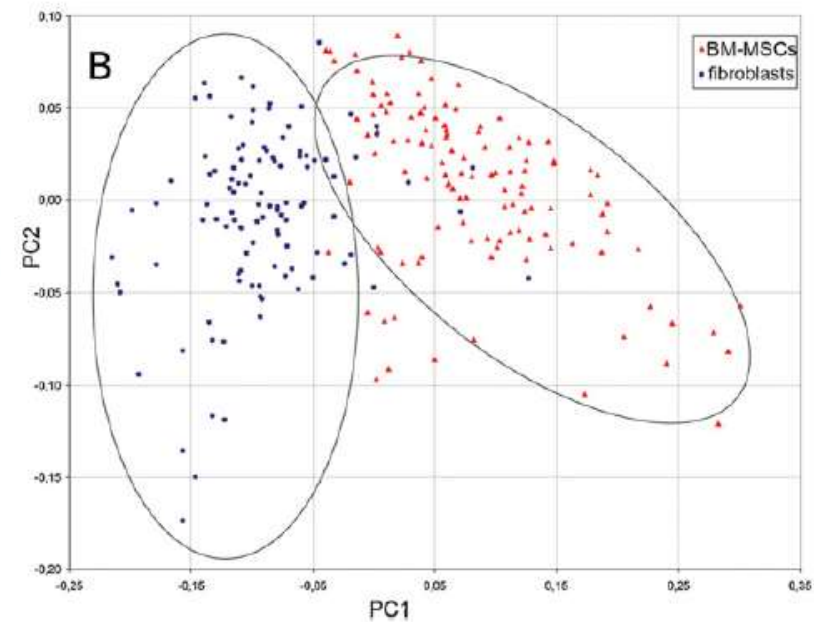
Differentiation of cell phenotypes

Results from Raman micro-spectroscopy:

- Spectral fingerprints are cell phenotype-specific
- PCA provides a clear discrimination of fibroblast and MSCs



M. Pudlas et al., Medical Laser
Application, 2011

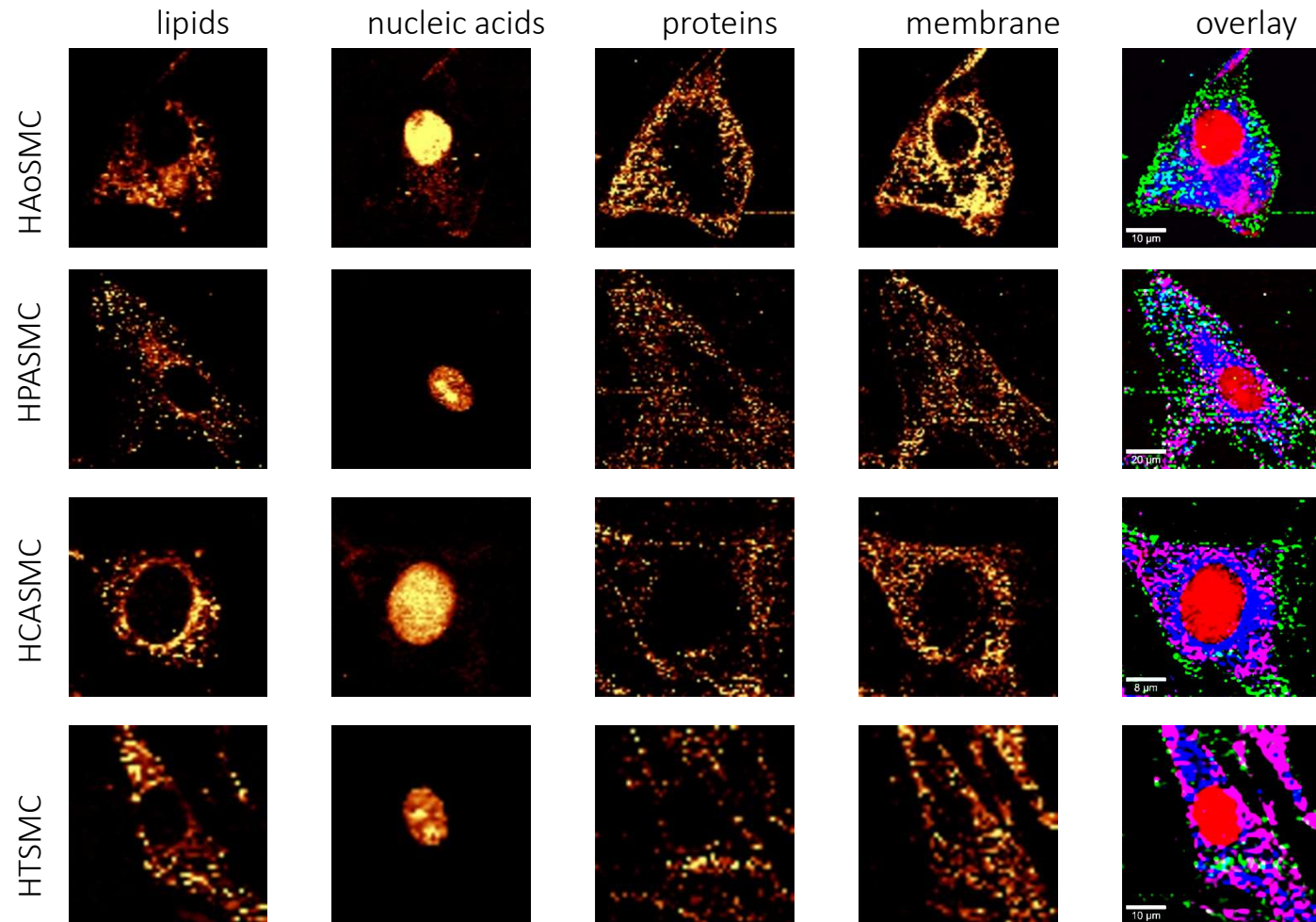


PC1 describes 64% of the total
variance

Eva Brauchle

Raman Imaging on single cells

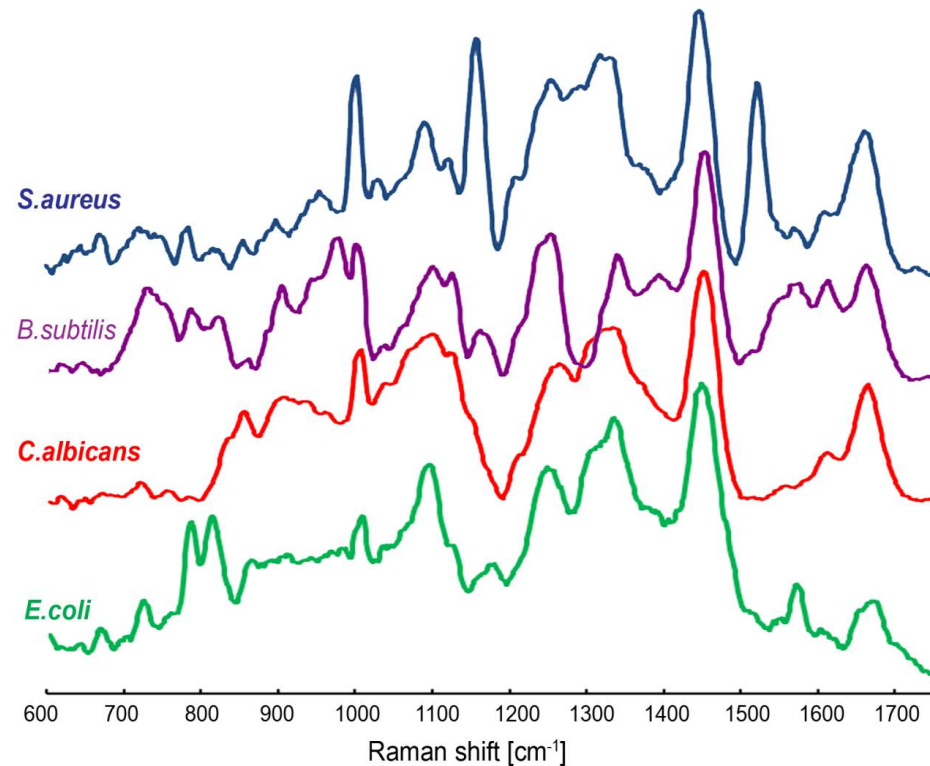
Raman images of smooth muscle cells isolated from different tissues



Eva Brauchle

Marzi et al. *In Prep*

Raman spectroscopy for discriminating bacterial species



Applications:

- Diagnosis of infections
- Quality control for food and pharmaceuticals

Advantages:

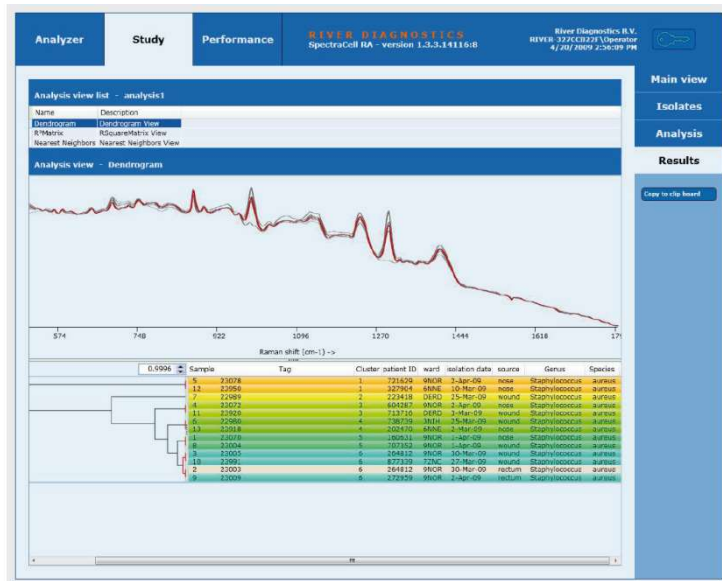
- Short incubation times (micro-colonies) or analysis on the single cell level

Raman spectroscopy

Raman spectroscopy for discriminating bacterial species

Epidemiology of nosocomial infections

River ® diagnostics
SpectraCell RA



Reviewing screen of test results of SpectraCell RA interface



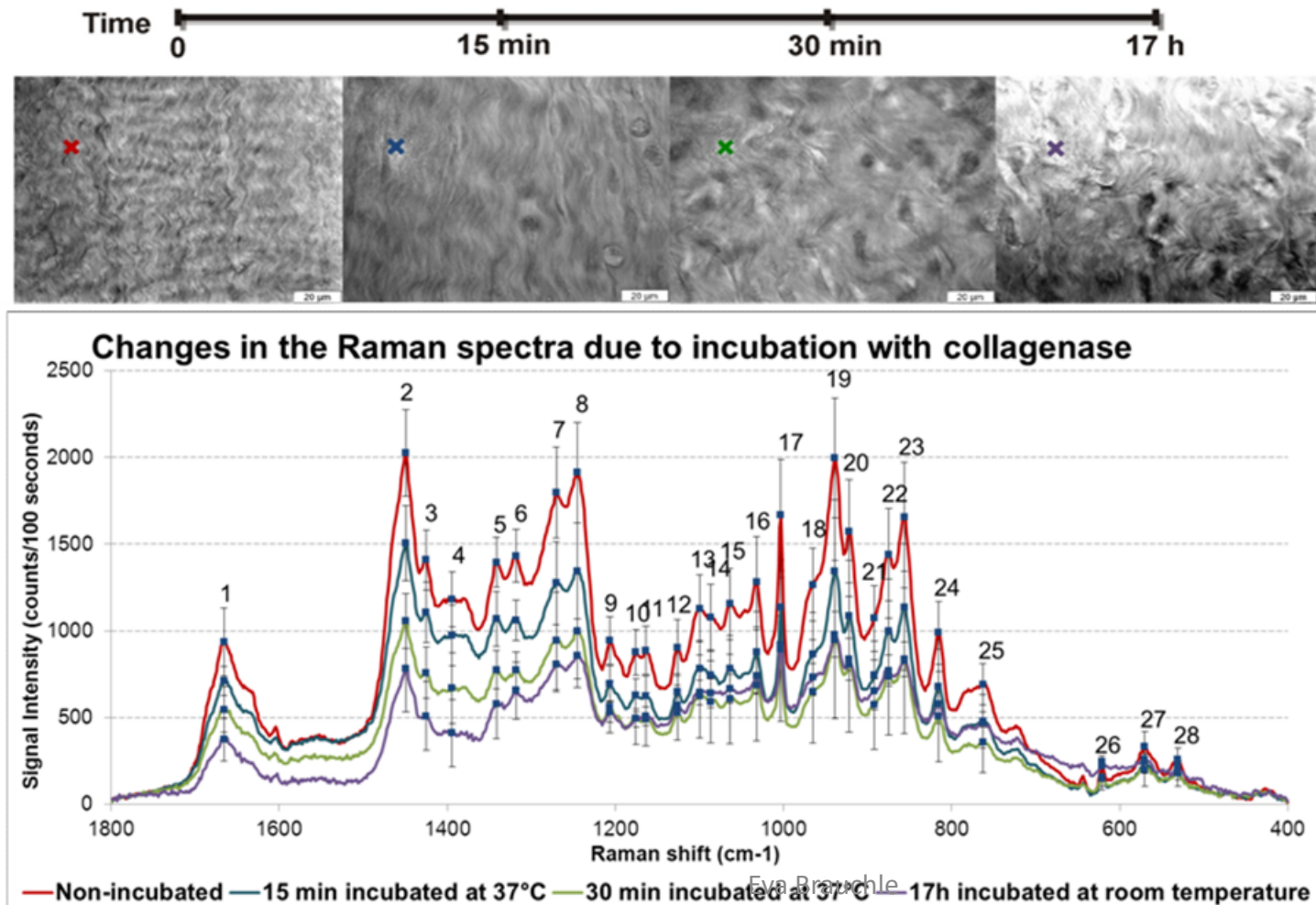
Eva Brauchle

<http://www.riverd.com/aboutspectra.html>

Raman micro-spectroscopy - Applications

Identification and analysis of extracellular matrix (ECM) structures

Degradation of collagen in porcine heart valves



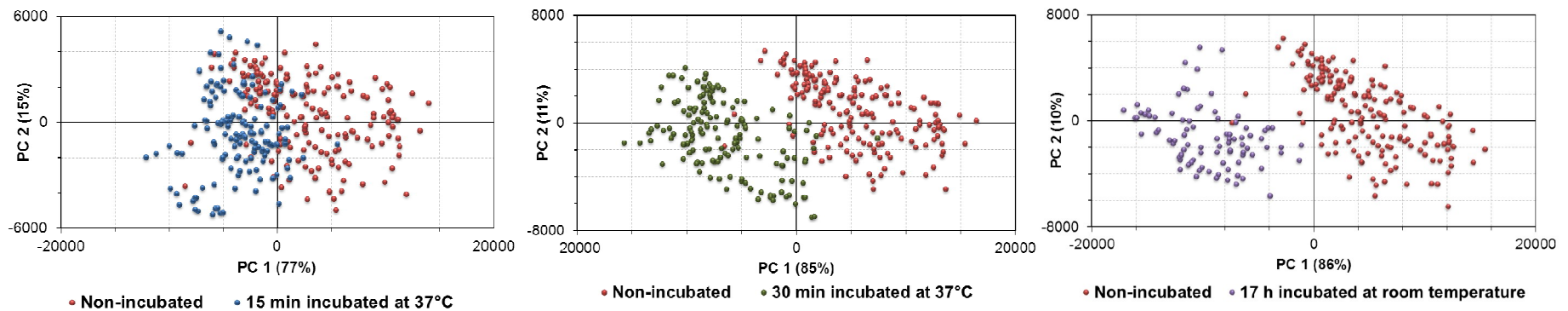
Votteler et al. 2012

Raman micro-spectroscopy - Applications

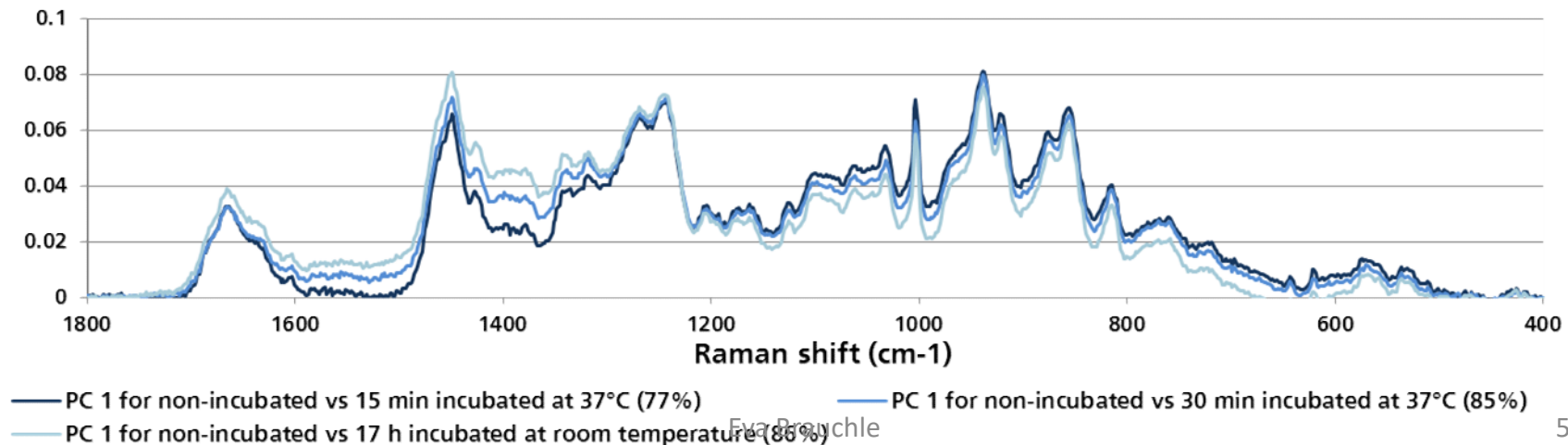
Identification and analysis of extracellular matrix (ECM) structures

Degradation of collagen in porcine heart valves

Scores

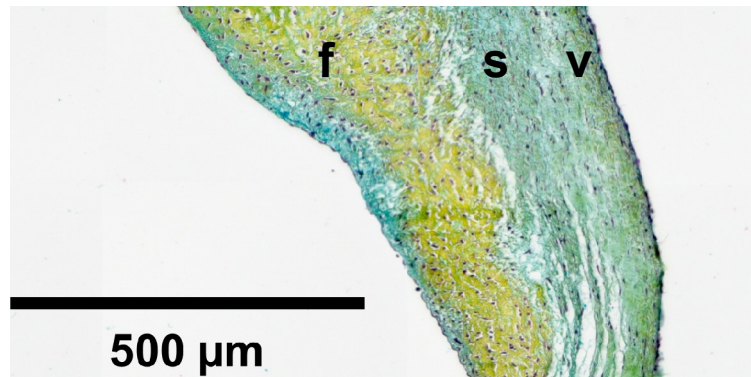


Loadings



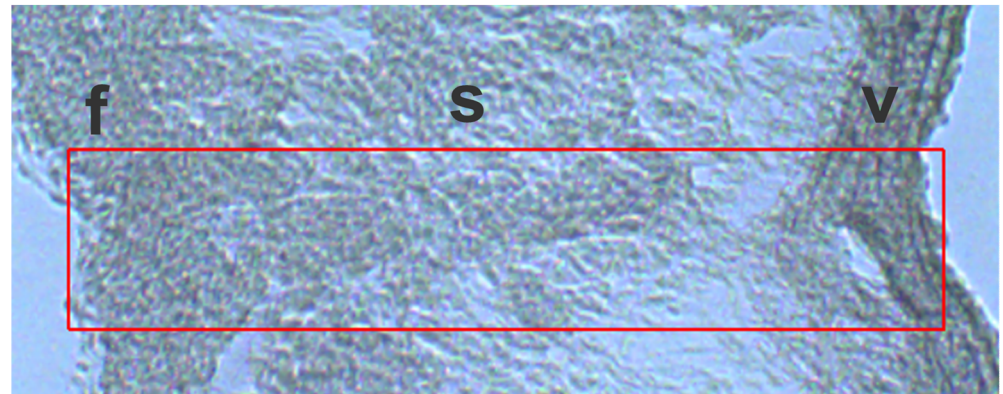
Raman Imaging of heart valve leaflets

Movat Pentachrome staining

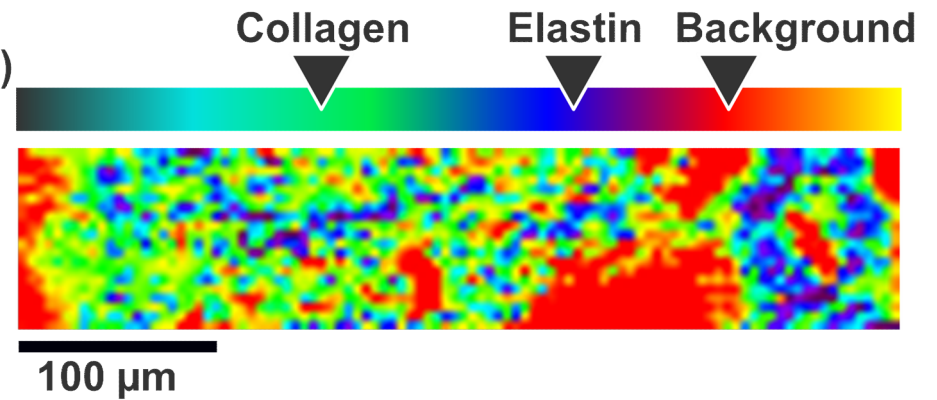


f: fibrosa
s: spongiosa
v: ventricularis

(a)

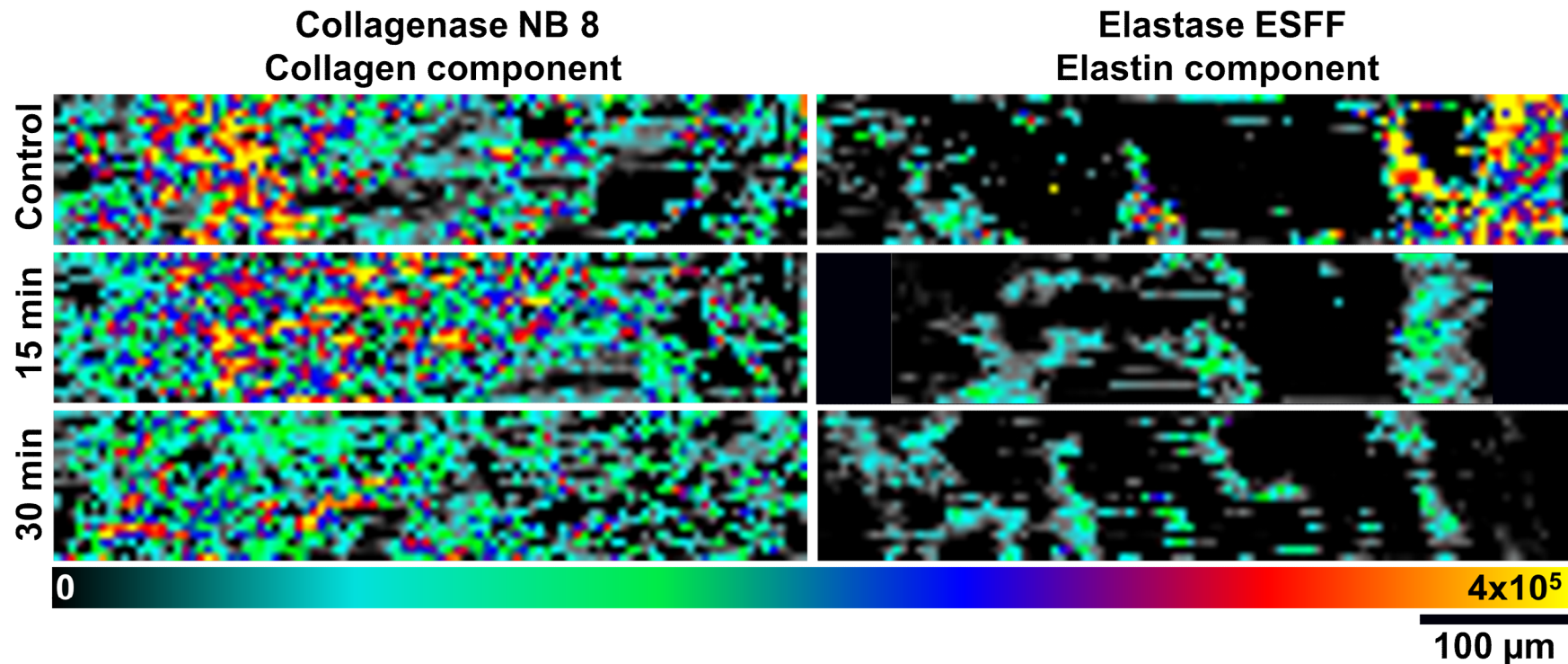


(b)



Raman Imaging of heart valve leaflets

Monitoring of ECM degradation



Color coded images based on the fitted component's intensity. The enzymatic digestion results in a drop in collagen (left panel) and elastin content (right panel) in the leaflet.

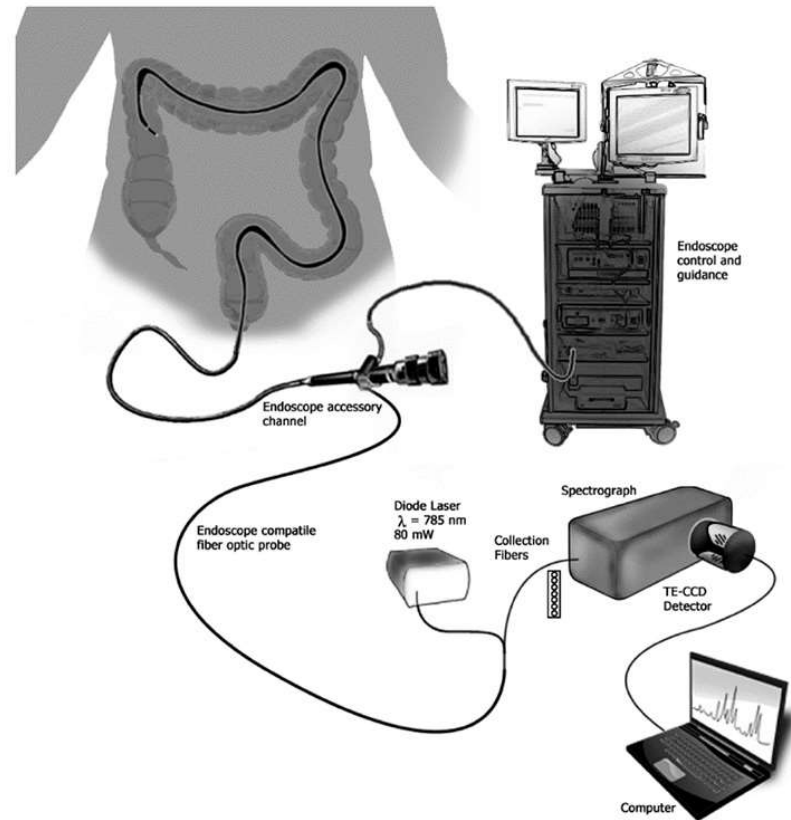
In vivo Raman

Skin Analysis



<http://www.riverd.com/skin-analysis>

Endoscopy



<https://www.osa-opn.org/news/raman-ibd/>

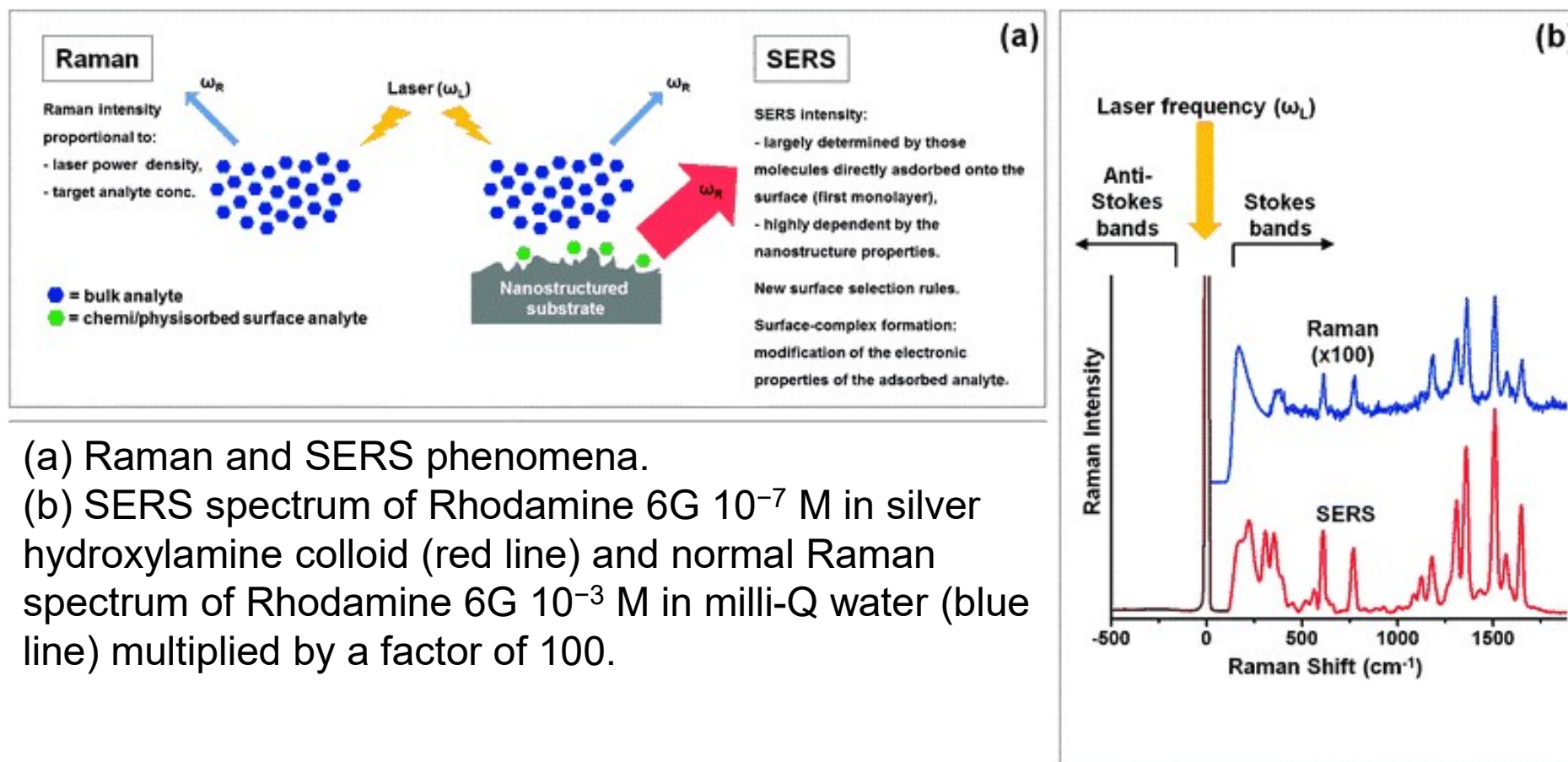
Limitations

- Raman scattering is a rare event
- Long measurement times and strong lasers are required (for biological samples), risk of sample burning
- Autofluorescence of the sample can obscure the Raman scattering

How to overcome these limitations?

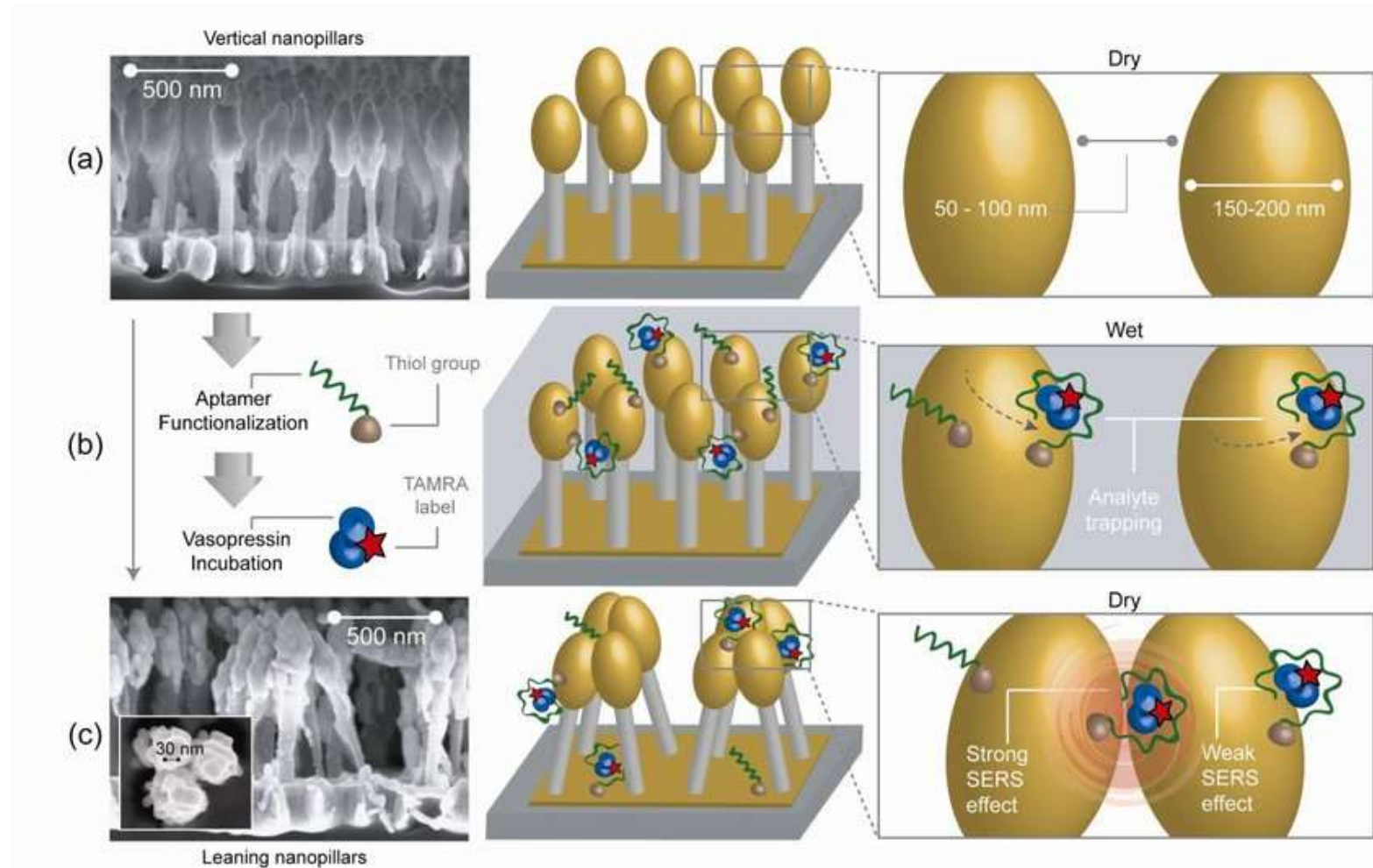
- Surface-enhanced Raman scattering (SERS)
- Coherent Anti-Stokes Raman scattering (CARS)

Surface-enhanced Raman scattering (SERS)



Guerrini and Graham, 2012

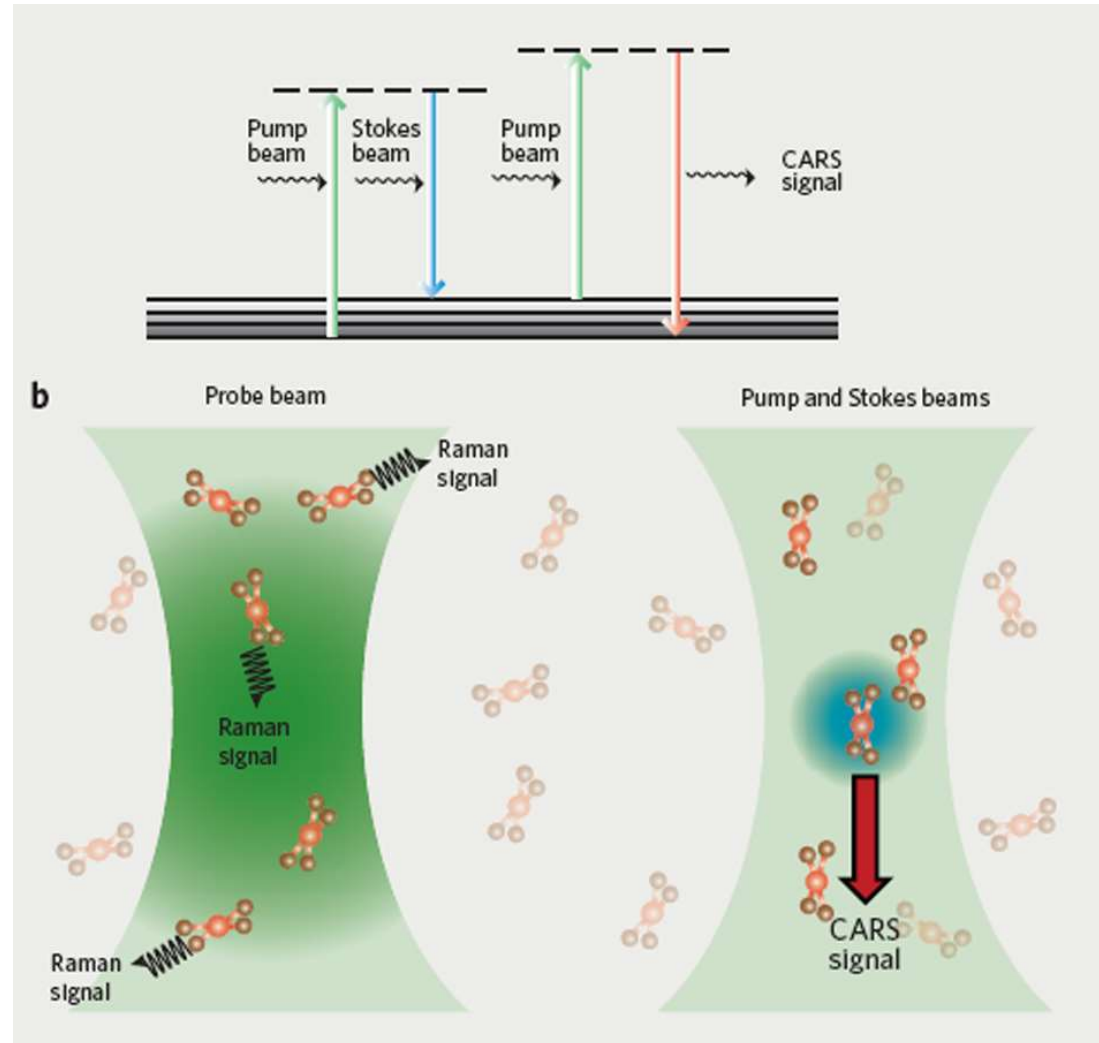
SERS on nanostructured surfaces



https://www.researchgate.net/figure/Schematics-of-the-functionalized-leaning-nanopillar-detection-method-a-Highly-packed_fig14_292967158

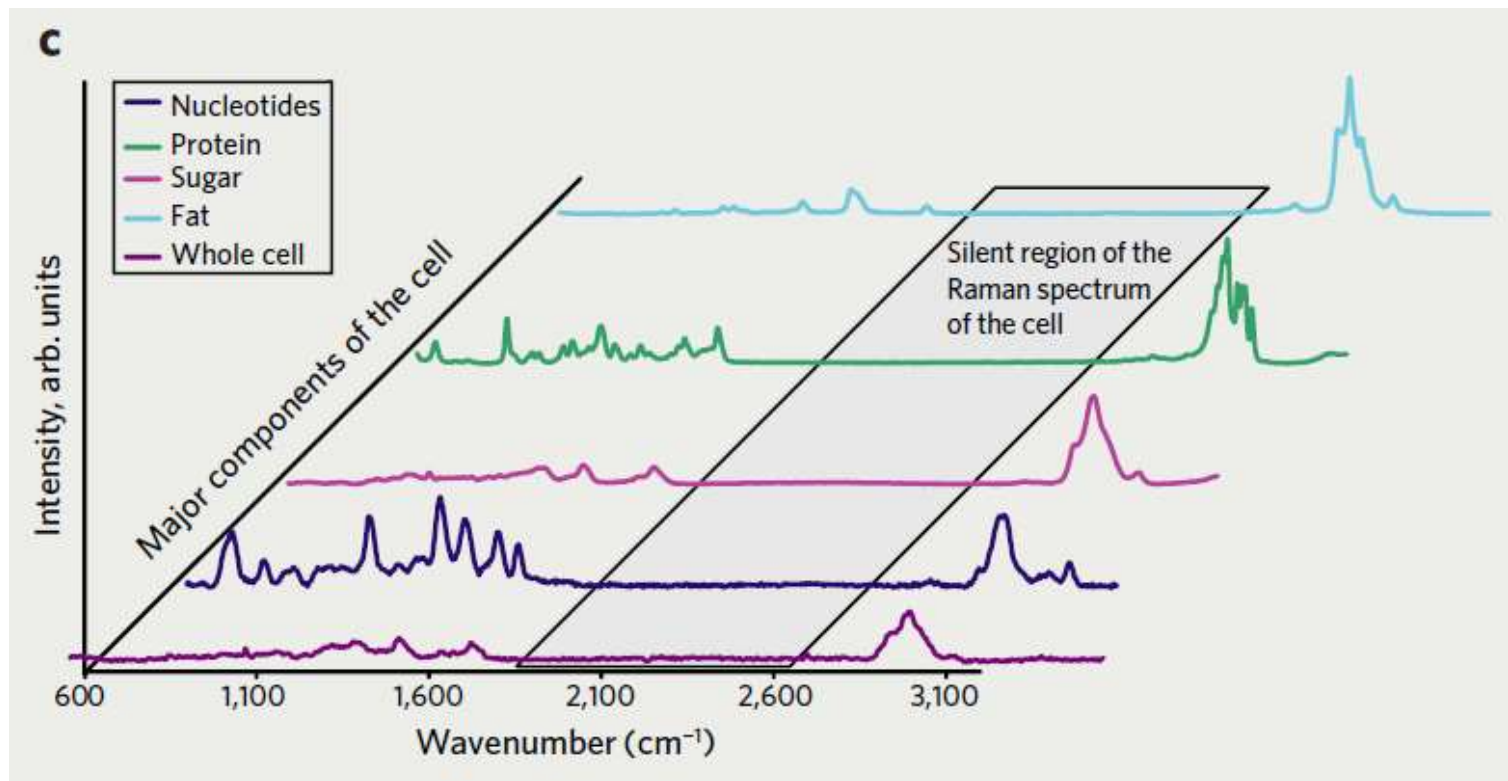
Coherent Anti-Stokes Raman Scattering (CARS)

- Non-linear Raman effect
- Use of two lasers with different wavelength
- Pump beam and Stokes beam interact with the same sample and generate polarization



Coherent Anti-Stokes Raman Scattering (CARS)

- Chemical information is consistent with spontaneous Raman spectroscopy
- Lipid signals are most frequently used for imaging

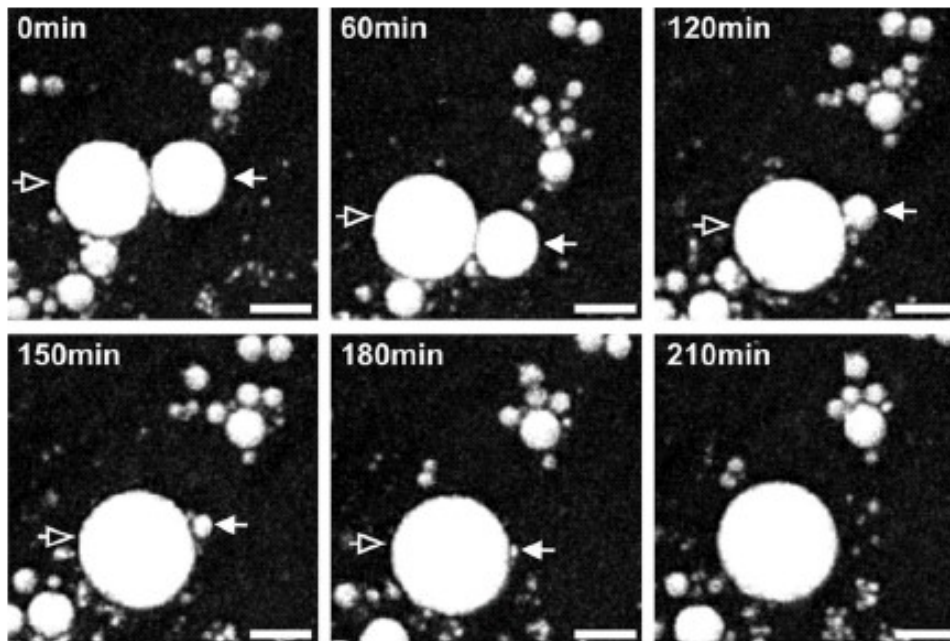


Summary Raman microscopy

- Optical detection of molecular vibrations in any sample
- Biological samples result in very complex spectra
- Multivariate analysis (PCA-based methods) on the spectra
- Imaging with biochemical contrast
- Marker-free method for cell/tissue monitoring in vitro
- Clinical applications

Coherent Anti-Stokes Raman Scattering (CARS)

- Dynamics of intracellular lipid vesicles



Paar, Jüngst et al. in J. Biol. Chem. 2012

Video:

<http://www.jbc.org/content/suppl/2012/02/06/M111.316794.DC1/jbc.M111.316794-3.wmv>

Eva Brauchle

Multiphoton Imaging

Prediction of multiphoton
excitation in her PhD in 1931



Maria Göppert-Mayer

Über Elementarakte mit zwei Quantensprüngen

Von Maria Göppert-Mayer

(Göttinger Dissertation)

(Mit 5 Figuren)

Einleitung

Der erste Teil dieser Arbeit beschäftigt sich mit dem Zusammenwirken zweier Lichtquanten in einem Elementarakt. Mit Hilfe der Diracschen Dispersionstheorie¹⁾ wird die Wahrscheinlichkeit eines dem Ramanefekt analogen Prozesses, nämlich der Simultanemission zweier Lichtquanten, berechnet. Es zeigt sich, daß eine Wahrscheinlichkeit dafür besteht, daß ein angeregtes Atom seine Anregungsenergie in zwei Lichtquanten aufteilt, deren Energien in Summe die Anregungsenergie ergeben, aber sonst beliebig sind. Fällt auf das Atom Licht, dessen Frequenz kleiner ist, als die entsprechende Eigenfrequenz des Atoms, so tritt außerdem noch eine erzwungene Doppelemmission hinzu, bei der das Atom seine Energie in ein Lichtquant der eingesandten und eins der Differenzfrequenz aufteilt. Kramers und Heisenberg²⁾ haben die Wahrscheinlichkeit dieses letzteren Prozesses korrespondenzmäßig berechnet.

Außerdem wird die Umkehrung dieses Prozesses betrachtet, nämlich der Fall, daß zwei Lichtquanten, deren Frequenzsumme gleich der Anregungsfrequenz des Atoms ist, zusammenwirken, um das Atom anzuregen.

Ferner wird untersucht, wie sich ein Atom gegenüber stoßenden Teilchen verhalten kann, wenn es gleichzeitig die Möglichkeit hat, spontan Licht zu emittieren. Oldenberg³⁾ findet experimentell eine Verbreiterung der Resonanzlinie des Quecksilbers, wenn er die angeregten Atome vielfach mit lang-

Multiphoton Microscopy

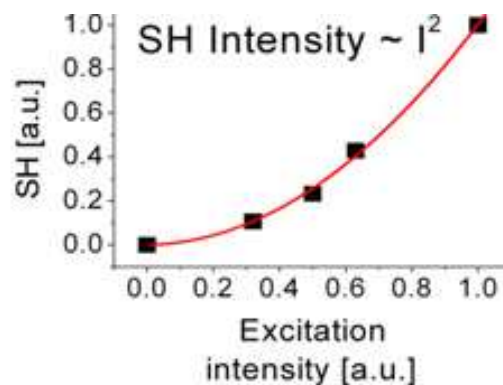
- Nonlinear optical process
- Excited region is limited to a subfemtoliter volume
- Requires high-speed femtosecond near-infrared pulsed laser systems
- Imaging of intrinsic fluorophores (marker-free)
- Enables in vivo deep tissue imaging
 - => **potential clinical application**
- It can be distinguished between different multiphoton processes:
 - Second harmonic generation (SHG)
 - Two-photon excited fluorescence (TPEF)



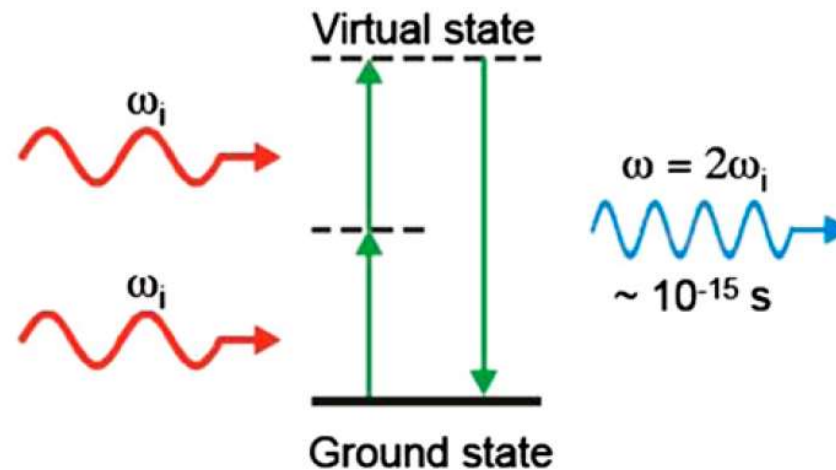
<http://www.jenlab.de/MPTflex-R.114.0.html>

Second harmonic generation (SHG)

- Two photons interact with non-linear material. One photon with exact the double frequency is emitted
- Virtual transition (no bleaching or photodamage)
- Energetically neutral
- Emission happens in femtoseconds
- SHG intensity is proportional to the incident number of photons in squares



M. Kozak et al. Physica E, 2012

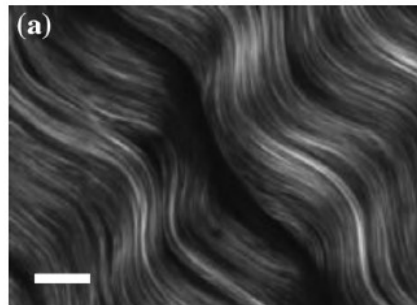


Eva Brauchle Pentazis et al., PNAS 2010

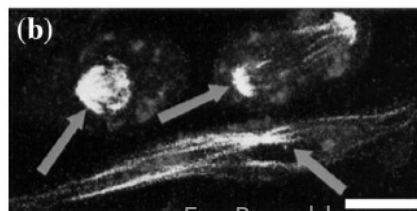
SHG imaging

- Imaging of the structural organization of macromolecules in tissues
- Endogenous SHG is known for molecules that form microfibers or fibrils (collagen, microtubules, myosin)
- These proteins have a cylindric organization of proteins in common

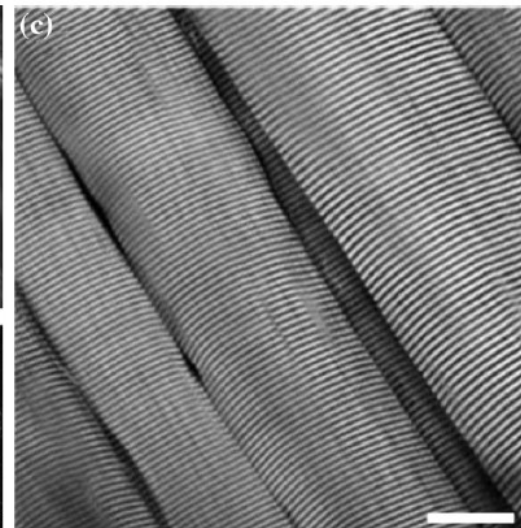
Collagen in a
rat tail



microtubules

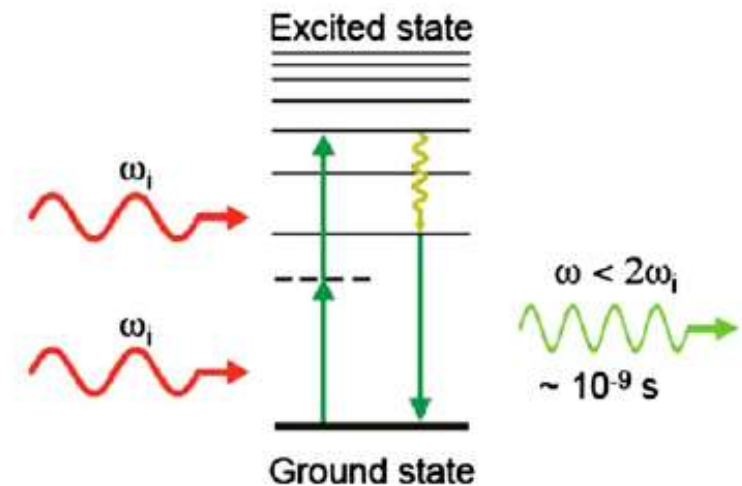


Skeletal muscle



Two-photon excited fluorescence (TPEF)

- Two photons (NIR range 700-1200 nm) are absorbed at the same time and can excite the emission of blue, red or green fluorescence
- Excitation frequency is lower than in SHG
- Molecules reach an excited stage
- Procedure happens in nanoseconds
- Energy is lost

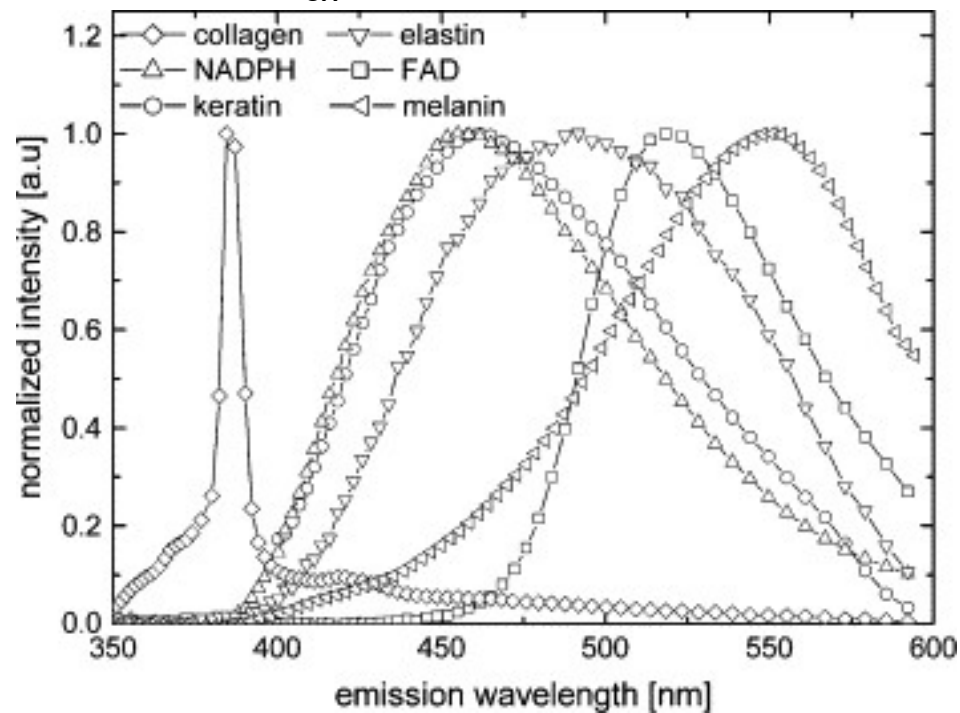


Pentazis et al., PNAS 2010

Two-photon excited fluorescence (TPEF)

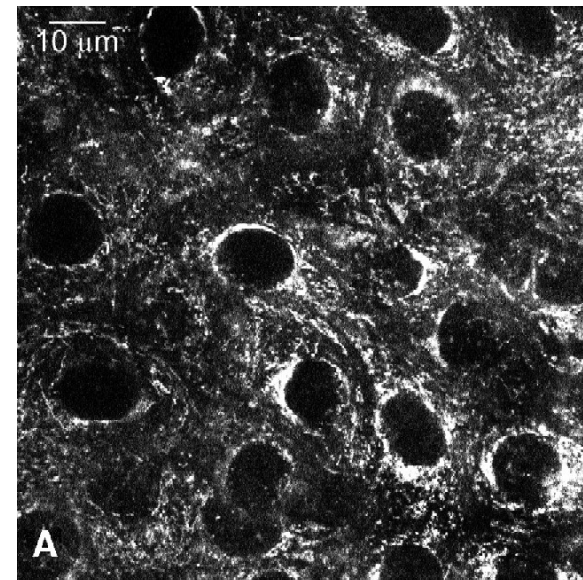
- Visualization of endogenous molecules

Emission spectra of different endogenous molecules $\lambda_{\text{ex}} = 764 \text{ nm}$



Palero, Biophys J. 2007

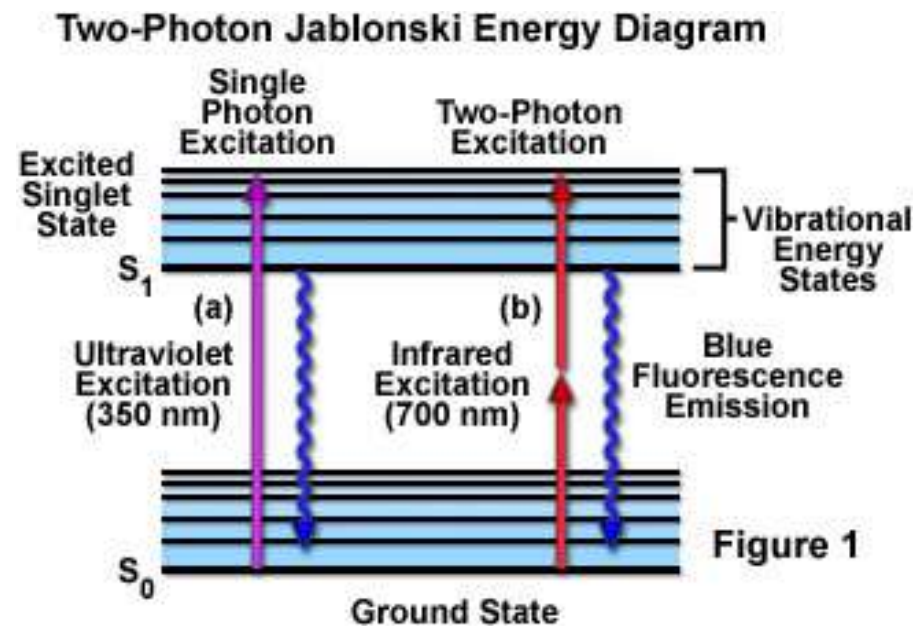
Excitation of NADP(H) $\lambda_{\text{ex}} = 720 \text{ nm}$ in dermal cells



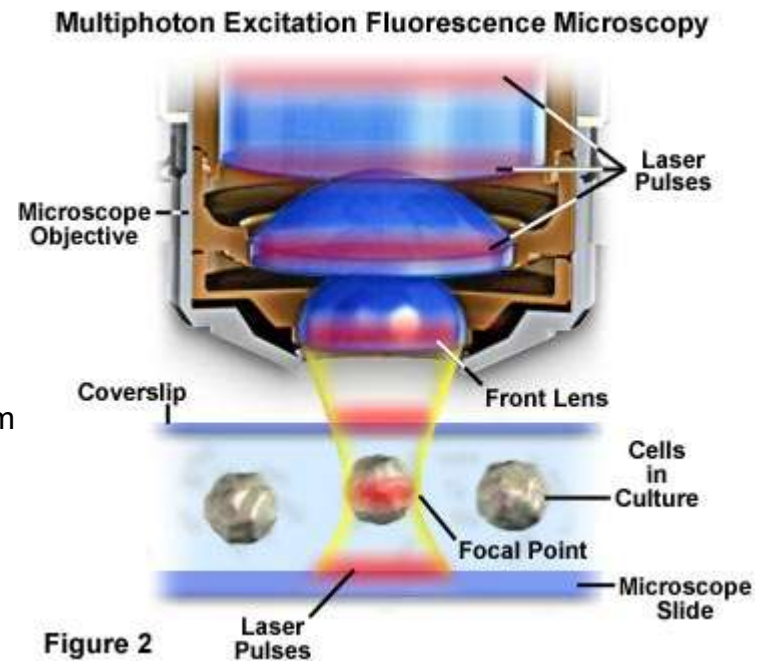
Schenke-Layland, Adv. Drug Del Reviews, 2008

Two photon-excited fluorescence (TPEF)

Advantages of TPEF compared to one photon-excited fluorescence



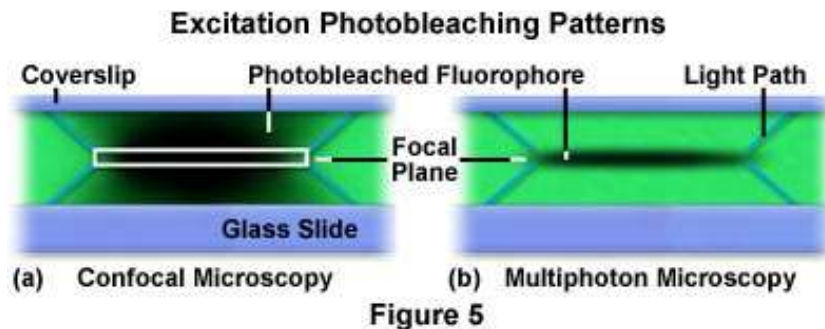
<http://www.microscopyu.com/articles/fluorescence/multiphoton/multiphotonintro.html>



Two-photon excited fluorescence (TPEF)

Advantages of TPEF compared to one photon-excited fluorescence

- Deeper penetration in tissue due to the use of NIR light
- Photo-damage and photo-bleaching



<http://www.olympusmicro.com/primer/techniques/fluorescence/multiphoton/multiphotonintro.html>

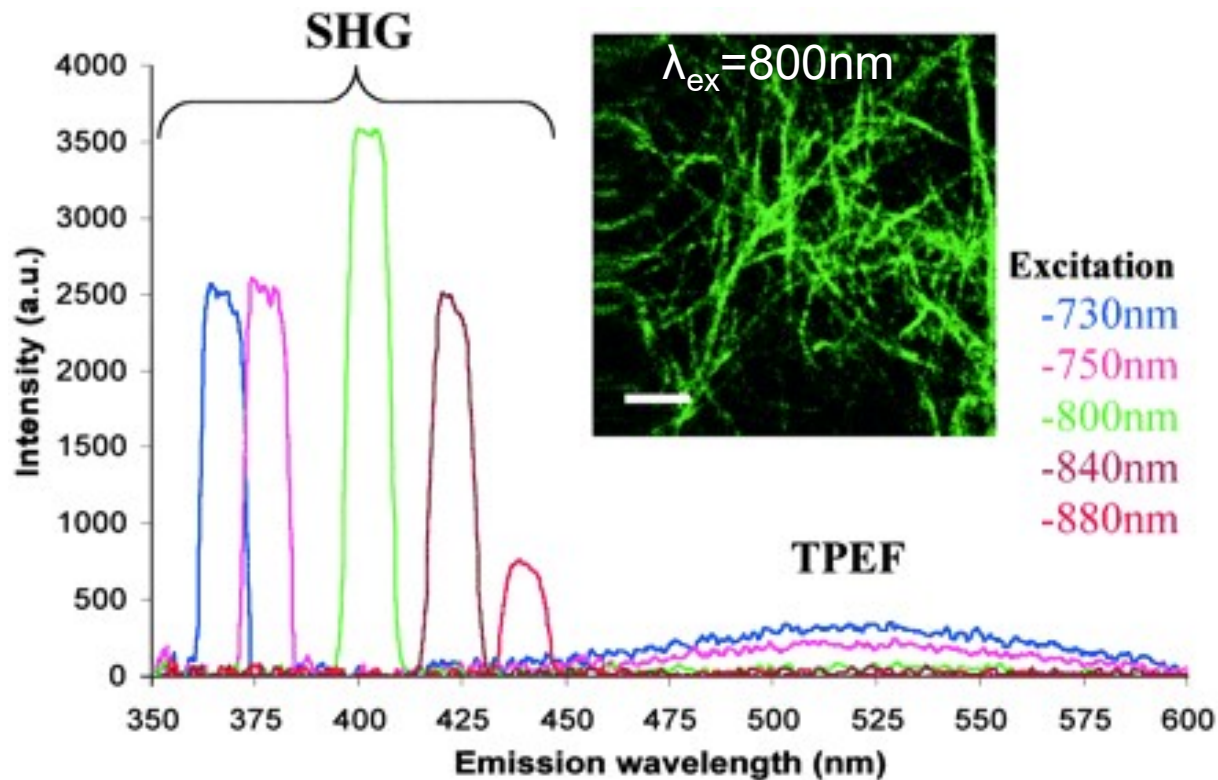
Fluorophore	Excitation λ (nm)	Emission λ (nm)	Fluorescence Lifetime τ (ns)
Keratin		525	
NAD(P)H	340	450–470	0.3–0.6*
Flavins	370, 450	530	5.2
Melanin	UV/VIS	440, 520, 575	0.2/1.9/7.9
Elastin	300–340	420–460	0.2–0.4/0.4–2.5
Collagen	300–340	420–460	0.2–0.4/0.4–2.5
SHG	720–960	360–480	0
Lipofuscin	UV/VIS	570–590	
PPIX	405 ... 630	635, 710	11

Schenke-Layland, J. Biophotonics 2008

Multiphotonen Tomography

- Combination of SHG and TPEF imaging

Emission spectra of a 3D tissue model



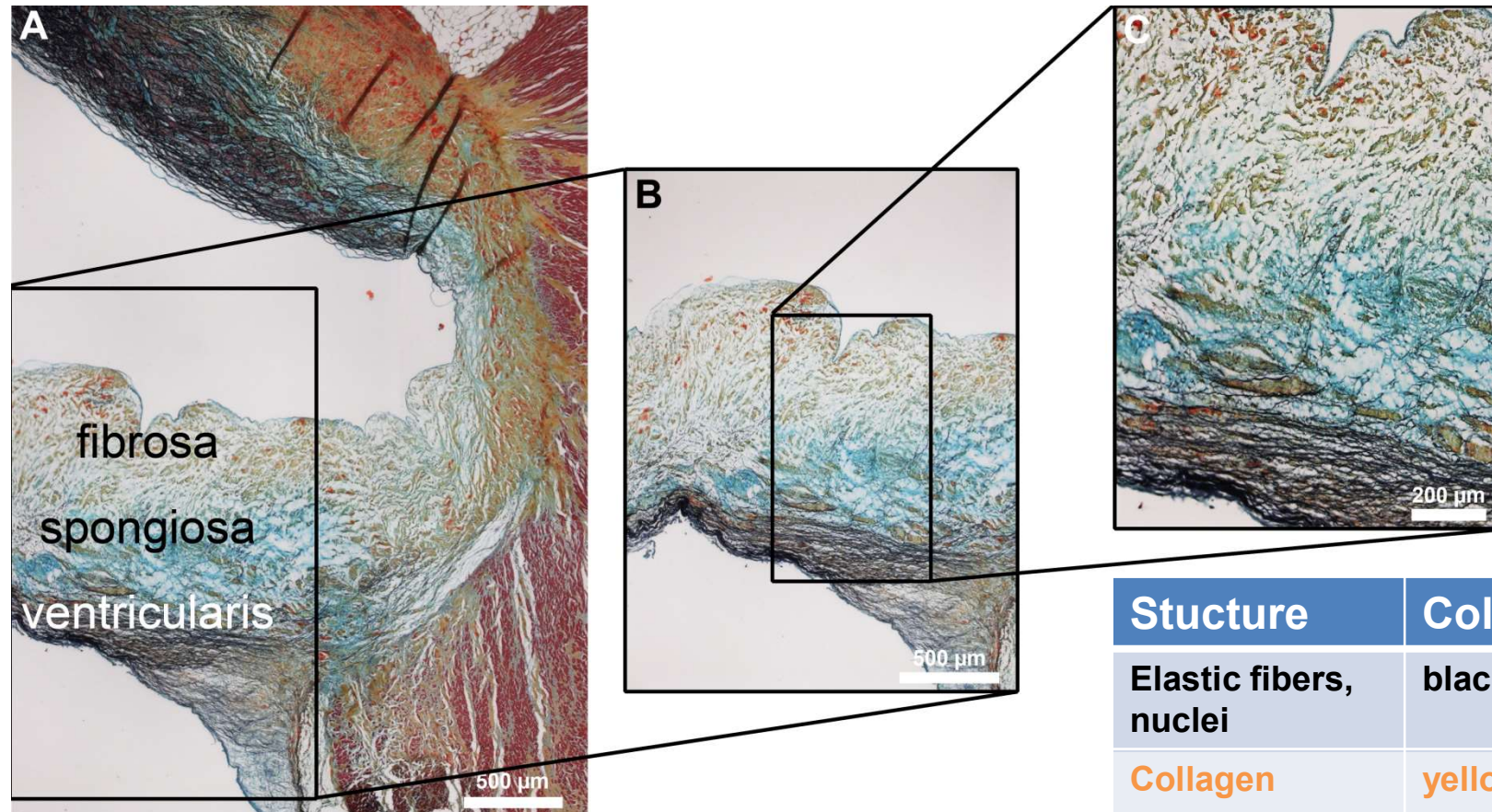
Zoumi, PNAS. 2002

Multiphoton Imaging

Intrinsic chromophores	Localization	One-photon (<i>excitation maxima/emission maxima</i>)	Multi-photon (<i>excitation maxima/emission maxima</i>)
NAD(P)H	Mitochondrial matrix	260/440 nm; 351/450 nm (Avi-Dor <i>et al.</i> , 1962; Scott <i>et al.</i> , 1969; Brandes & Bers, 1999; Shuttleworth <i>et al.</i> , 2003).	730–780/460–480 nm (Xu & Webb, 1996; König, 2000a; Wang <i>et al.</i> , 2008a).
Flavoprotein	Mitochondrial matrix	376/450 nm; 365/500 nm; 450/515 nm (Richards-Kortum & Sevick-Muraca, 1996; van den Berg <i>et al.</i> , 1998).	900/510–650 nm (Masters <i>et al.</i> , 1997; Huang <i>et al.</i> , 2002; Ramanujan <i>et al.</i> , 2005).
Elastin	Extracellular matrix. Main fluorescent component of elastic fibres.	315/405 nm; 350/420 nm; 410/500 nm; 450/520 nm (Deyl <i>et al.</i> , 1980; Richards-Kortum & Sevick-Muraca, 1996).	730–760/460–480 nm (Xu & Webb, 1996; Schenke-Layland <i>et al.</i> , 2004; König <i>et al.</i> , 2005a).
Lipofuscin	Heart muscle, adrenals, skin, RPE and nerve cells. Arranged around the nucleus.	364/450 nm; 488 nm/?; 568 nm/?; 633 nm/? (Richards-Kortum & Sevick-Muraca, 1996; Marmorstein <i>et al.</i> , 2002).	800/500 nm to 550 nm (Bindewald-Wittich <i>et al.</i> , 2006; Han <i>et al.</i> , 2006).
Melanin	Skin, hair and RPE	340/440–520 nm; 430 nm/? (Mosca <i>et al.</i> , 1999; Elleder & Borovanský, 2001).	800/550 nm (Teuchner <i>et al.</i> , 2000; Hoffmann <i>et al.</i> , 2001).
Porphyrins	Erythrocytes	400/650–670 nm (König <i>et al.</i> , 1992; Schneckenburger <i>et al.</i> , 1993; Ricchelli, 1995).	800/590 nm (König, 2000a; Mathai <i>et al.</i> , 2007).
Lipoamide dehydrogenase	Mitochondrial matrix	363 and 456 nm/?; 296/330 and 520 nm (Fukuzawa & Aki, 1983; Huang <i>et al.</i> , 2002).	900/510–650 nm (Masters <i>et al.</i> , 1997; Huang <i>et al.</i> , 2002; Ramanujan <i>et al.</i> , 2005).
Keratin	Epithelial cells	277/382 nm (Pena <i>et al.</i> , 2005).	700–900/460–550 nm (760/475 nm; 860/515 nm) (Pena <i>et al.</i> , 2005; Wu & Qu, 2005).
Collagen	Extracellular matrix	265/310 nm; 280/385 nm; 330/390 nm; 450/530 nm (Richards-Kortum & Sevick-Muraca, 1996; Georgakoudi <i>et al.</i> , 2002).	Excitation: 800–860 nm; emission: one half of the excitation wavelength (Zoumi <i>et al.</i> , 2002; König & Riemann, 2003; Zipfel <i>et al.</i> , 2003b; Wang <i>et al.</i> , 2008a).

ECM in heart valves

Movats pentachrome staining



Stucture	Color
Elastic fibers, nuclei	black, blue
Collagen	yellow
Proteoglycans	Light blue, green
Fibrin, muscle	red

Multiphoton Tomography

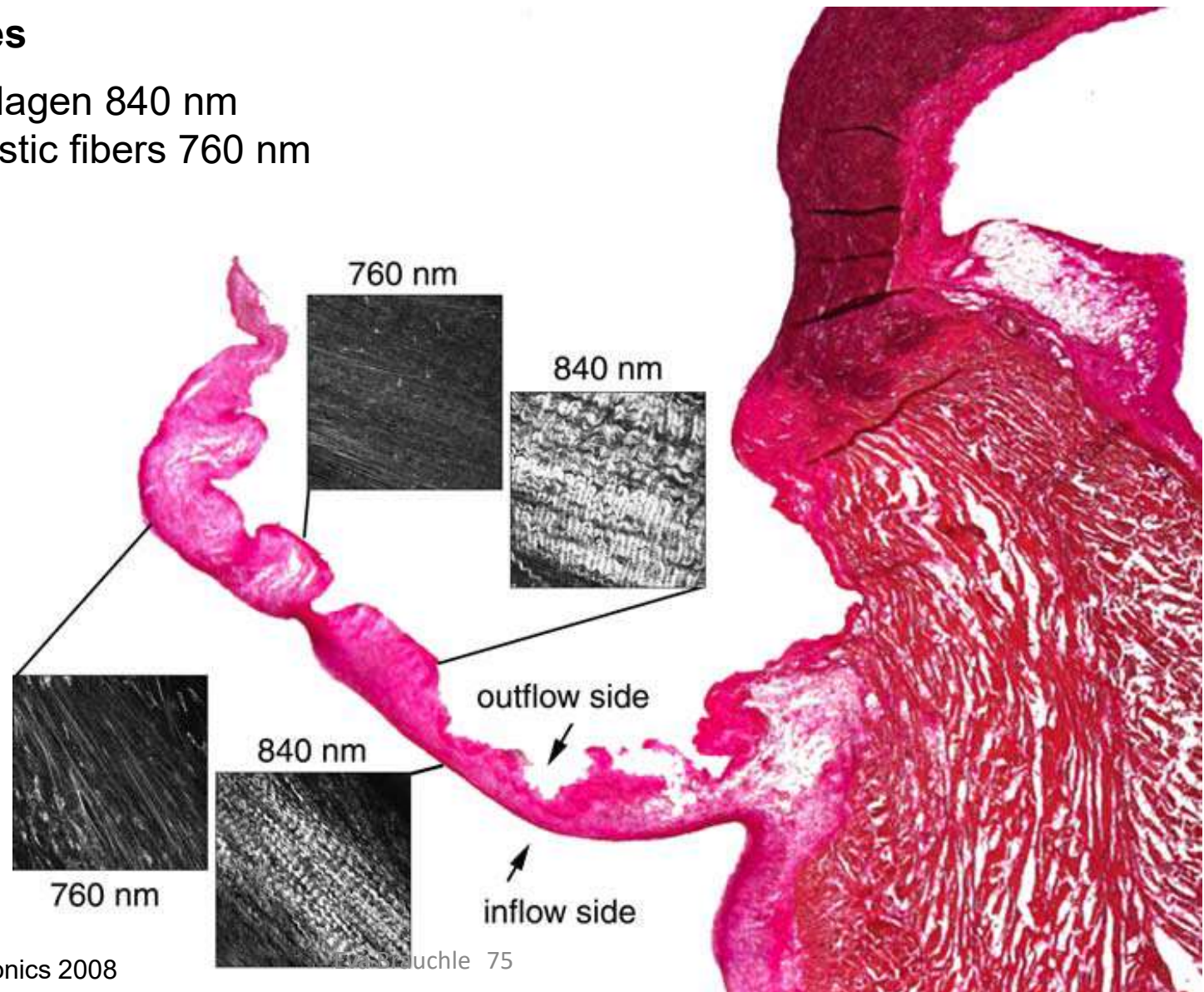
ECM in heart valves

SHG: collagen 840 nm
TPEF: elastic fibers 760 nm

Video



Tec.mov



Multiphoton Tomography

Its potential application in monitoring of the conservation process of donor heart valves

- Conventional cryopreservation due to **freezing (CFC)**
- Cryopreservation due to **vitrification (IFC)**

IFC is characterized by:

1. No formation of ice crystals
2. Slow freezing process
3. Low concentration of anti-freezing agents

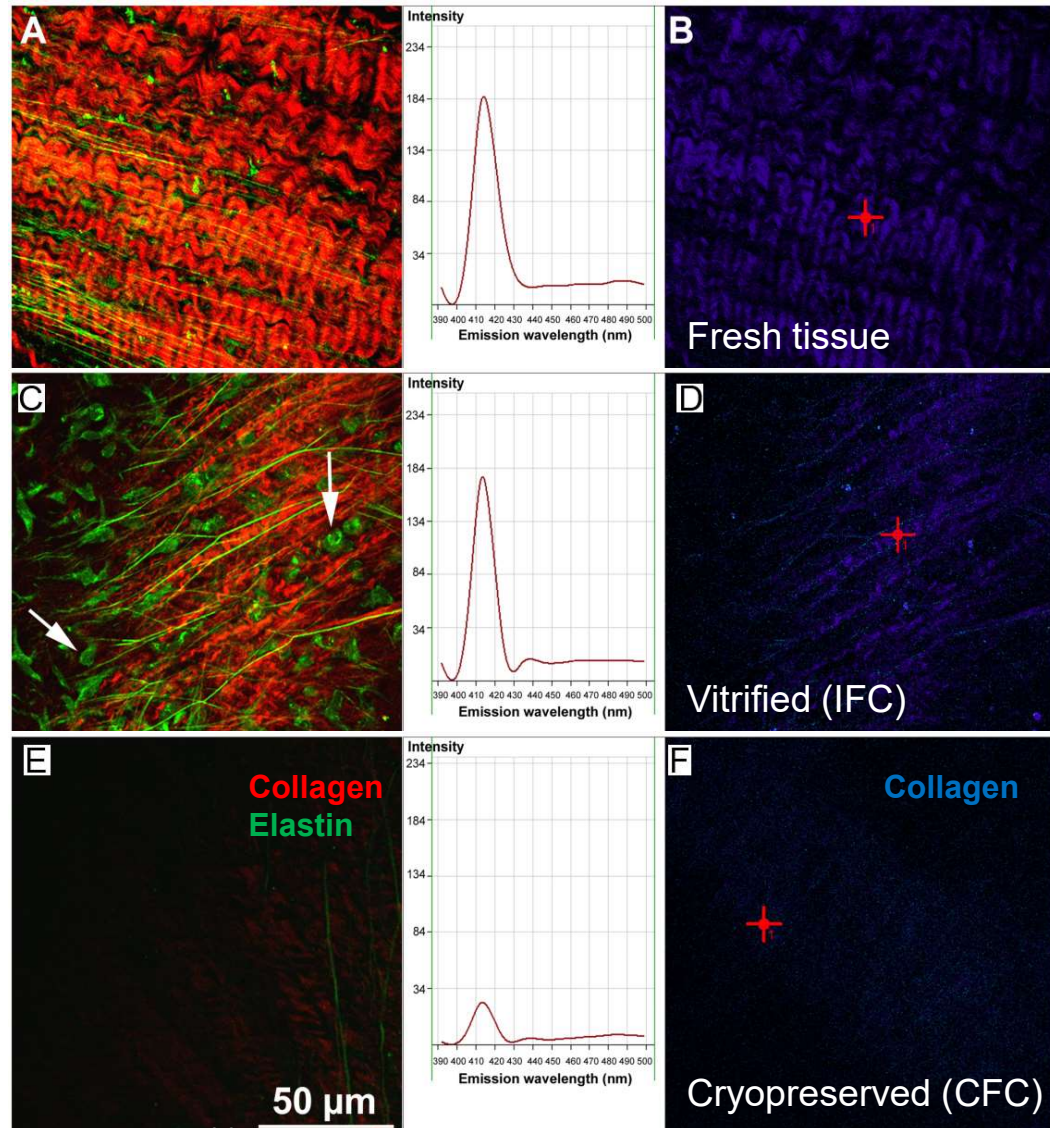


Multiphoton Tomography: Heart valves

Collagen-rich outflow side

Comparison of ECM in a valve after IFC and CFC

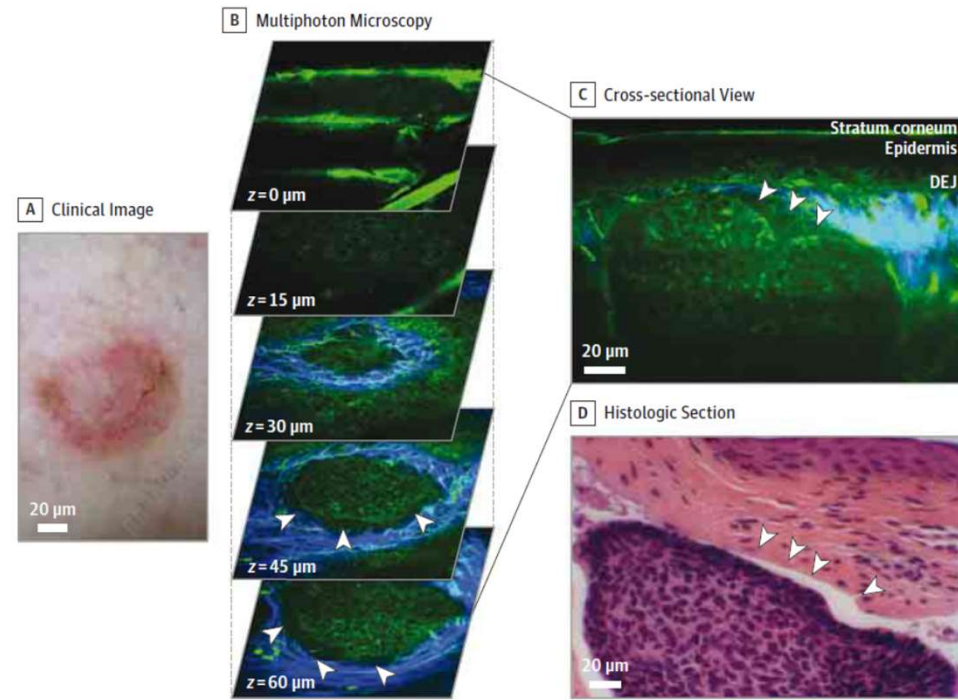
Monitoring of ECM degradation and remodeling in vivo



In vivo Multiphoton Tomography of Basal Cell Carcinoma



Balu et al. JAMA Dermatology 2015



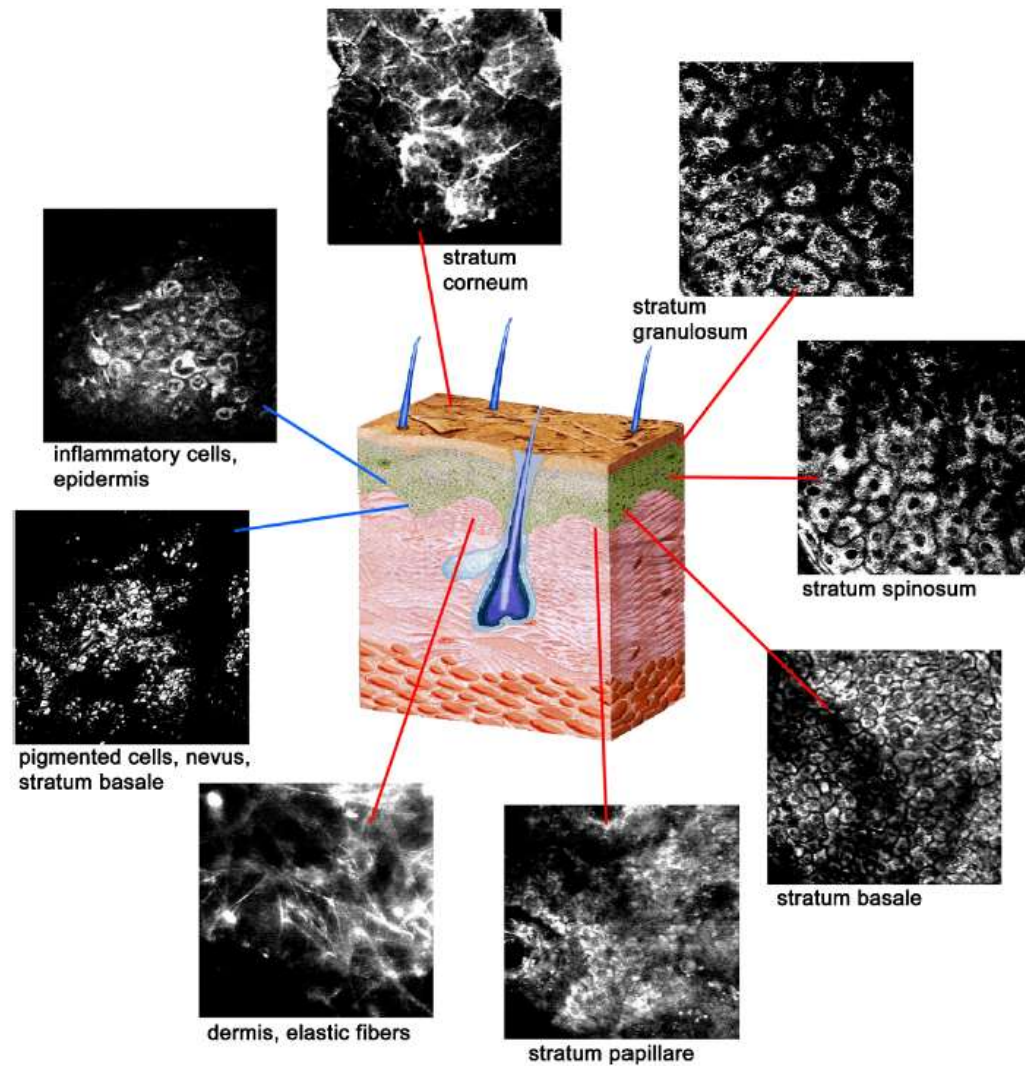
Balu et al. JAMA Dermatology 2015

Collagen (blue)
Basoloid cells (green)
Elastin (green)

Multiphoton Tomography

Skin Layers

$$\lambda_{\text{ex}} = 740 \text{ nm}$$

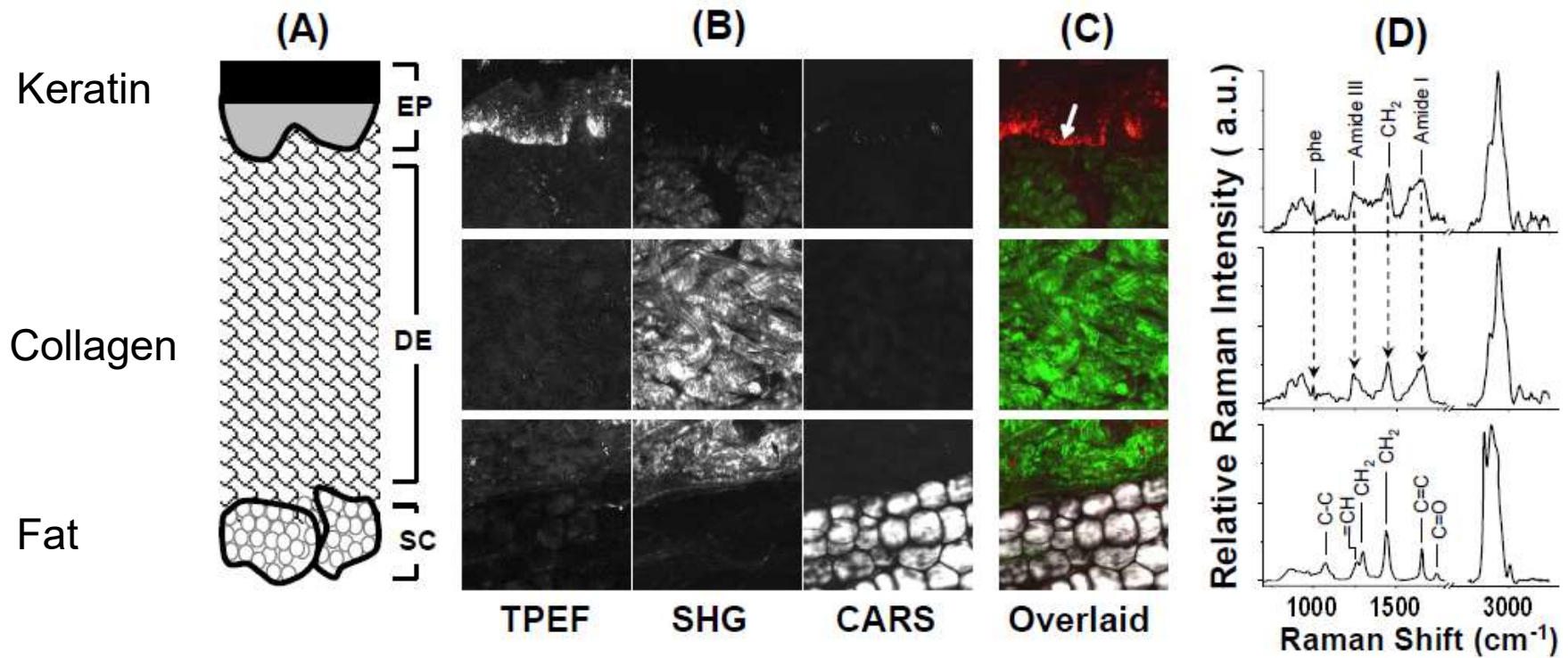


Summary Multiphoton Imaging

- Near-infrared laser for excitation
- Signal detection in visible/UV range
- SHG is limited to specific proteins
- TPEF has broad emission spectra (less specific)
- High-resolution depth scanning of tissues

Combination of imaging methods

Cross-section of skin



Jhan, Optics Express, 2008

Thank you for your Attention