

Department for Medical Technologies & Regenerative Medicine

AG Schenke-Layland

Advanced microscopy & non-invasive imaging

Dr. Julia Marzi

julia.marzi@uni-tuebingen.de

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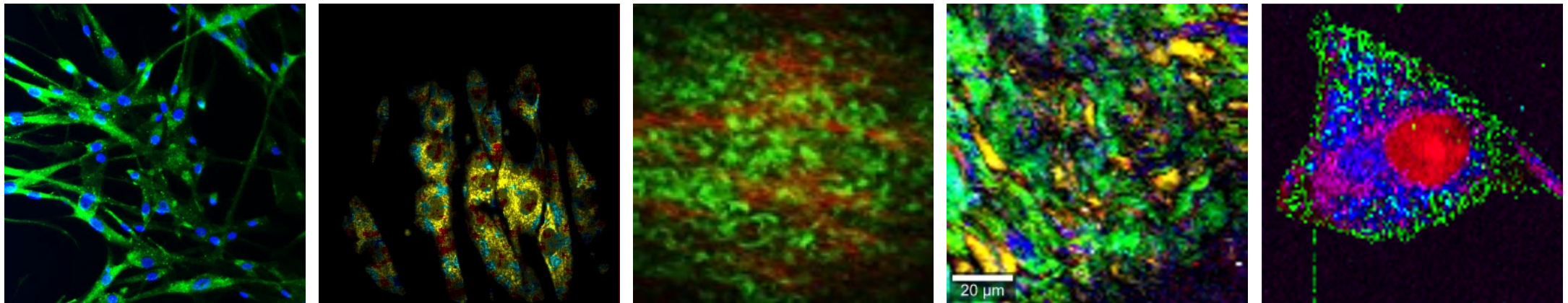
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Advanced microscopy & non-invasive imaging

- 1) Implant testing
- 2) Introduction to microscopy
- 3) Fluorescence lifetime imaging
- 4) Multiphoton microscopy
- 5) Raman microspectroscopy



Implant testing

How do material design & properties influence in vivo performance or failure?

Material characterization & mechanical properties

Material-tissue interaction

Cytotoxicity, Viability

Degradation

Functionality

Safety

>> in vitro testing, animal models, in-human performance

In vitro & ex vivo testing

Ex vivo testing

Mechanical & surface properties

Biomechanical testing

Design

In vitro testing

2D & 3D models

3R principle: Replace, reduce, refine – alternatives to animal testing

- >> organ-on-a-chip technology: models that recapitulate human physiology
 - dynamic conditions – blood flow simulation, exchange of nutrients & drugs
 - physiological cell density
 - personalization by patient-derived tissue models (cells, microtumors, organoids)

Animal models

In vivo preclinical testing to bridge the gap between lab scale and translation into clinical practice

Strong regulations, confined to the 3R principle

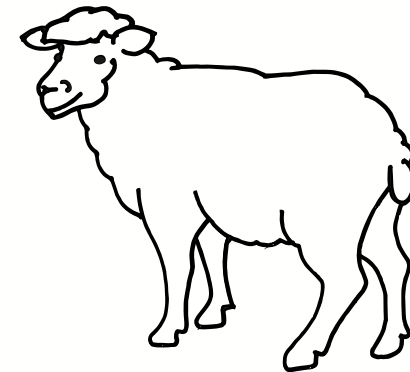
Rodent models: mouse, rat, rabbit

Large animal models: dog, pig, goat, sheep, horse, primates

Selection of the animal model depends on the research question

proof-of-concept, degradation, safety

biocompatibility, biomechanics, long-term repair/regeneration



Limitations

>> human physiology, anatomy and immunology, mechanical loads

Clinical testing

In-human performance & safety

Medical device clinical trials are required for all Class III medical devices and implants (EU MDR)

>> clinical evidence, clinical performance, clinical benefit ..

Only an implant or an ATMP (advanced therapeutic medicinal product)?

Tissue engineered products or combined ATMPs

CE-marked, used within the intended purpose or additional invasiveness / patient burdensome procedure?

Post-market surveillance

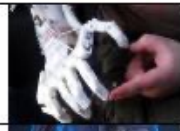
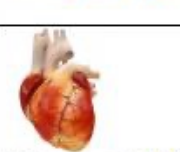
Quality by design

Trend towards individualized, patient-specific products e.g. by additive manufacturing

‘Quality cannot be tested into products - it should be built-in or should be by design.’

In situ tools needed which allow for

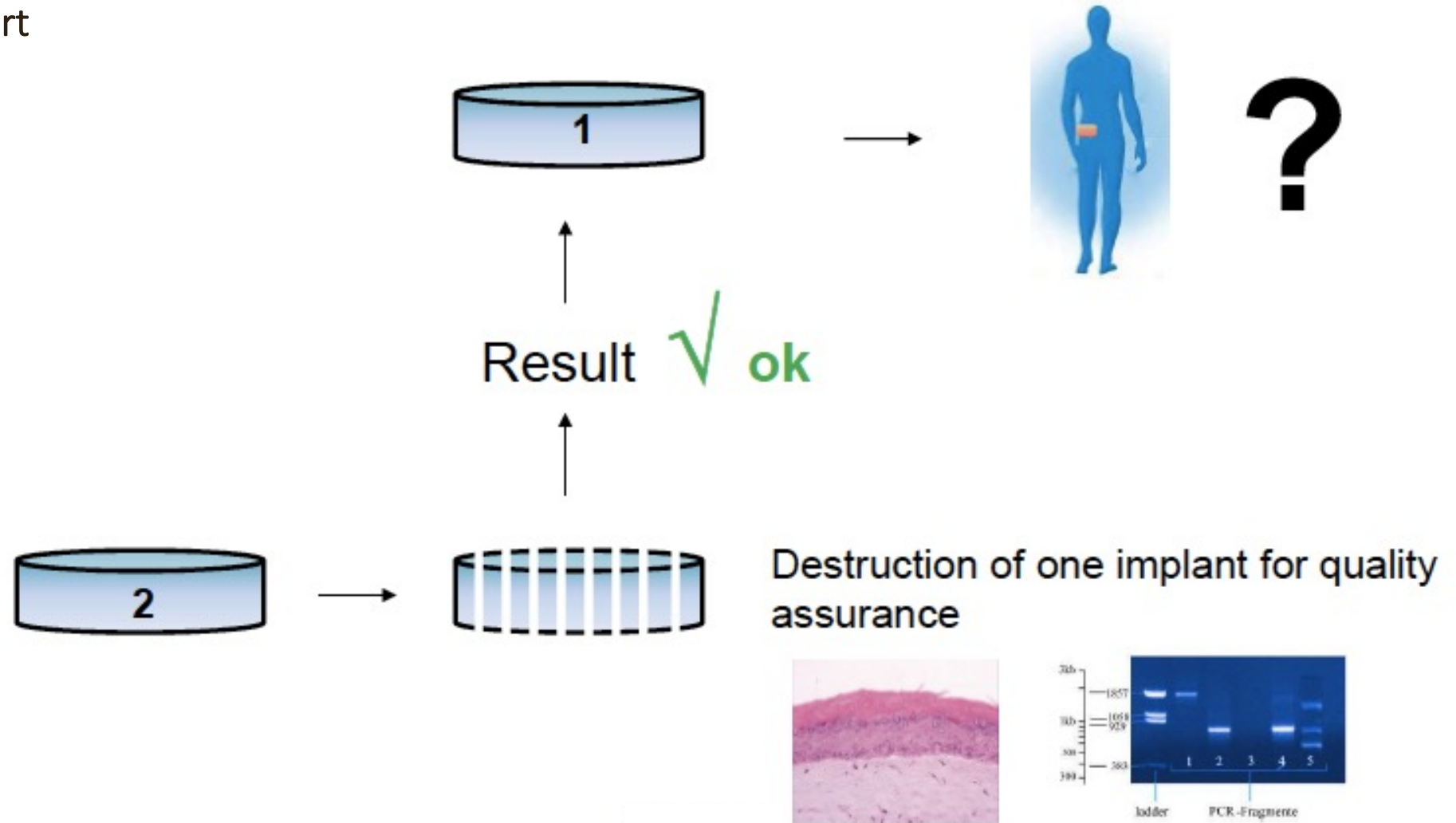
better understanding of structure-function relations
monitoring biological processes
non-destructive testing
real-time quality assessment

Personalized hearing aid		The hearing aid is one of the most successful printed products in the world. Over 10 million have been custom printed to date and every hearing aid company uses AM.
Customized invisible aligners		Over the last 17 years Align Technologies has manufactured more than 149 million unique aligners using AM, each comprised of hundreds of thousands of unique tooth measurements, captured digitally.
Affordable Prosthetics		Wearable mechanical braces are well suited for 3D printing because they are devices that are ideally customized to fit each patient.
Custom designed implants		3D printing enables customized and tailor-made implants. The implant's shape and fasteners placements are also unique and custom, created based on the patient's individual bone structure.
Medical models		3D printed medical models are widely being used to facilitate visualization and learning.
Bioprinting and tissue engineering		3-D bioprinting has been used by researchers at Cornell University to fabricate living heart valves that possess the same anatomical architecture as the original valve.
Intricate Scaffolds		The Wake Forest Institute for Regenerative Medicine uses AM to print scaffolds for body parts that can be covered with cells and then grow into noses, bones, ears, etc.
Cranium Replacement		The OsteoFab Patient Specific Cranial Device (OPSCD) is made from PEKK (a biocompatible bone-like material that will not interfere with x-ray scanning through) by AM. 3D printed implants may one day be widely used

Implant testing

State-of-the-art

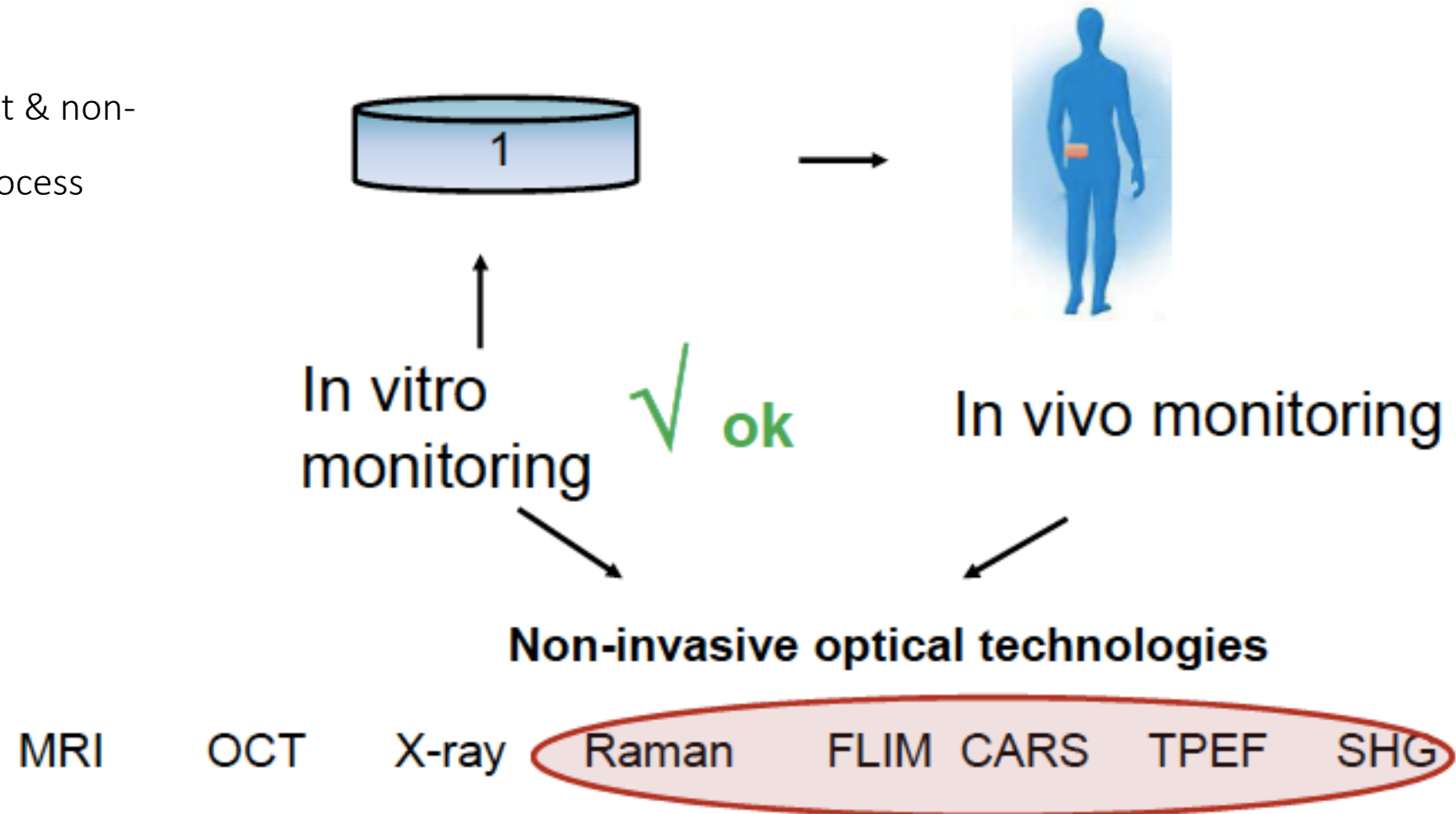
Two implants



Implant testing

The future

One single implant & non-invasive online process control



Implant testing

Non-invasive imaging

Intrinsic biomarkers

No application of dyes

No tissue preprocessing/staining

Advantages for biomedical imaging

Potential in vivo application

Real-time & in situ

Non-destructive

Less time consuming sample preparation

Clinical imaging

MRI

X-Ray

Tomography (CT, SPECT, PET)

Ultrasound

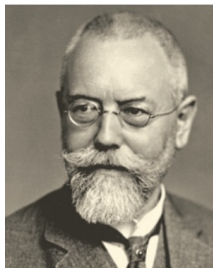
Elastography

Introduction – Microscopy History

Microscope in 1872



Fraunhofer



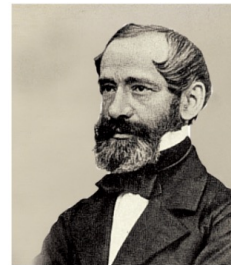
Köhler



Schott

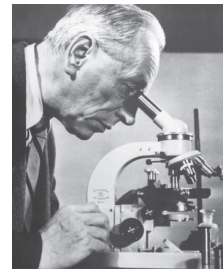


Abbe



Zeiss

$$d = \frac{\lambda}{2n \sin \alpha}$$



Zernike

Laser scanning microscope, today



Göppert-Mayer

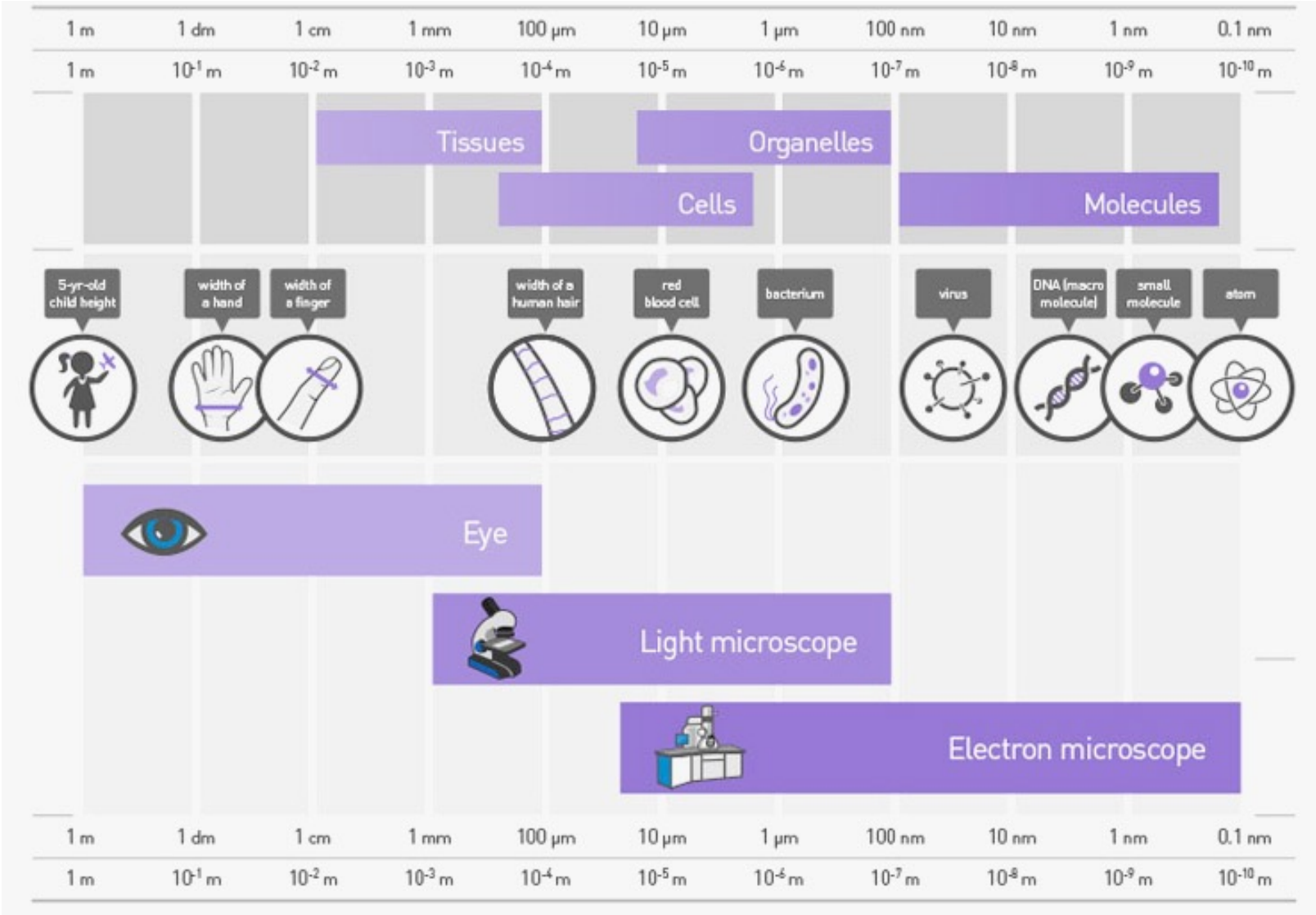


Stokes



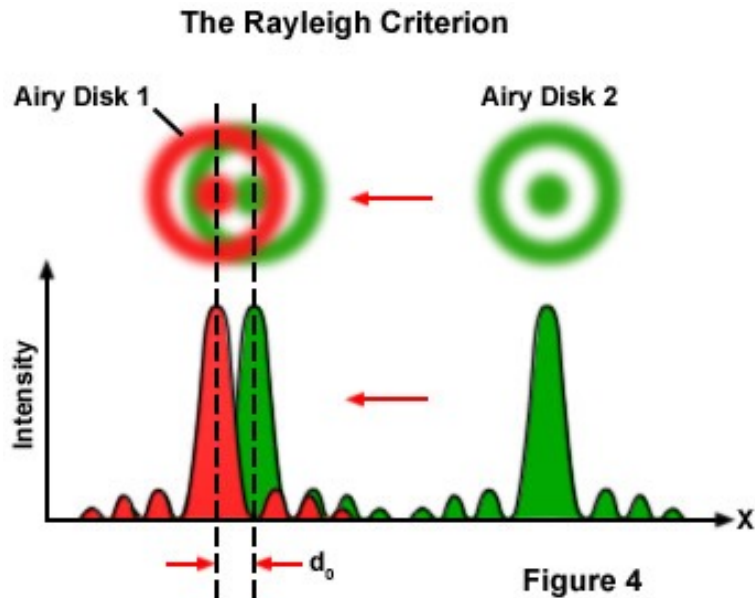
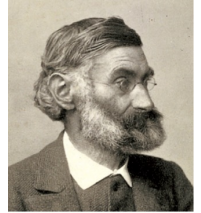
Förster

Introduction - Resolution



Introduction - Resolution

Shortest distance d_0 between two points that can still be distinguished as separate entities

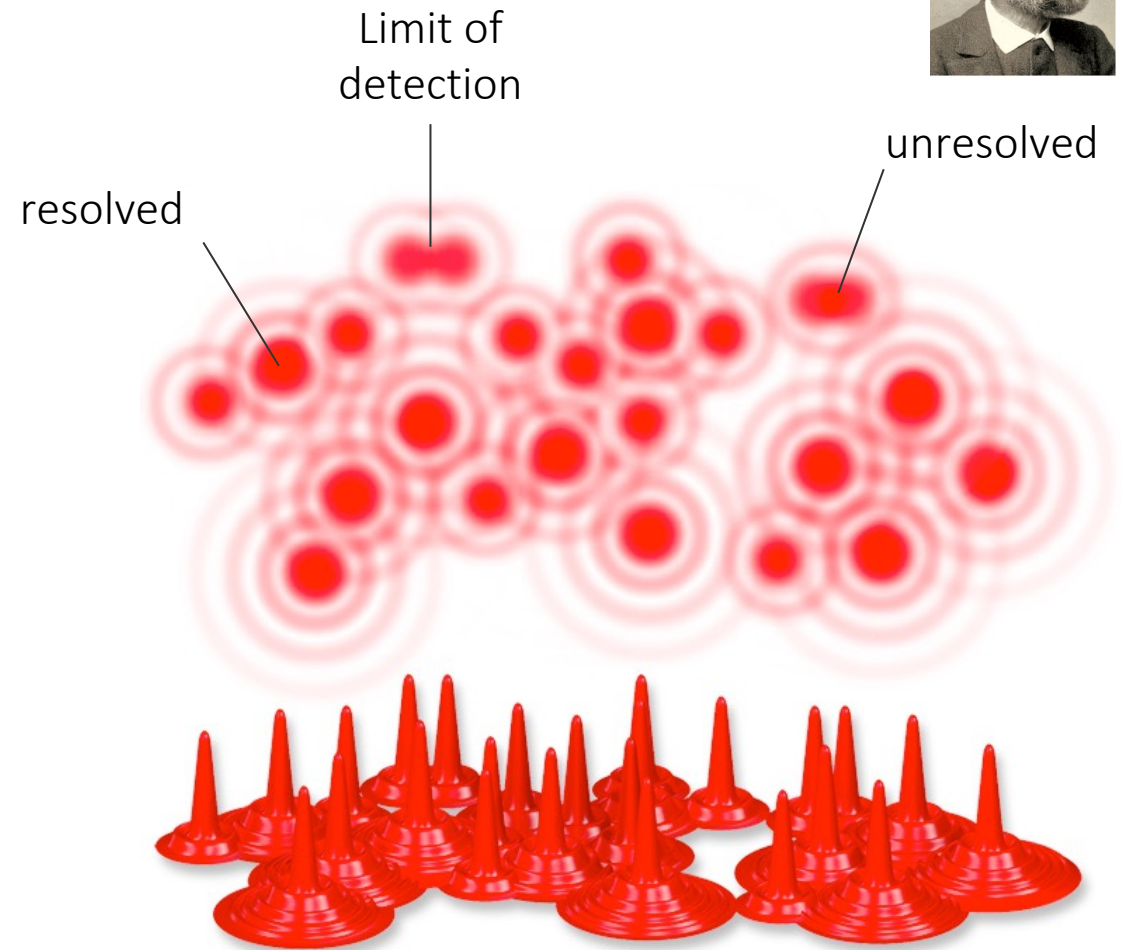


Abbe formula:

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

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

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



Introduction - Numerical Aperture

Numerical Aperture

N.A. = “opening” of an objective

the smaller the object the more light is diffracted

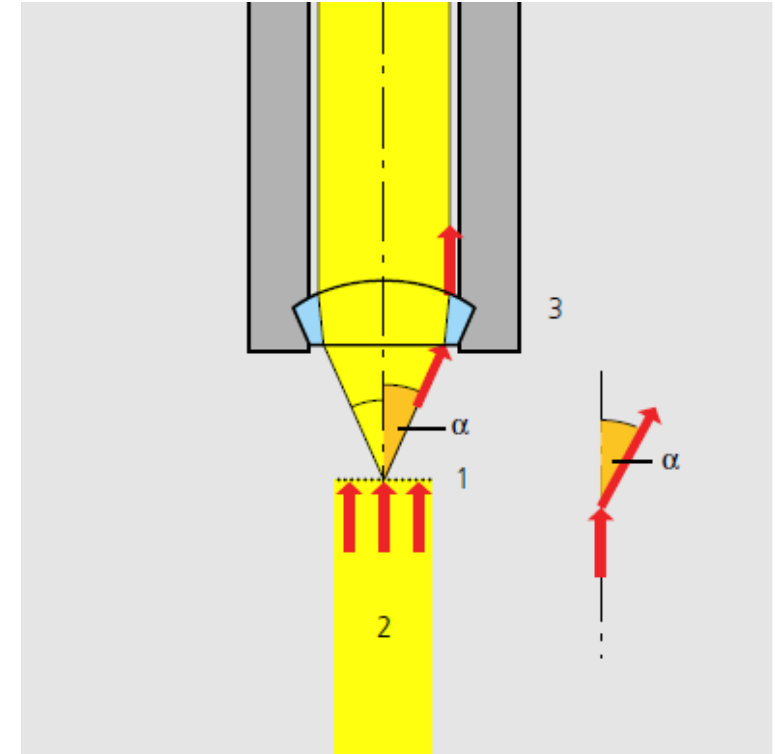
to collect sharp images from small objects, the objective needs to collect as much diffracted light as possible

Numerical Aperture = 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)



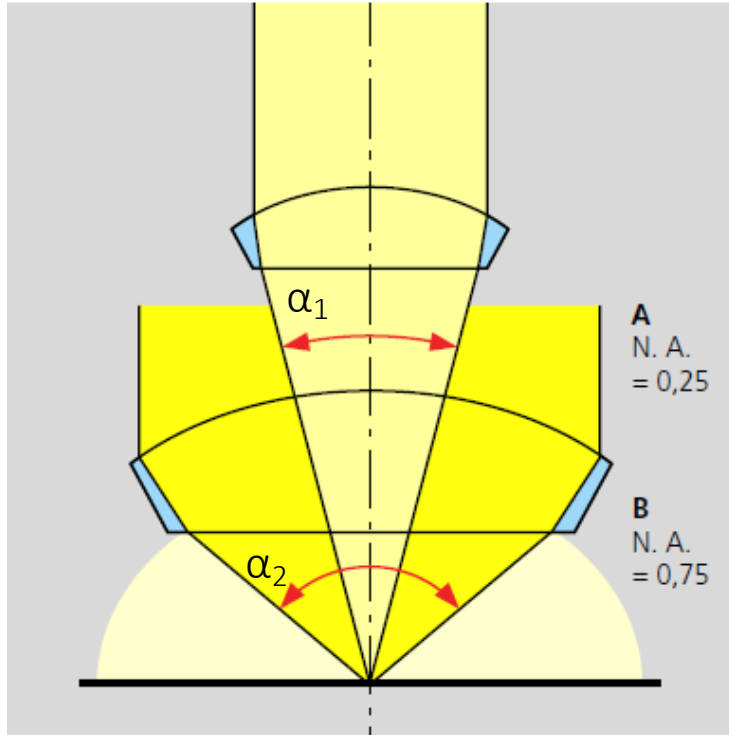
- 1 Object
- 2 Incident light
- 3 Objective

Introduction - Numerical Aperture

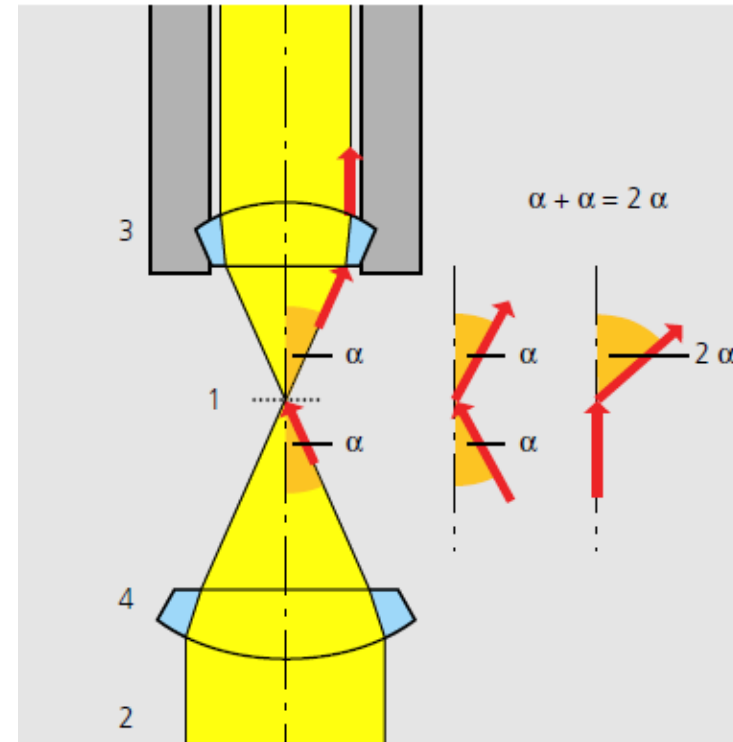
Increasing the resolution

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

Increase N.A. of the objective



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

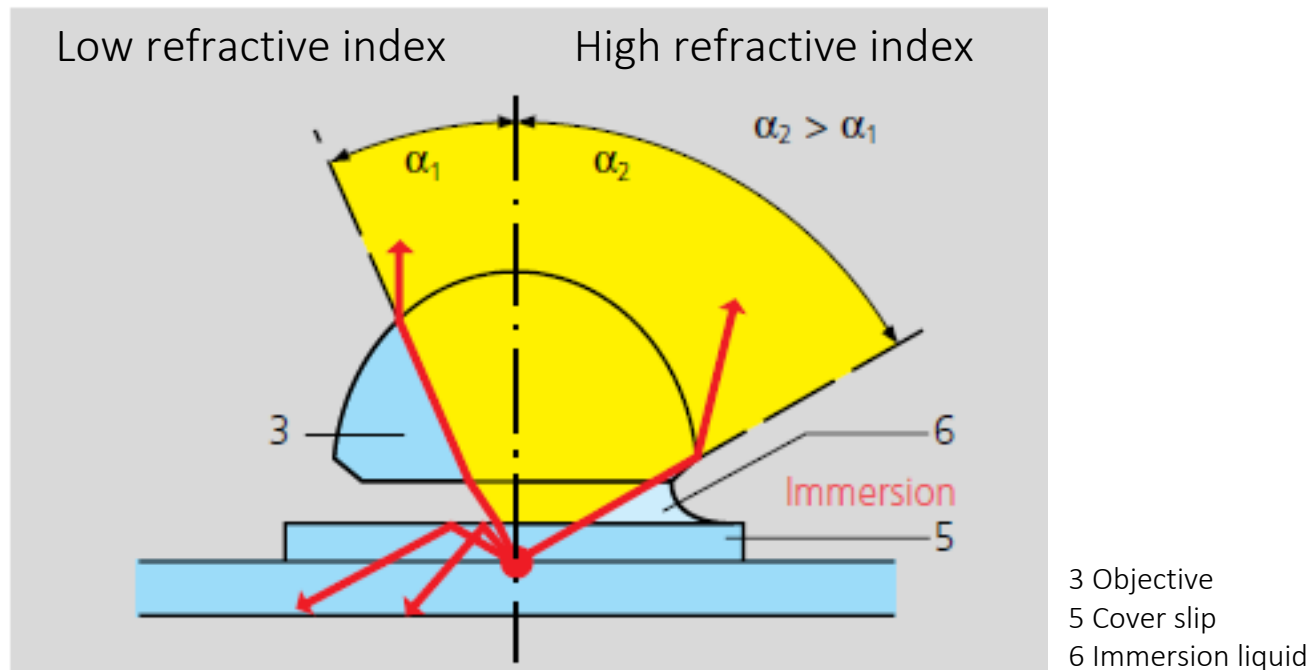


Introduction – Refractive index

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



Refractive indices n
of immersion liquids

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

Introduction – Image contrast

Contrast

Difference between the signal in one pixel and that in another

Information about the shape of the specimen

Difference in signal intensity between details of interest and “background”

Various physical and digital “filters”

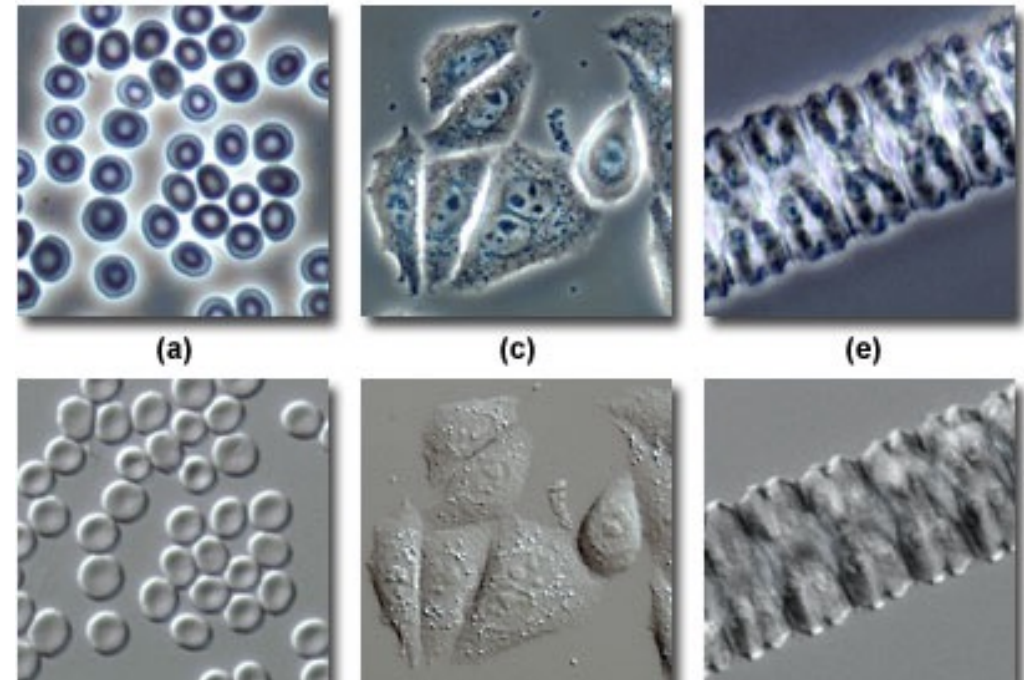
Contrast enhancing techniques

Köhler illumination

Phase contrast

Differential interference contrast (DIC)

Halos in Phase Contrast and DIC Microscopy



Introduction – Image contrast

Phase contrast

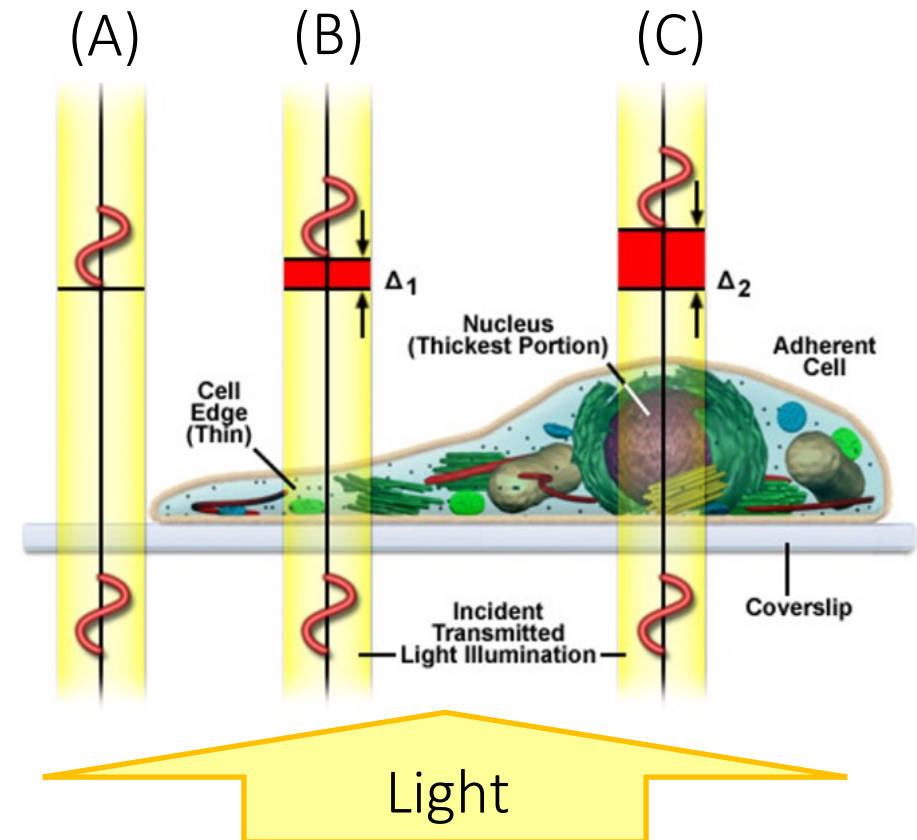
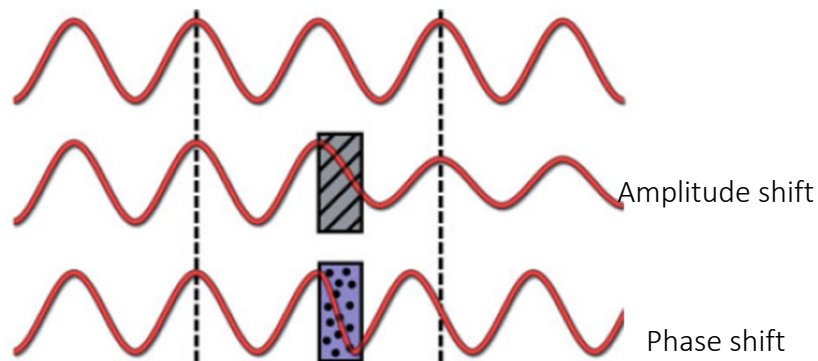
Small differences between the refractive indices of the aqueous solution (A) the cell periphery (B) and the nucleus (C)

Setup: condenser with annuli matched to a phase ring

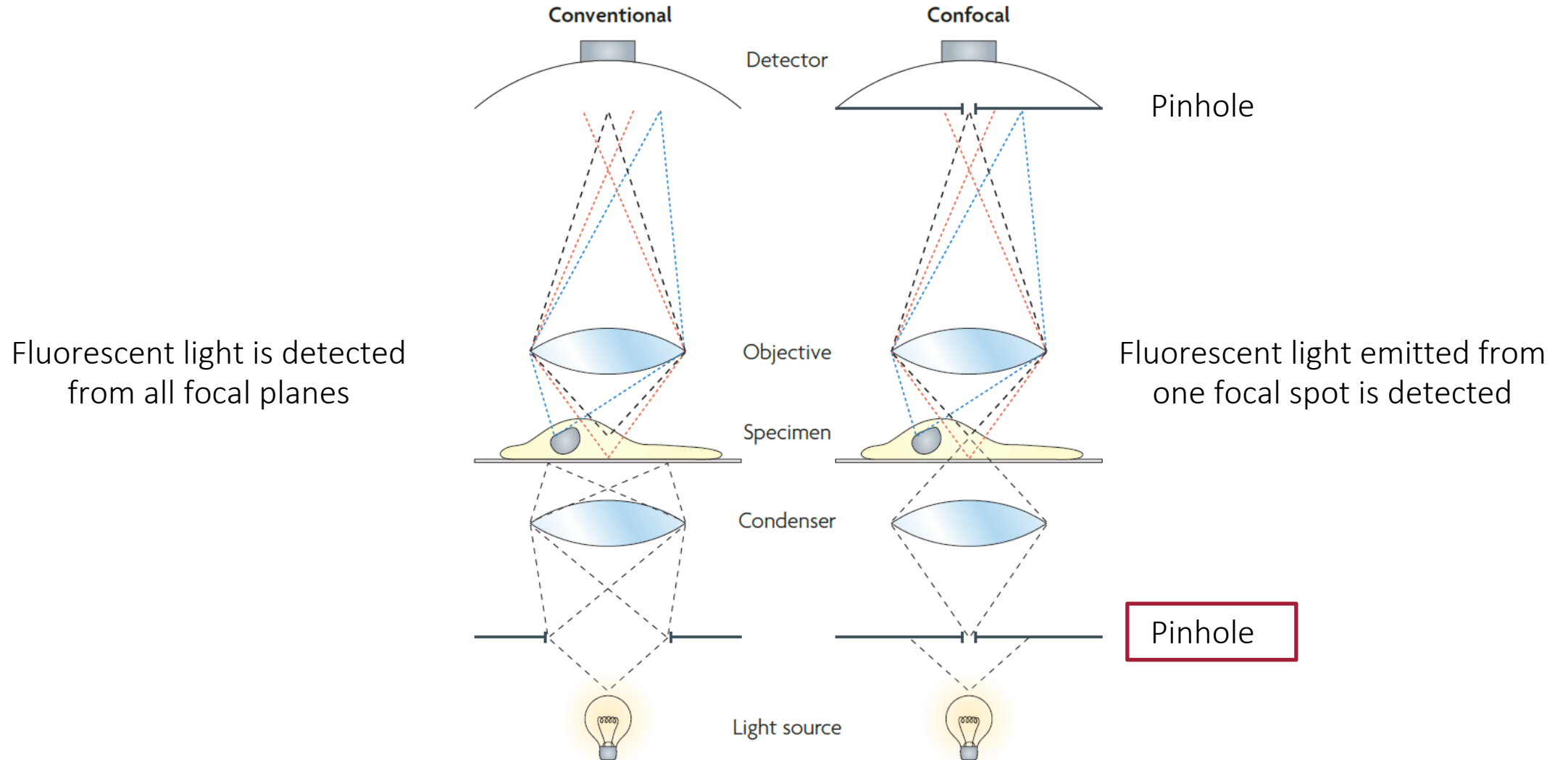
Shift of light rays are translated into intensity shifts

Ideal for thin specimens with low absorption

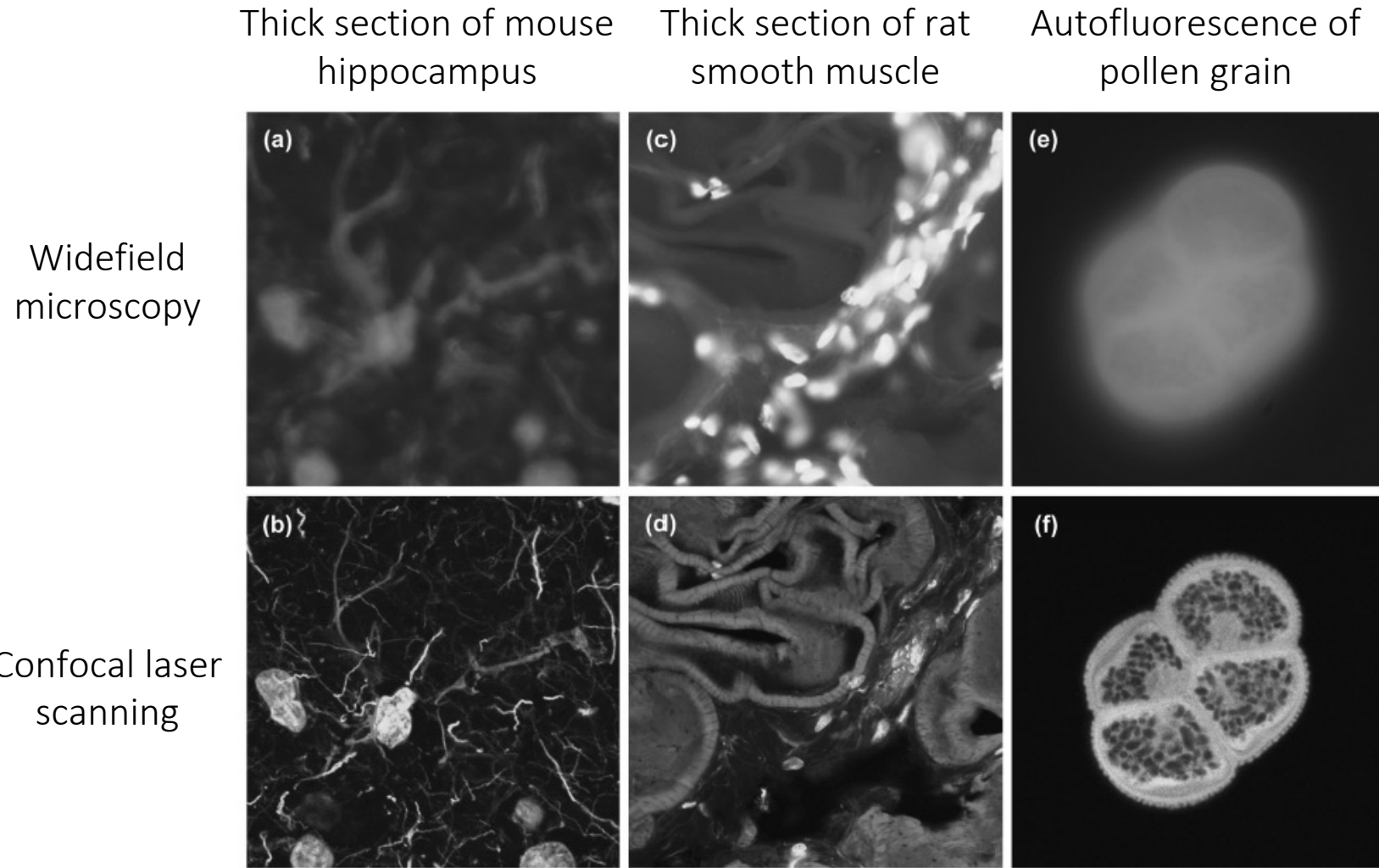
-> cells



Introduction - Confocality



Introduction - Confocality



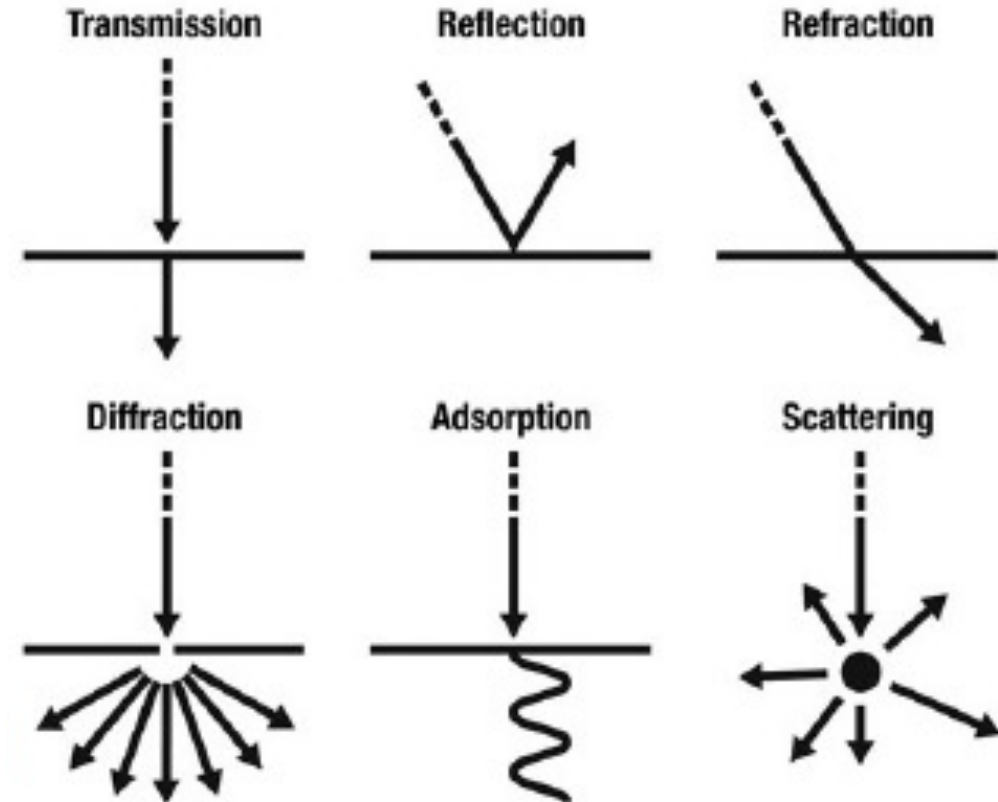
Introduction – Light-matter interaction

Light-matter interaction

Results in transmission, reflection, refraction, diffraction, absorption or scattering

Changes in photon energy are measured and represented in a spectrum

Microspectroscopy:
microscopy + spectroscopy



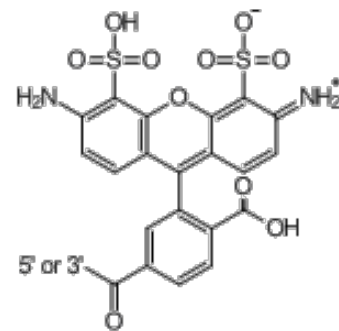
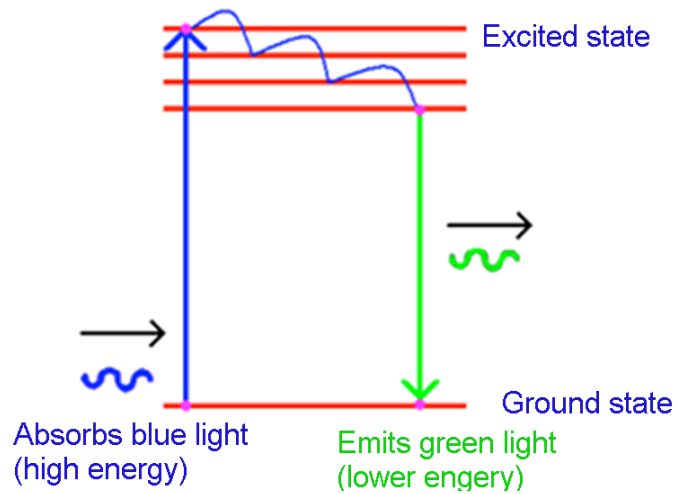
Introduction – Fluorescence Microscopy

Fluorescence microscopy

Luminescent process in which molecules emit light

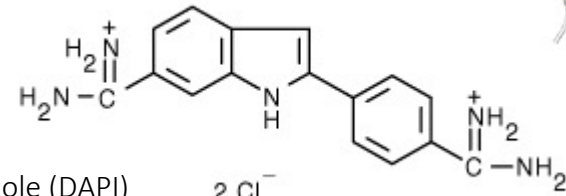
Fluorophores absorb light of one wavelength and emit (fluoresce) light of another wavelength

Autofluorescence or via fluorescent dyes



Alexa488

4',6-diamidino-2-phenylindole (DAPI)



Green fluorescent protein (GFP)



Introduction – Limitations

Wide-field

Thickness of specimens

No z resolution

Information about colocalization is insufficient

Confocal

Autofluorescence within the specimens

Limited penetration depth/long acquisition times

Quantification of fluorescence signal is limited

Application of fluorescence dyes

Destructive sample preparation

Use of multiple dyes is limited

No discrimination of dyes with similar excitation and emission ranges

Bleaching of fluorophores

Introduction – Non-invasive imaging

Non-invasive, label-free microscopic imaging

Fluorescence lifetime imaging (FLIM)

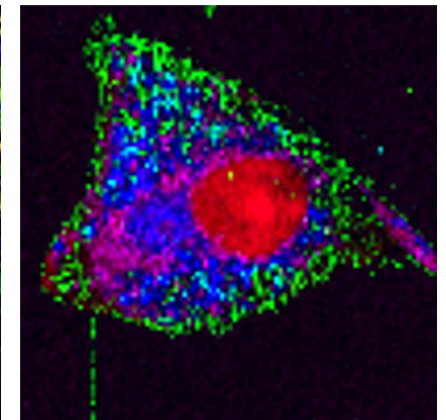
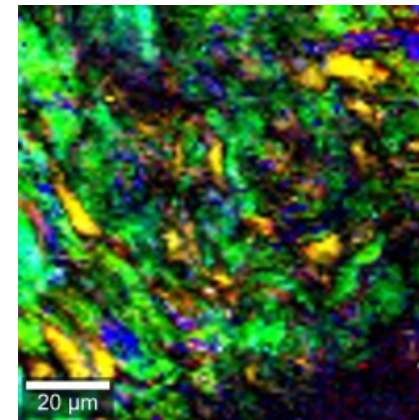
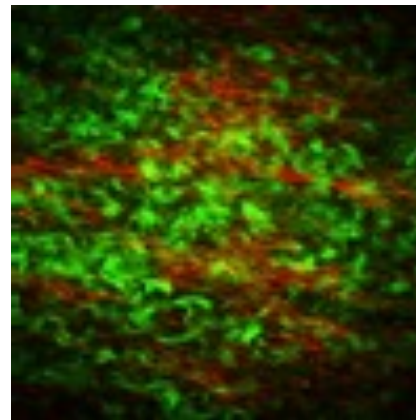
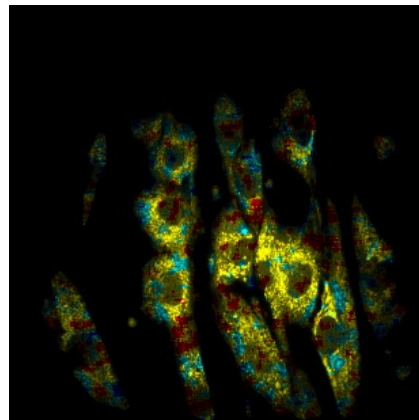
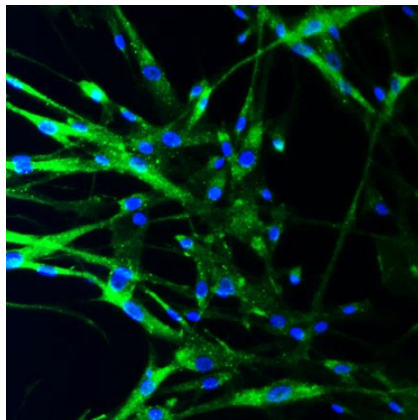
Multiphoton imaging

Raman imaging

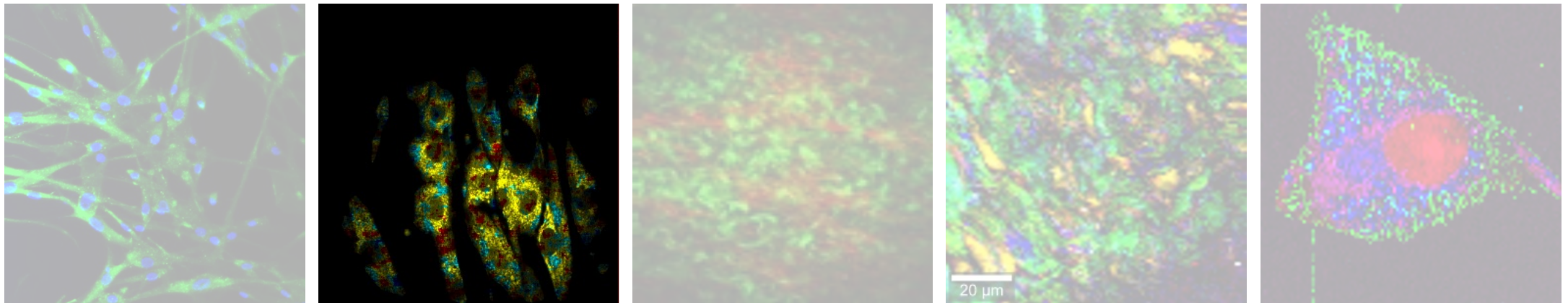


Endogenous fluorophores

Further techniques: Scanning Ion Conductance, Optical Coherence, Holotomography, PLIM



Fluorescence Lifetime Imaging (FLIM)

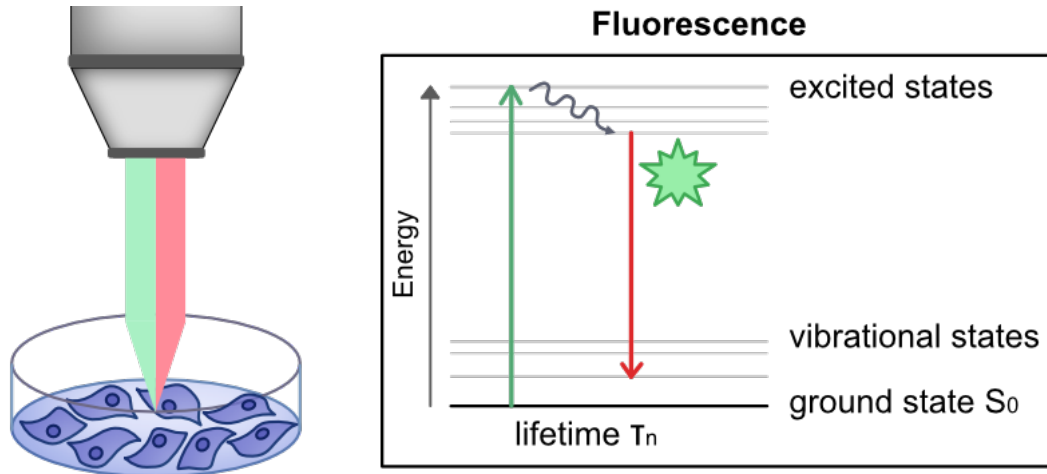


FLIM - Basics

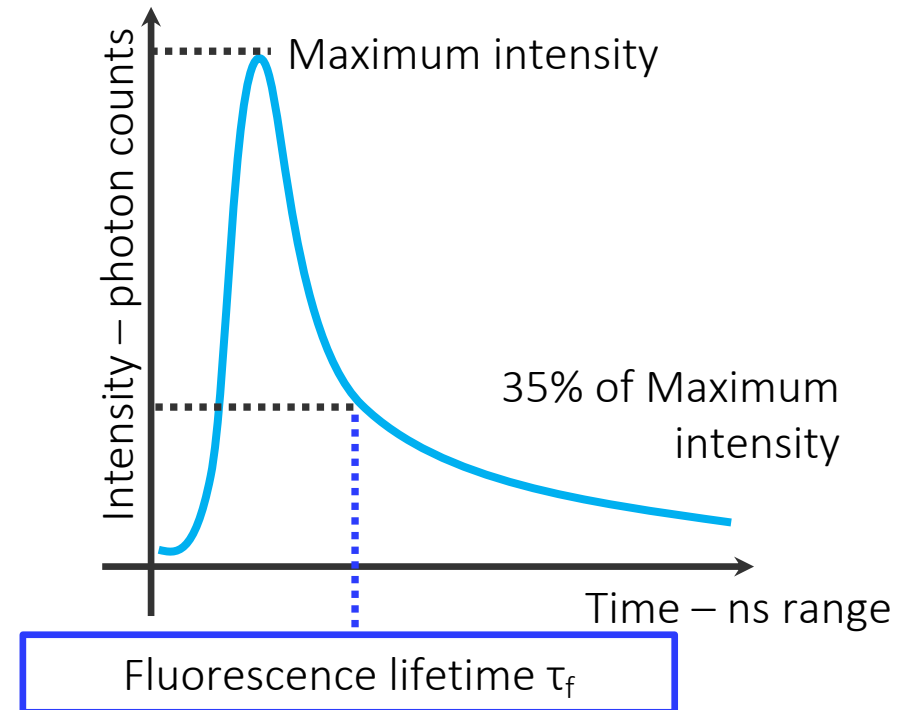
Every fluorescent molecule has an average time that it spends in the excited state followed after excitation

>> fluorescence life time τ

Simplified Jablonski energy diagram



Fluorescence decay curve



FLIM - Basics

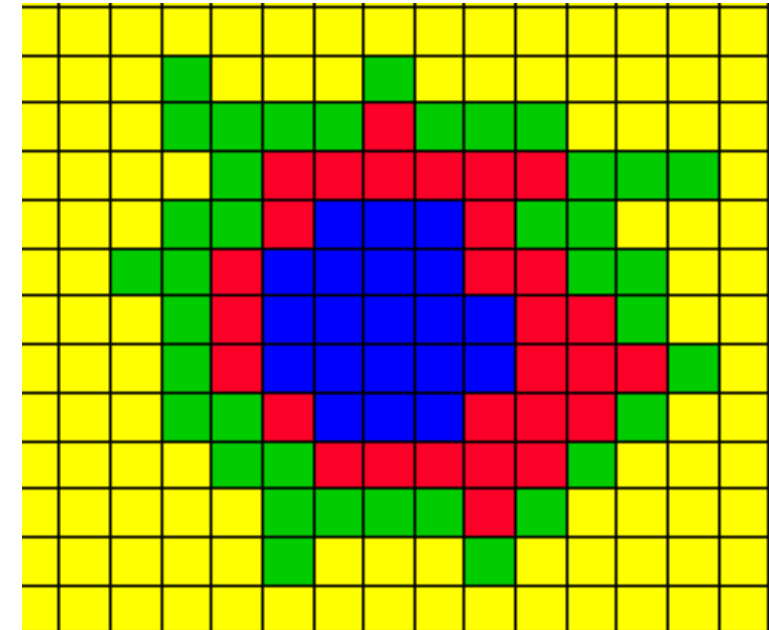
FLIM uses contrasts based upon fluorescence lifetimes for image construction

Lifetime τ_f is specific for a fluorophore

Based on τ_f multiple dyes can be distinguished in one channel at high detector sensitivity (multiexponential decay fitting)

FLIM can be used for:

- Ion concentration imaging
- Chemical microenvironment (pH, protein-binding...)
- Quantitative fluorescence measurements



Fluorescence lifetime image

Wavelength -color
lock-up table



τ_1 τ_2 τ_3 τ_4

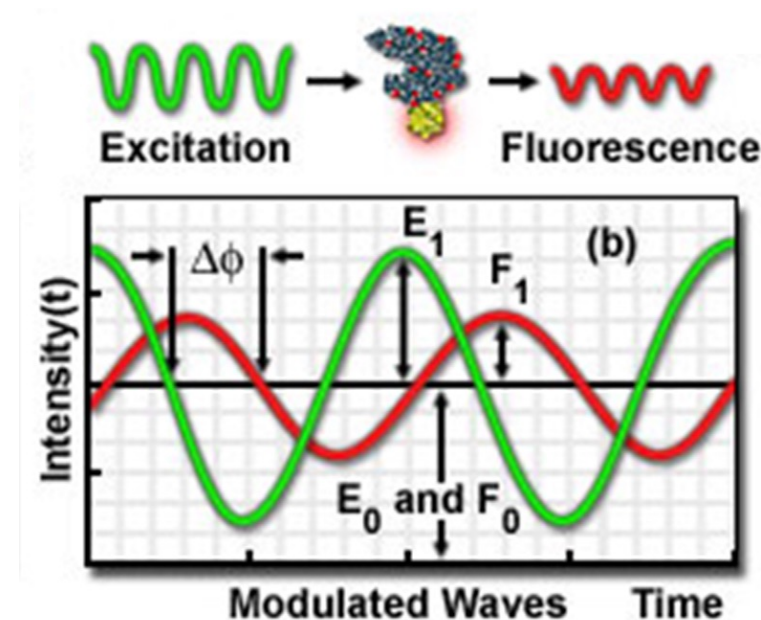
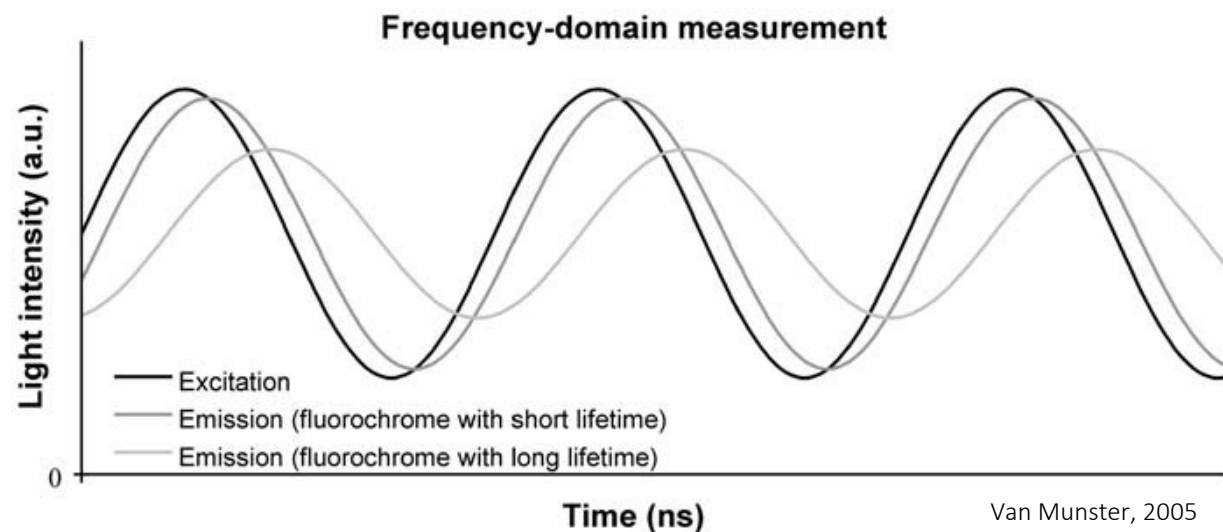
FLIM – Detection modes

Frequency-domain FLIM

>> Measurement of the phase shift $\Delta\phi$ of fluorescent light with respect to the phase of a modulated excitation source

Commonly used with wide-field microscopy

Emission is delayed relative to the excitation wave



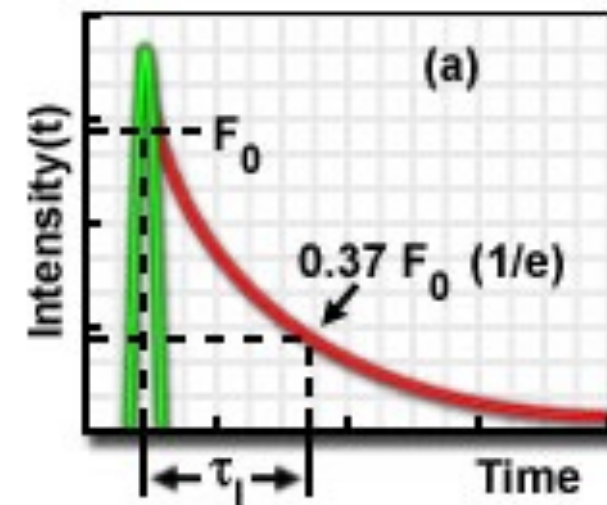
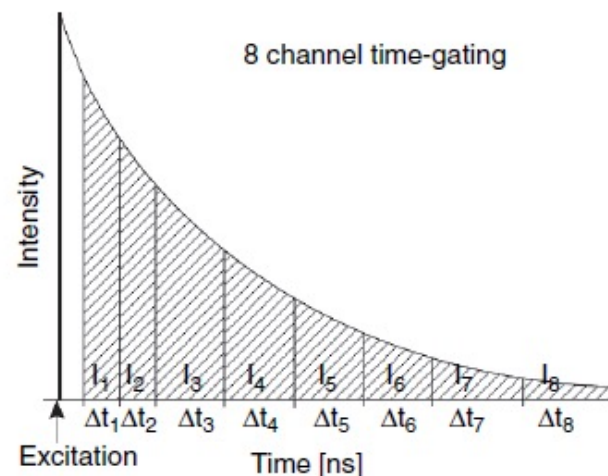
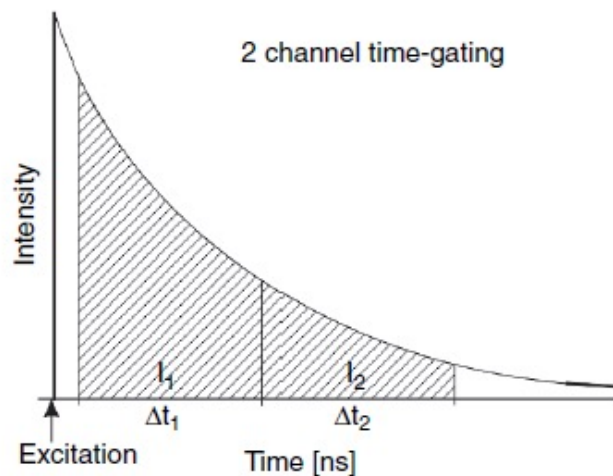
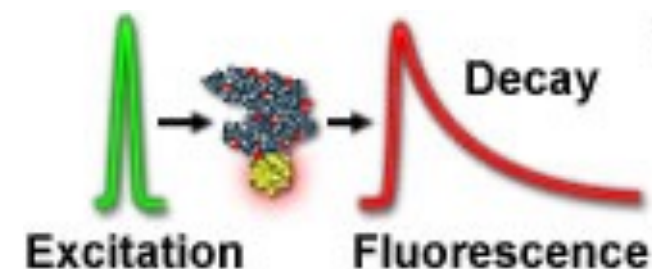
FLIM – Detection modes

Time-domain FLIM

>> Measurement of the time-dependency of fluorescence intensity with respect to a short excitation pulse

Use of a pulsed light source

Time-correlated single photon counting (TCSPC) or time gating



Pawley: Biological confocal microscopy

FLIM – endogenous & exogenous markers

Fluorophore	Solvent	Excitation max [nm]	Emission max [nm]	Lifetime [ns]
Endogenous				
Tryptophan	Water	295	353	3.1
Tyrosine	Water	275	304	3.6
Phenylalanine	Water	260	282	6.8
NADH	Water	350	460	0.4
FAD	Water	450	525	4.7
FMN	Water	450	525	2.3
Exogenous				
ATTO 565	Water	561	585	3.4
Rhodamine B	Water	562	583	1.68
Cy3	PBS	548	562	0.3
Cy5	PBS	646	664	1
DAPI	TRIS/EDTA	341	496	0.16
FITC	PB pH 7.8	494	518	4.1
Fluorescein	PB pH 7.5	495	517	4
GFP	Buffer pH 8	498	516	3.2
Hoechst	TRIS/EDTA	336	471	0.35
Lucifer yellow	Water	428	535	5.7

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

FLIM - NADH

FLIM of NADH – ATP production

Reduced Nicotinamide adenine dinucleotide (phosphate)

Important cofactor in cell metabolism

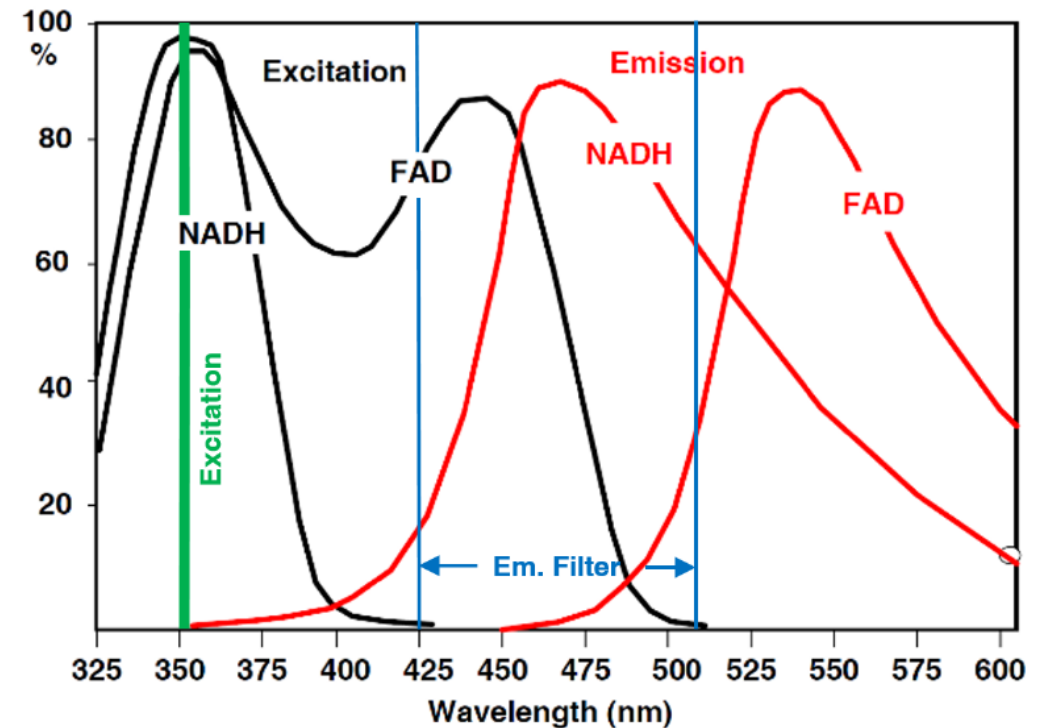
Free NAD(P)H: short lifetime τ_1

Protein-bound NAD(P)H: long lifetime τ_2

Typical values: $\tau_1 \sim 700$ ps and $\tau_2 \sim 2600$ ps

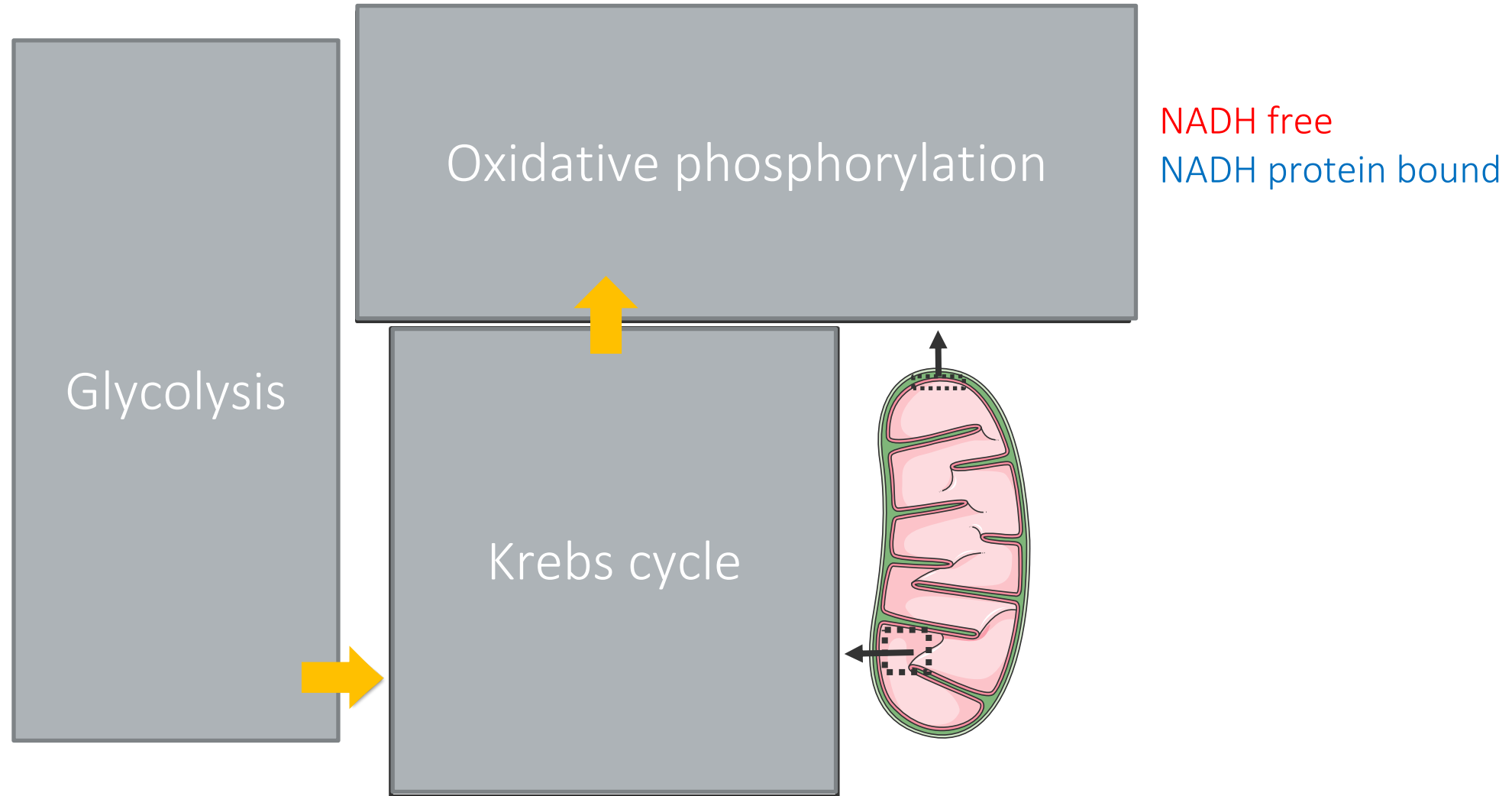
Hypothesis:

lifetime and contribution changes with disease



FLIM - NADH

FLIM of NADH – ATP production



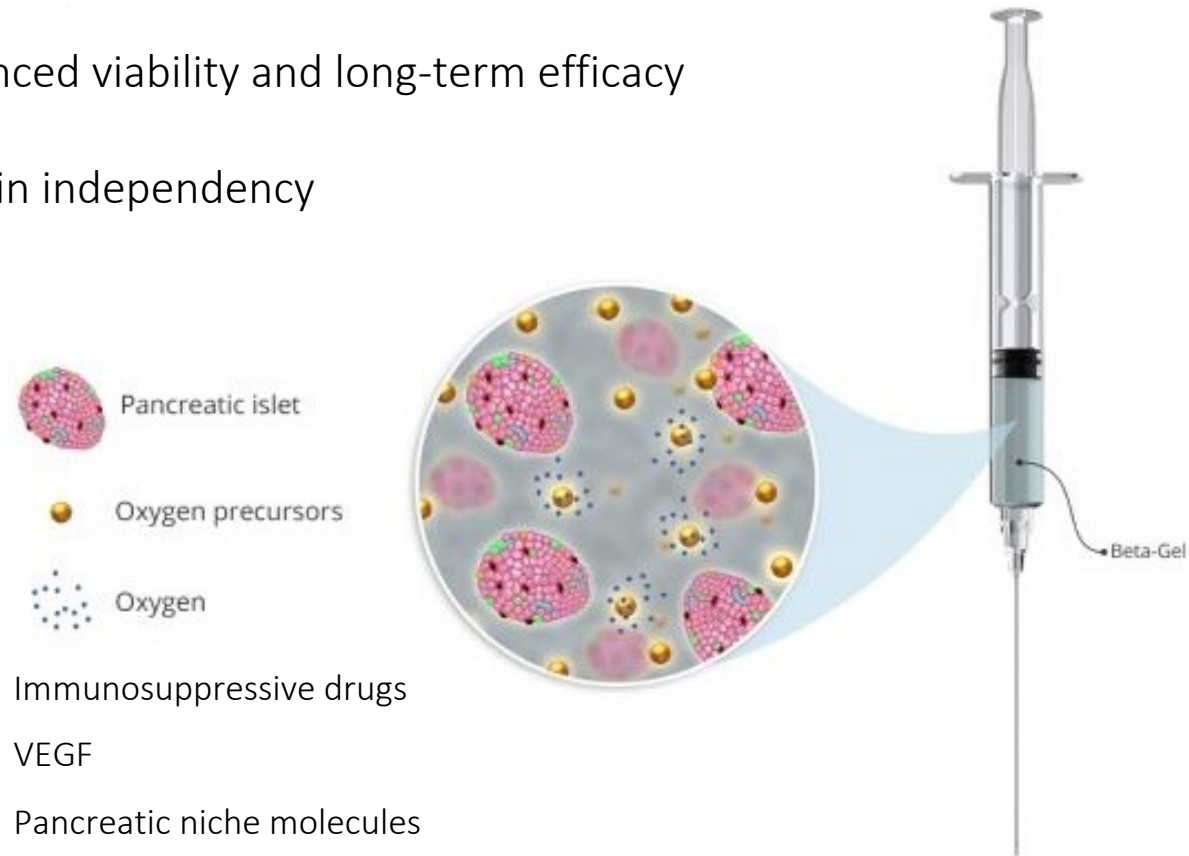
FLIM

FLIM parameters enable the metabolic evaluation of the inquired specimen

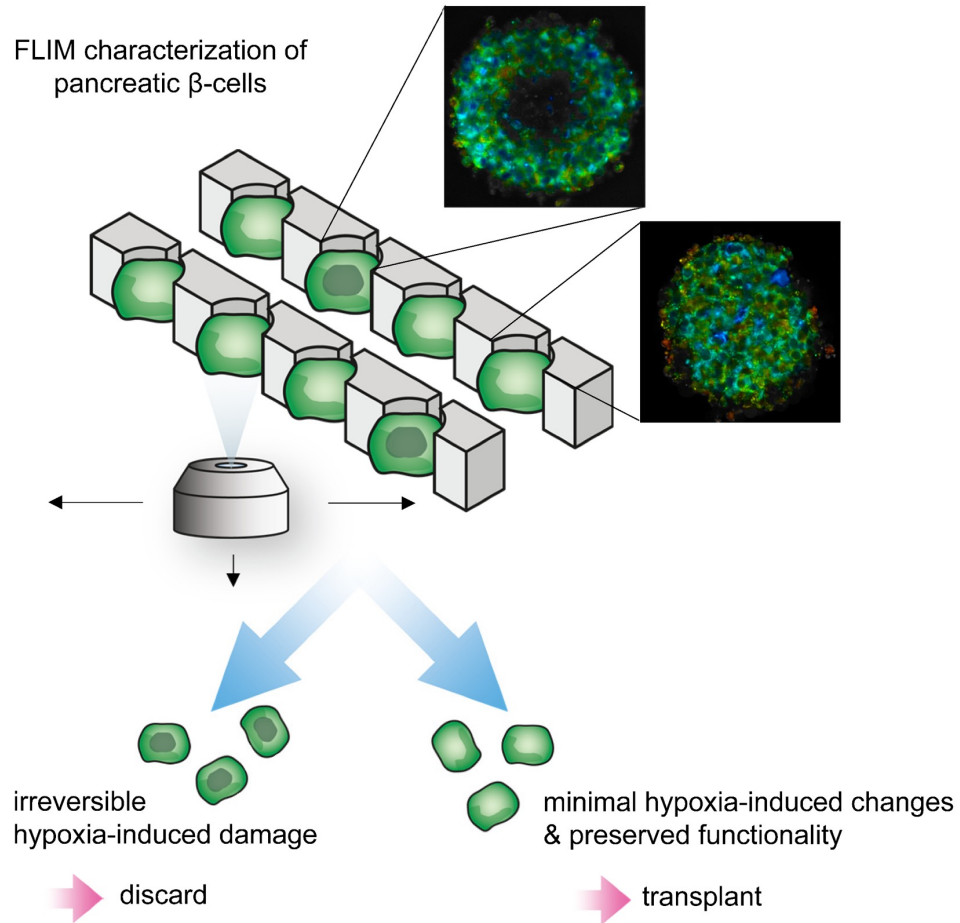
DRIVE

Diabetes-Reversing Implants for enhanced viability and long-term efficacy

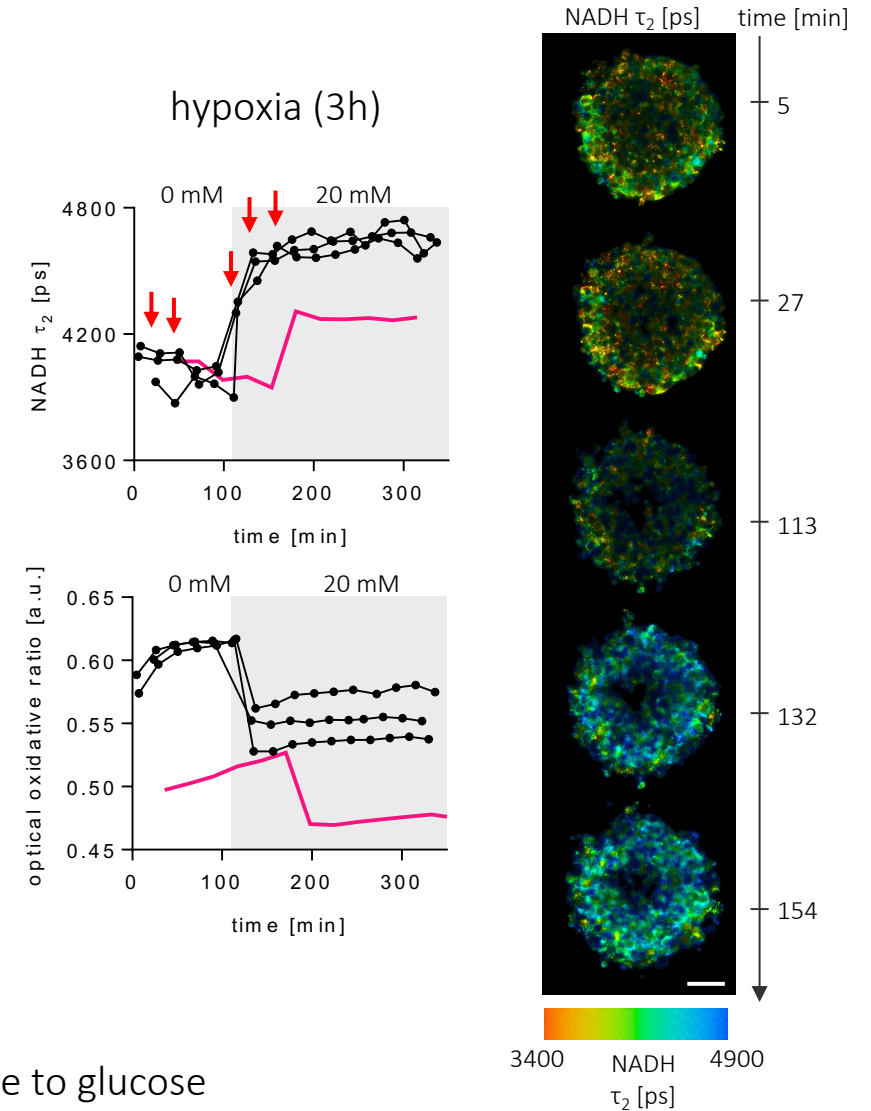
>> Islet transplantation < 5 years insulin independency



FLIM – Hypoxia in pseudoislets



>> Hypoxia “overload” as metabolic response to glucose



FLIM - Summary

Intensity-independent information from fluorescent molecules

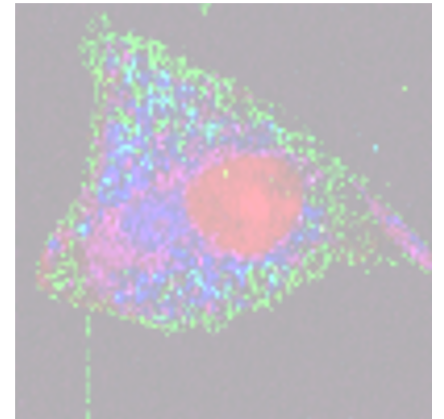
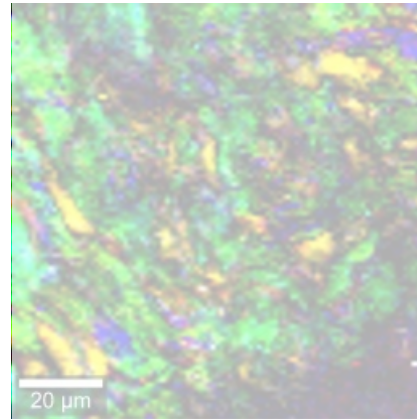
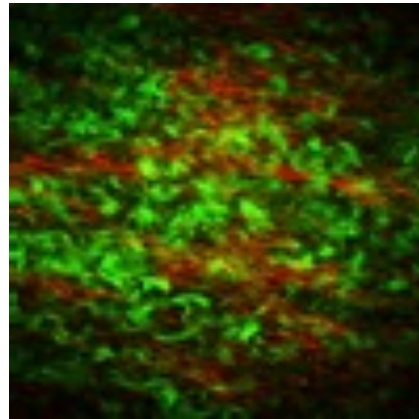
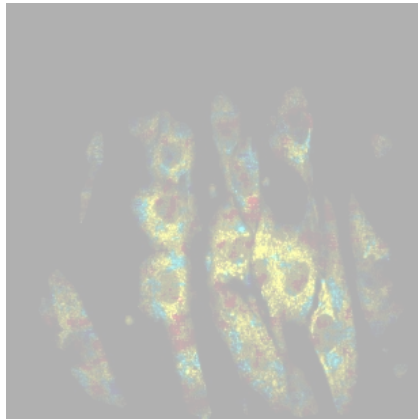
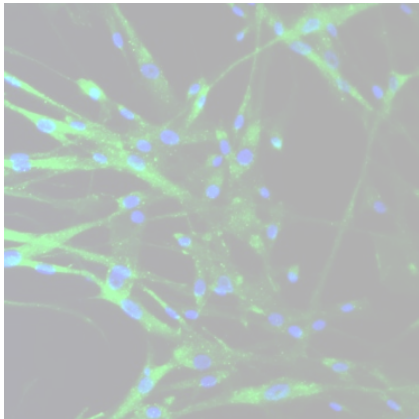
Imaging of protein-bound and free metabolites

>> Label-free metabolic profiling of cells

Sensitive to the fluorophores microenvironment

>> new ways for life scientists to detect, visualize, and investigate structure and function in biological systems

Multiphoton Microscopy



Multiphoton microscopy - History



Maria Göppert-Mayer

Maria Göppert-Mayer predicted multiphoton absorption in 1931

Practical proof was not possible by that time

In 1961 first laser systems enabled to proof her theory

Über Elementarakte mit zwei Quantensprüngen

Von Maria Göppert-Mayer

(Göttinger Dissertation)

(Mit 5 Figuren)

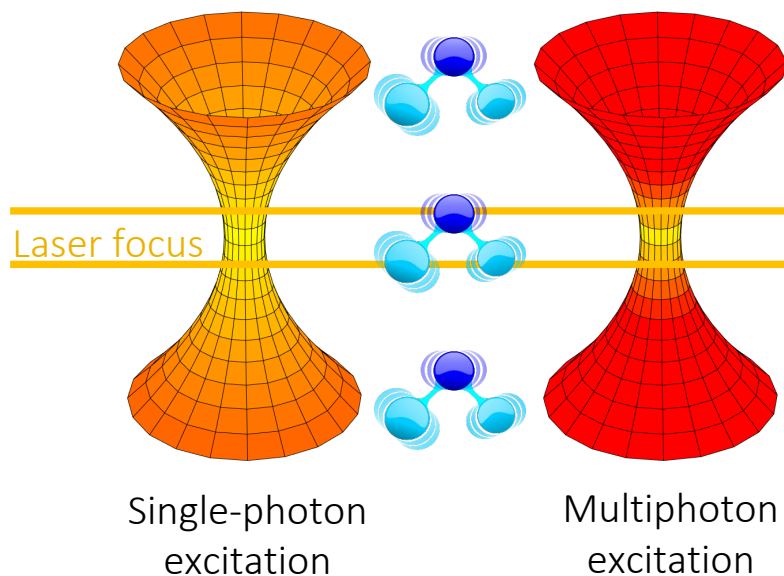
Einleitung

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 Ramaneffekt 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 Doppelemission 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 – Resolution & Background



Nonlinear optical process

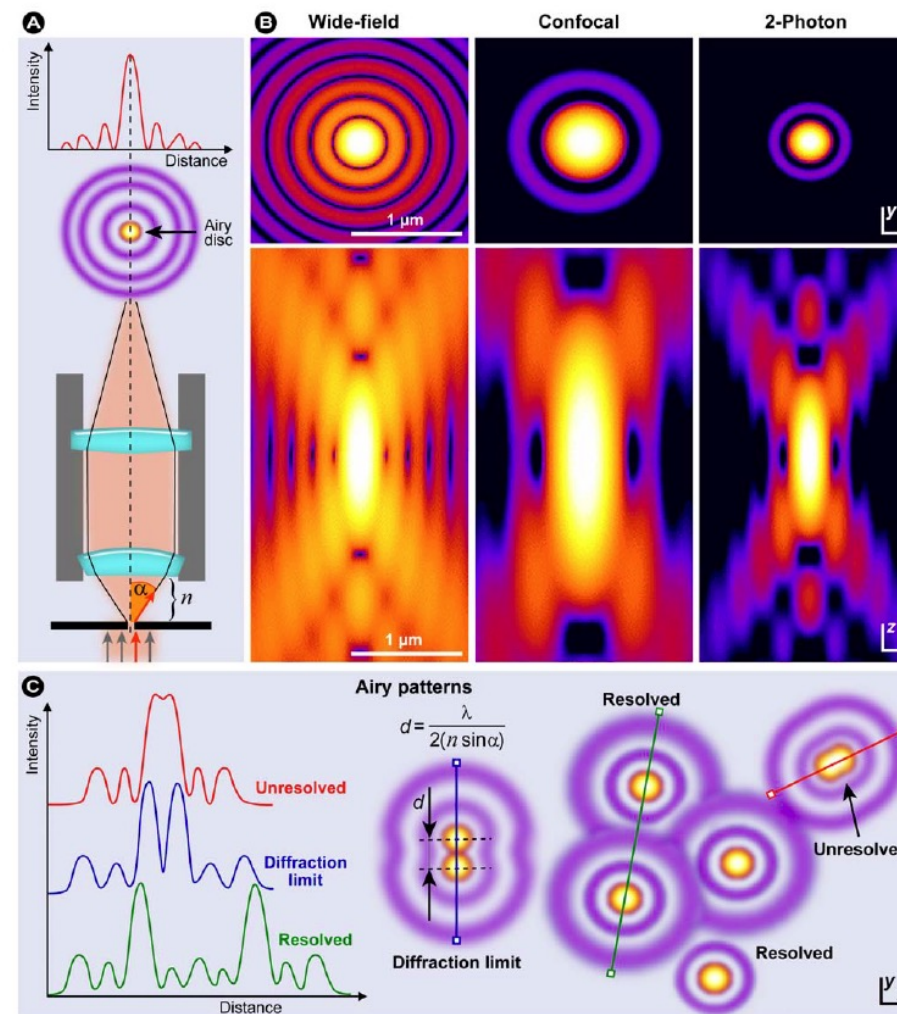
Simultaneous absorption of photons of the same wavelength

Excited region is limited to a subfemtoliter volume

Fluorescence is only generated in the focal plane

No pinhole needed

No absorption in out-of-plane areas



Multiphoton microscopy - SHG

Second harmonic generation (SHG)

Two photons interact with non-linear material

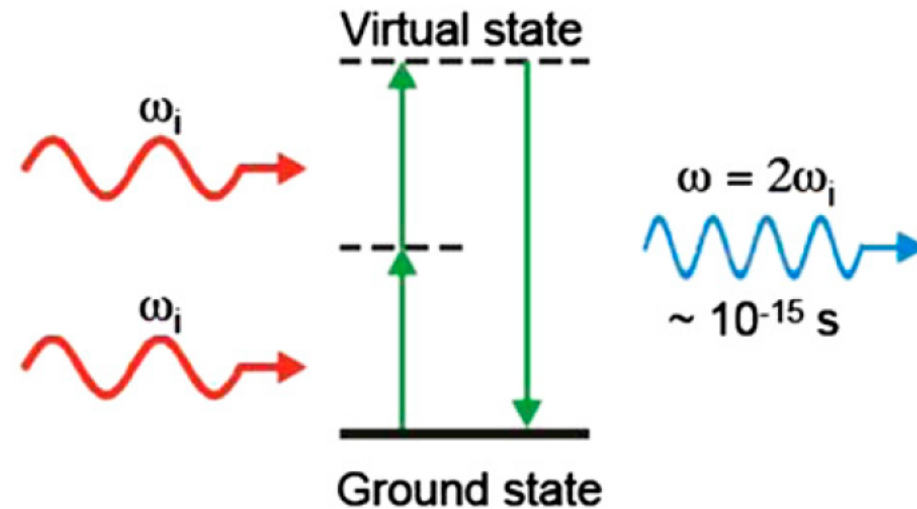
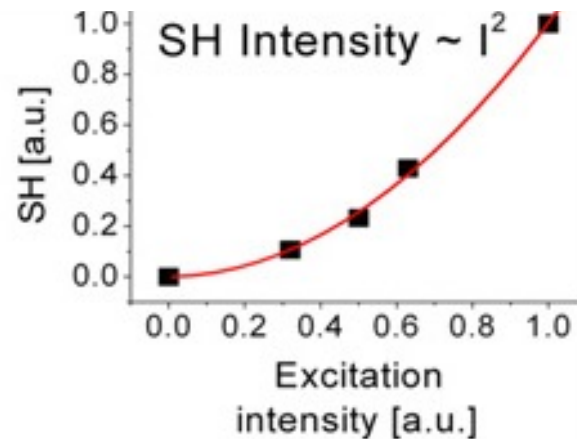
>> One photon with exact the double frequency is emitted

Virtual transition >> no bleaching

SHG is energetically neutral >> no loss of energy

SHG intensity is proportional to the square of the incident light intensity

SHG-process happens within femtoseconds



Multiphoton microscopy – SHG

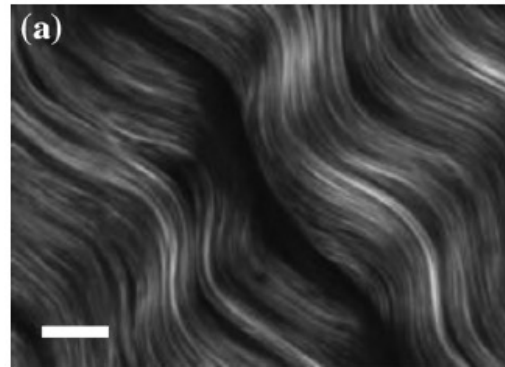
SHG imaging

Imaging of structural macromolecular organization in tissues

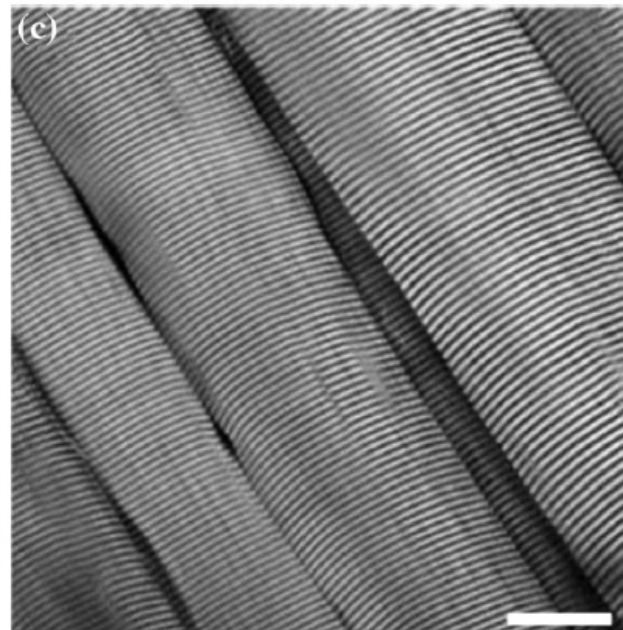
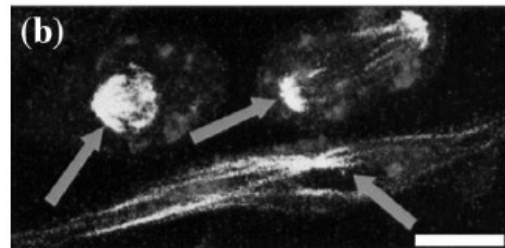
Endogenous SHG is known for certain non-centric symmetric molecules that form microfibers and fibrils (collagens, microtubuli and myosin)

A cylindric structural organization is common in all SHG-active molecules

collagen in a rat tail



microtubuli



Skeletal muscle fibers

Multiphoton microscopy - TPEF

Two-photon excited fluorescence (TPEF)

Two photons with near-infrared frequency (700-1200 nm) are absorbed simultaneously and excite together the emission of blue, red or green fluorescence

Excitation frequency is lower than in SHG imaging

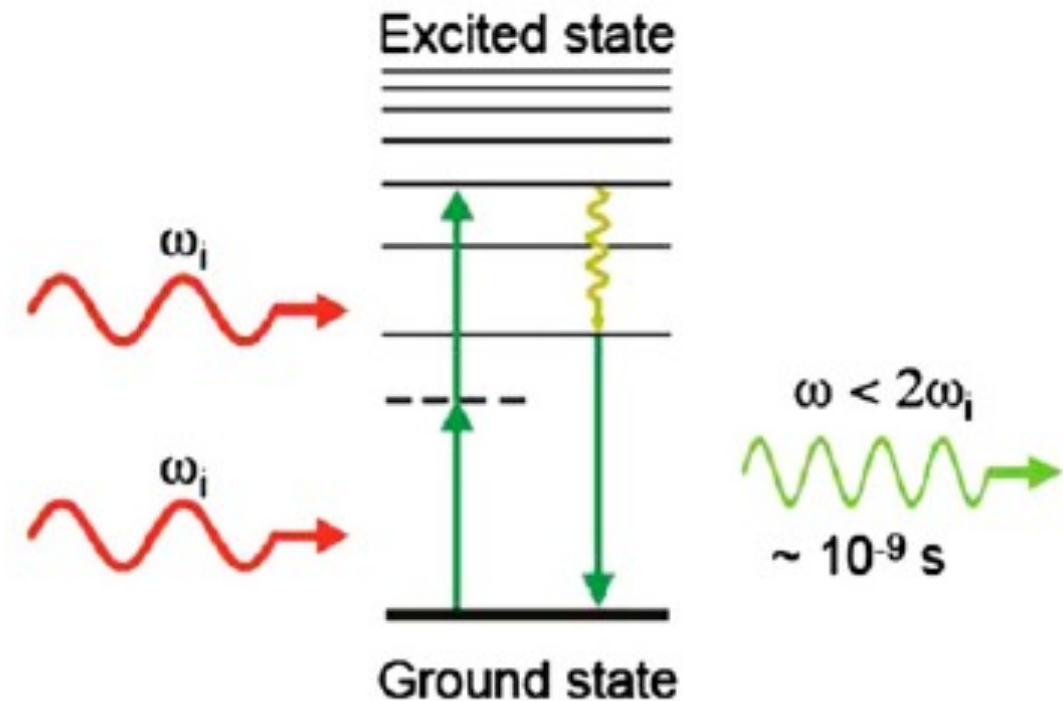
Molecules experience an excited stage

TPEF happens within nanoseconds

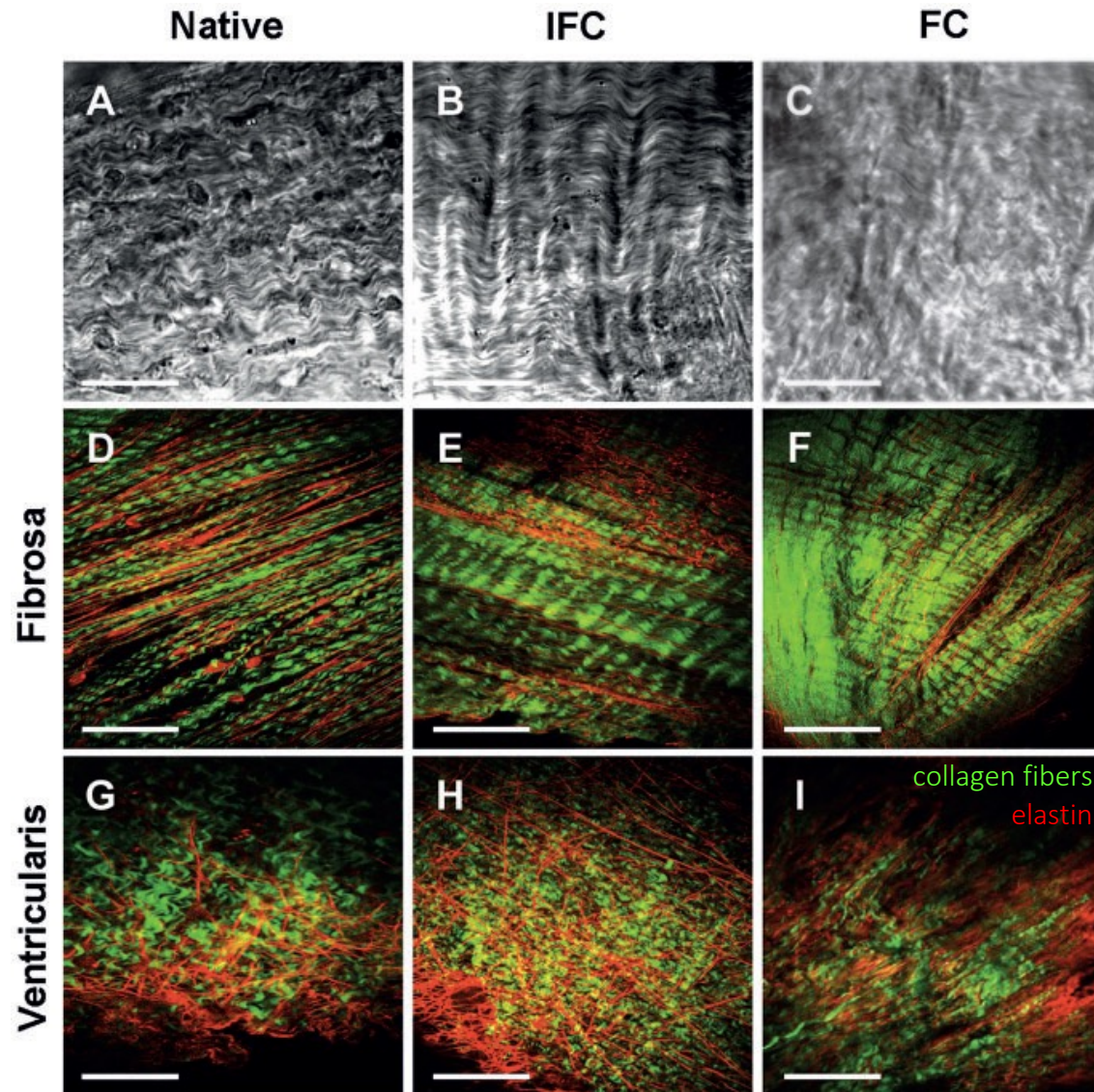
TPEF is connected with a loss of energy

Accessible to all 'autofluorescent' molecules

e.g. elastin, NAD(P)H



Multiphoton microscopy – Transplanted heart valves



green:
 λ_{ex} 800 nm
SHG signal of collagen fibers

red:
 λ_{ex} 740 nm
TPEF of elastic fibers & cells

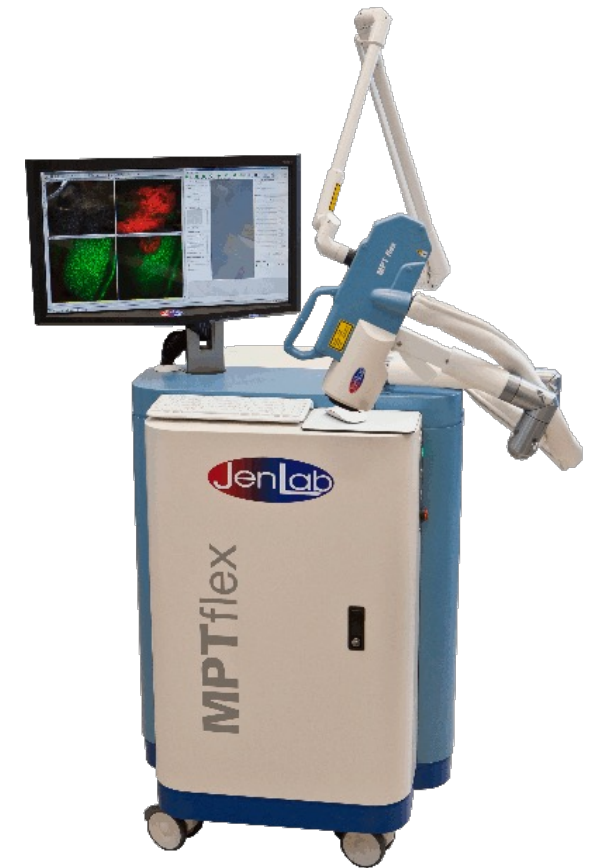
collagen fibers
elastin

Multiphoton Imaging - Summary

Label-free imaging of intrinsically fluorescent & SHG-active molecules

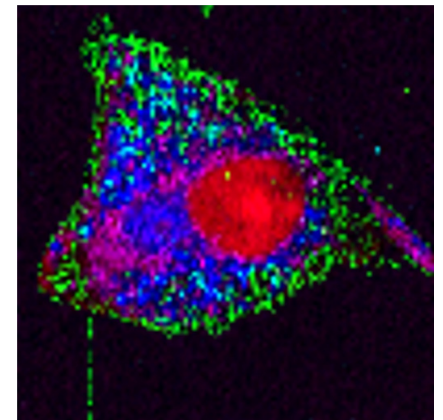
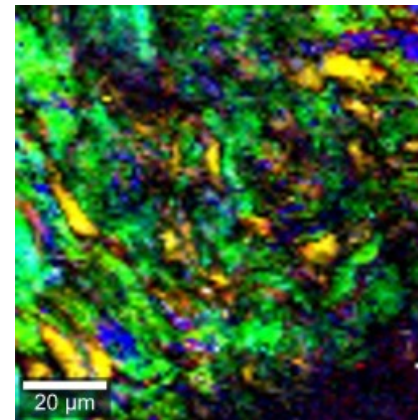
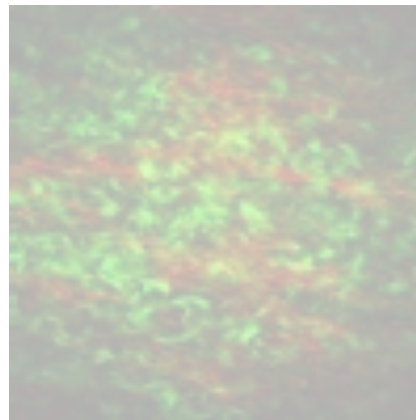
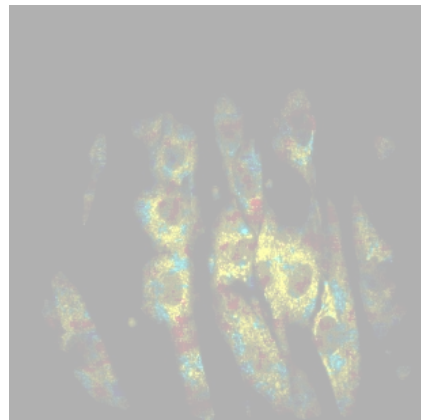
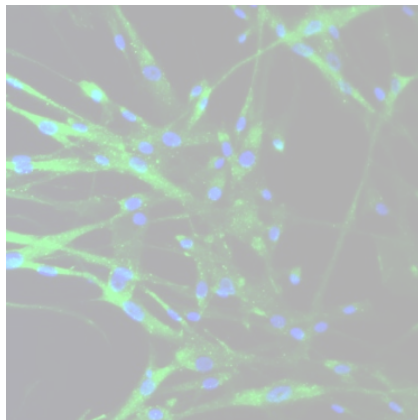
Advantages in measurements of thick tissue specimens

- >> deeper penetration (in vivo deep tissue imaging)
- >> less fluorescence background
- >> less phototoxicity, photobleaching
- >> less scattering



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

Raman Microspectroscopy



Raman microspectroscopy – Basics

Light scattering

One photon is absorbed another one is emitted at the same time

Most emitted photons have the same energy as the incident light (**elastic scattering**)

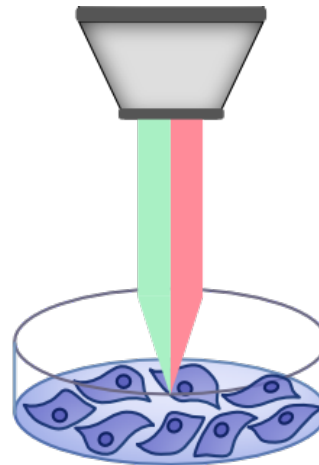
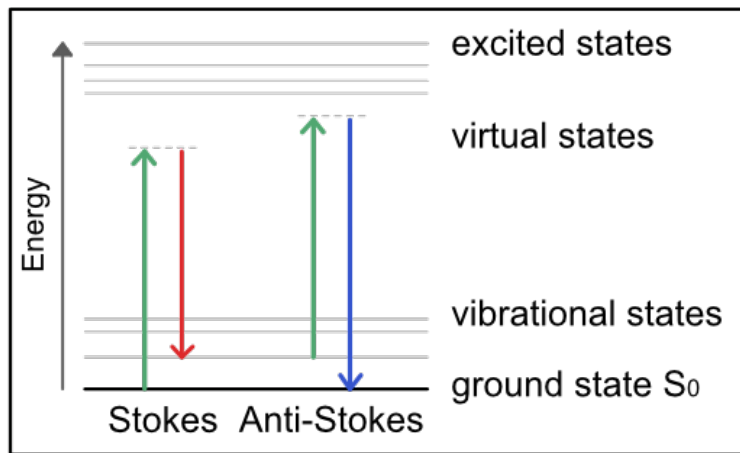
few photons are shifted in their frequency (inelastic scattering)

Stokes scattering refers to photons shifted towards red (lower energy)

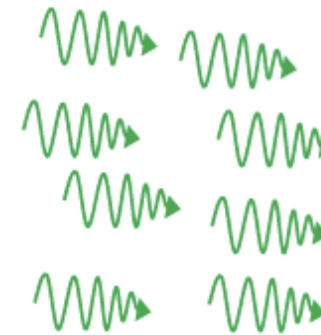
Photons with an increased energy correspond to **Anti-Stokes scattering**



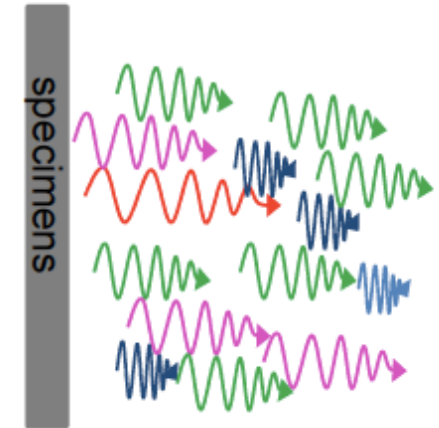
Chandra. Venkata Raman
(* 1887, † 1970)



Incident photons

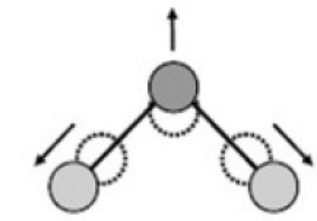


Scattered photons

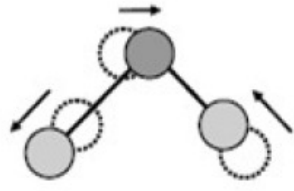


Raman microspectroscopy

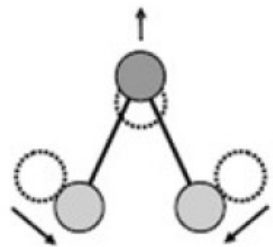
>> spectral analysis of the wavelength and intensity of inelastic scattered light from molecules



Symmetric vibrations

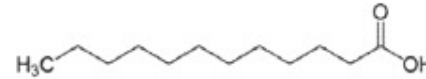


Assymmetric vibrations

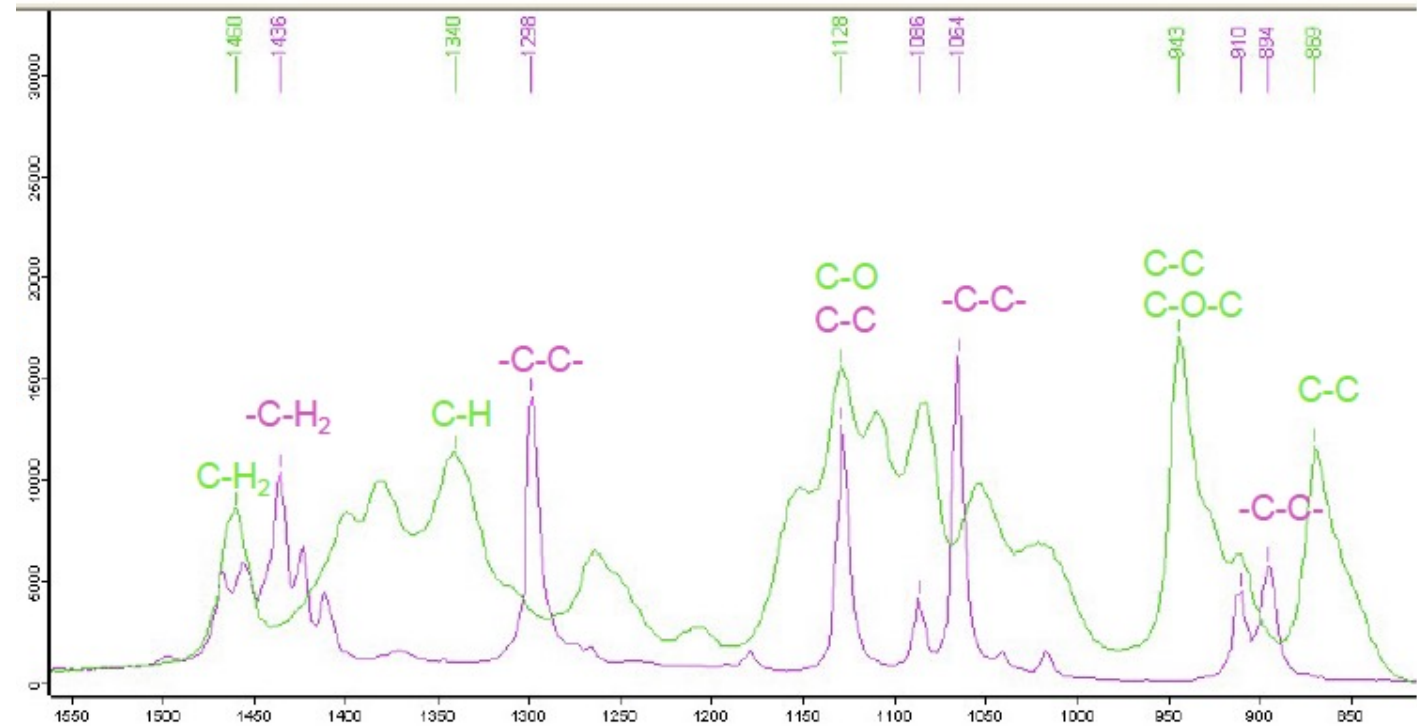
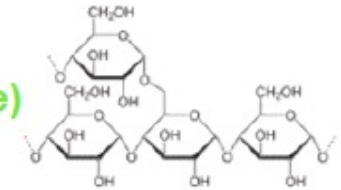


Deformation

Fatty acid



Starch
(carbohydrate)



Raman microspectroscopy - Instrumentation

Basic setup

Light microscope

upright or inverted

sample stage

Light source

Laser (NIR, green)

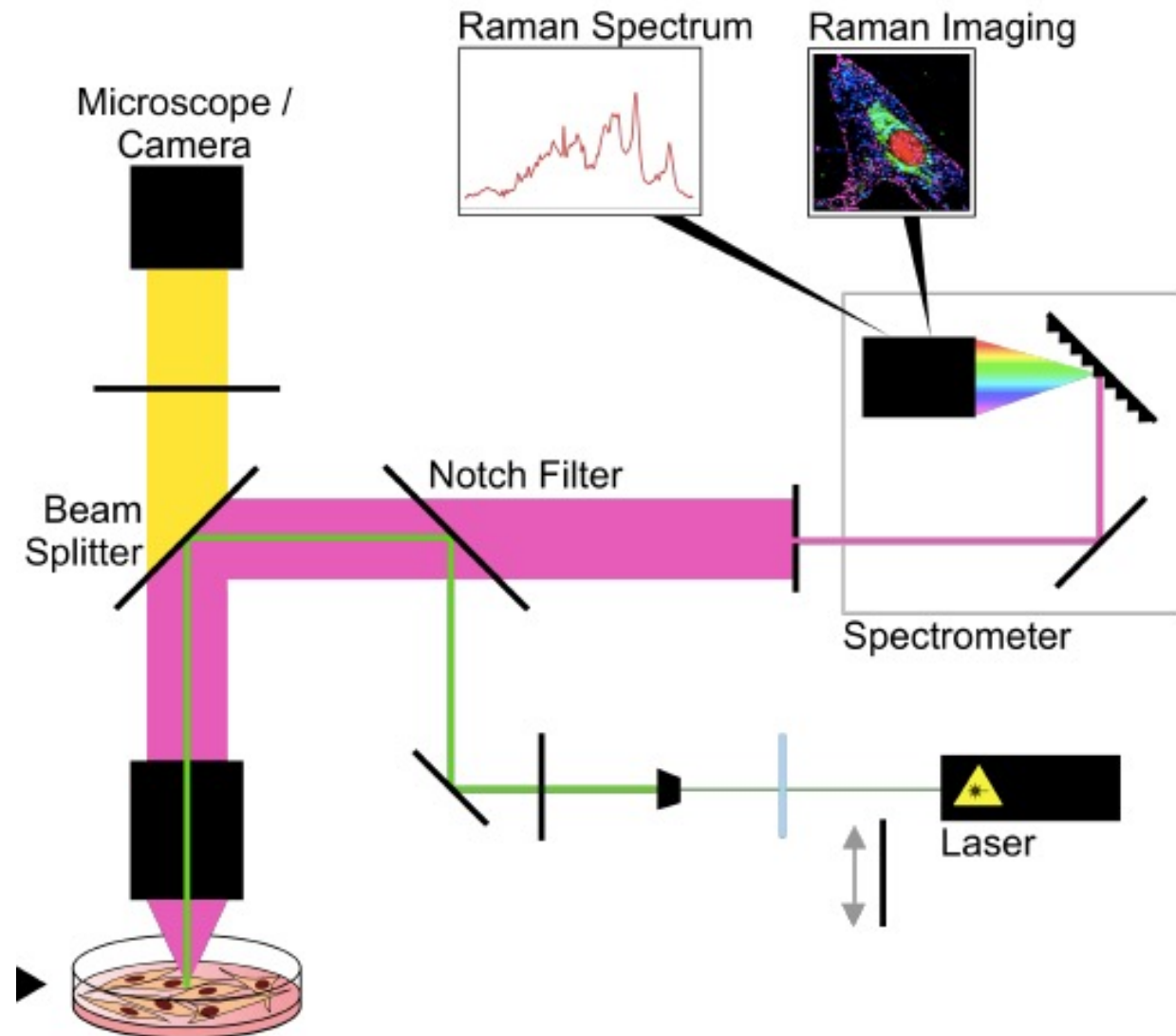
Filter/Mirror system to split the photons

beam splitter

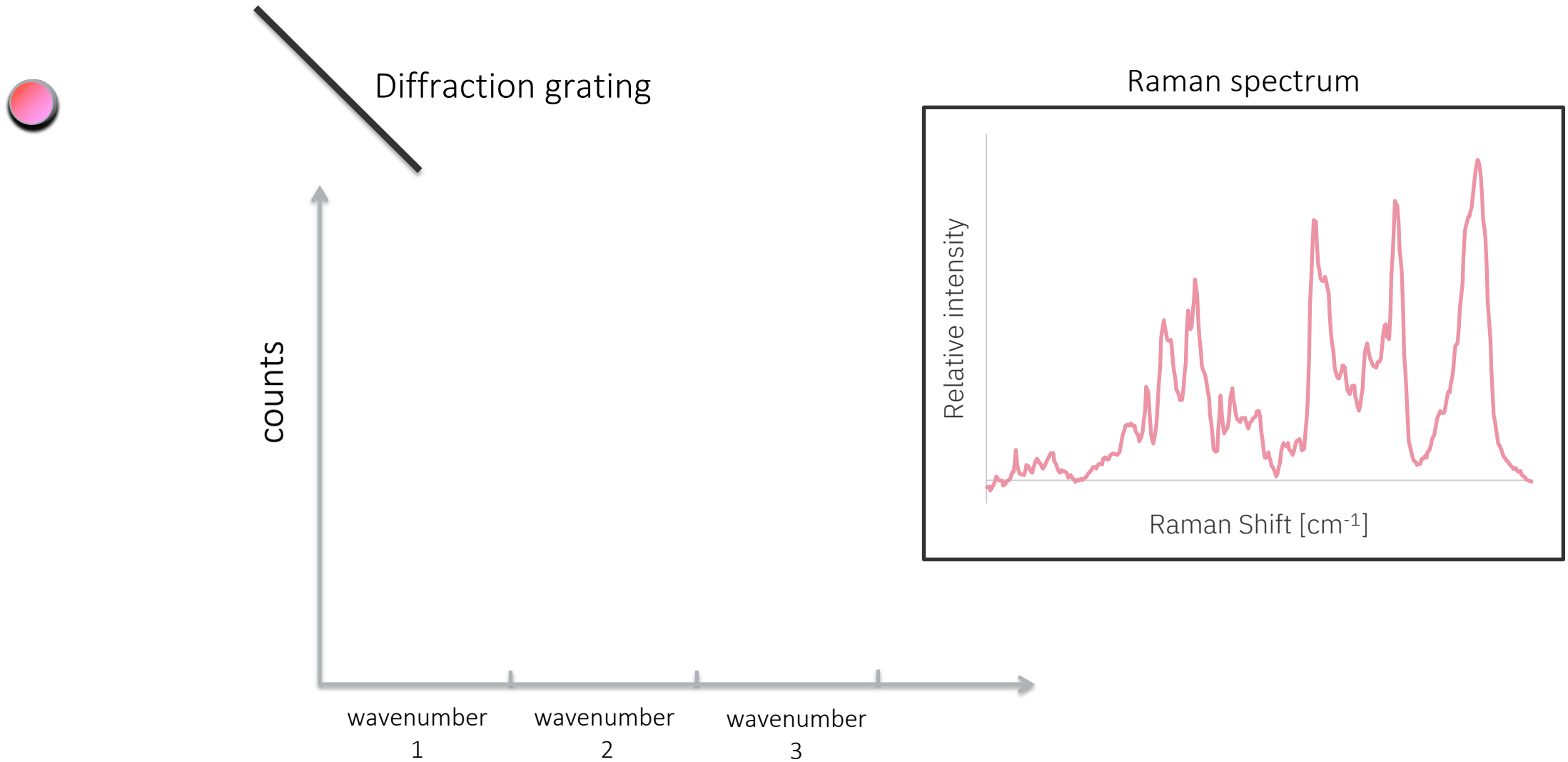
notch filter

Spectrometer

Detector (CCD camera)



Raman microspectroscopy - Basics



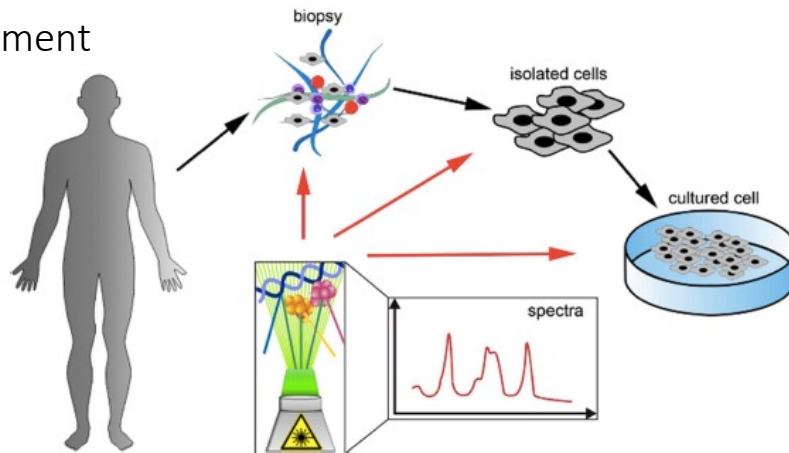
Raman microspectroscopy

Advantages

- non-invasive & marker free
- various samples (chemicals, materials, biological samples)
- non-destructive
- no sample preparation
- Imaging AND molecular analysis
- in situ measurement

Limitations

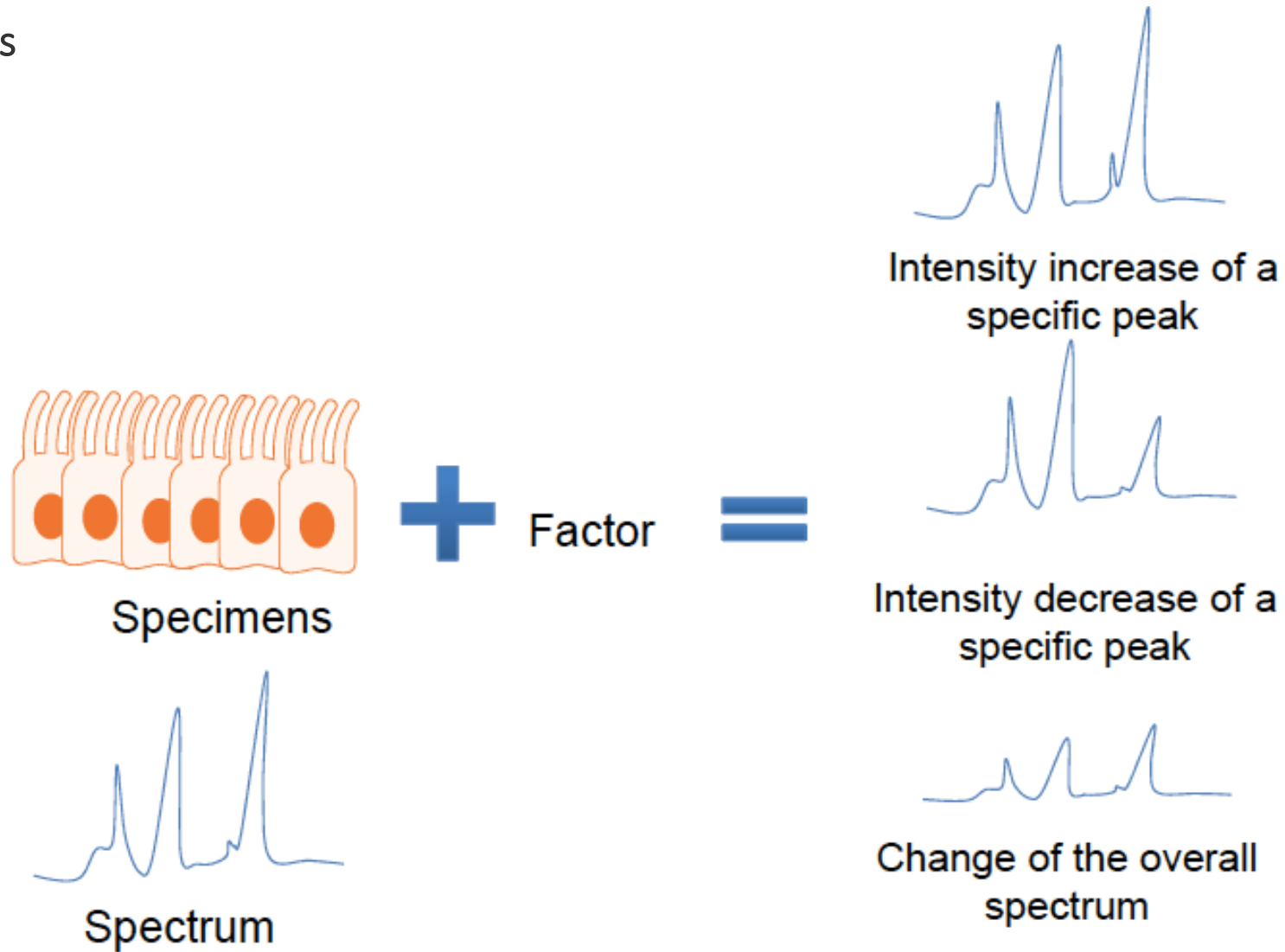
- Raman scattering is a rare event
- Long measurement times and strong lasers are required (for biological samples)
- Risk of sample burning
- Autofluorescence of the sample can obscure the Raman scattering
- Lack of comprehensive database for biomolecules



>> ideal conditions for real-time quality control of tissue engineered products

Raman microspectroscopy – Analysis

Spectral analysis



Raman microspectroscopy – Cell Analysis

Analysis of bacterial species

Applications:

Quality control for food and pharmaceuticals

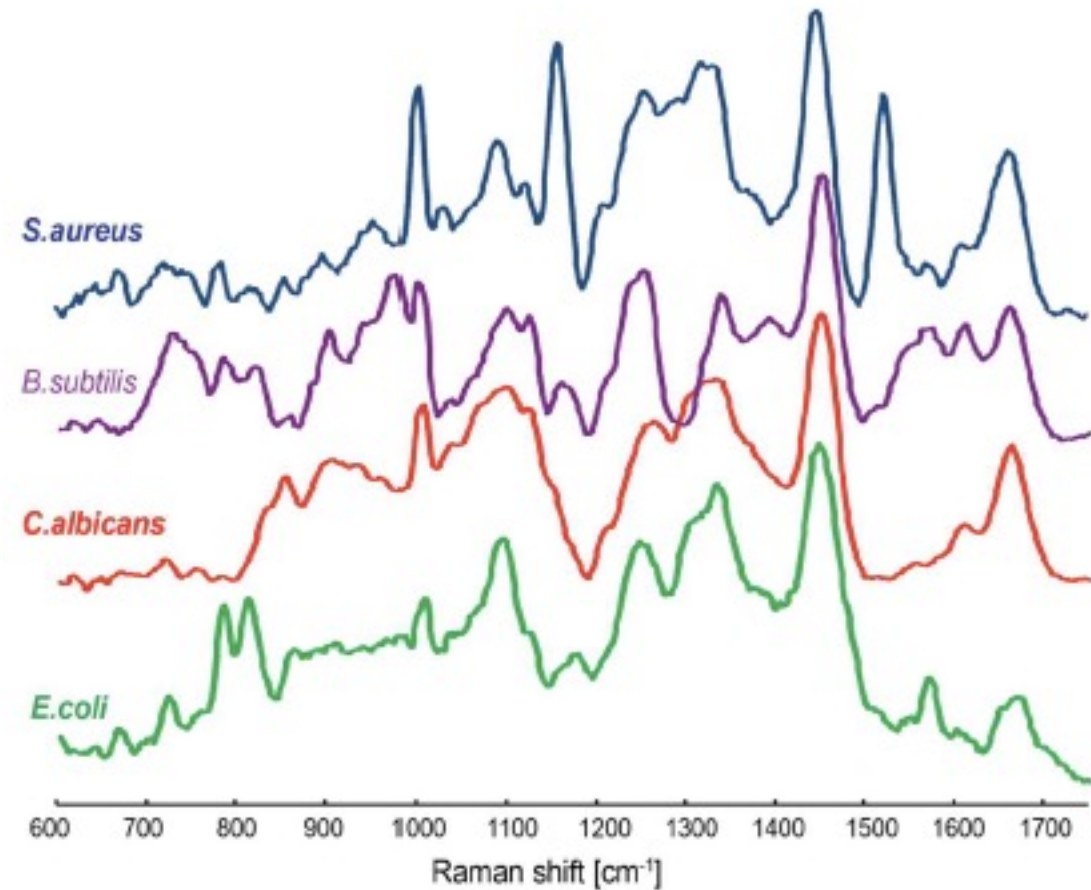
Diagnosis of infections

e.g. epidemiology of nosocomial infections
(River® diagnostics, SpectraCell RA)

Advantages:

Short incubation times (microcolonies)

analysis on the single cell level



Raman microspectroscopy

Spectral mapping

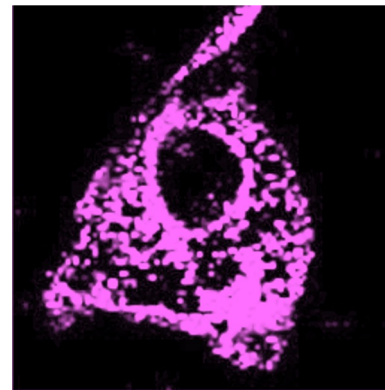
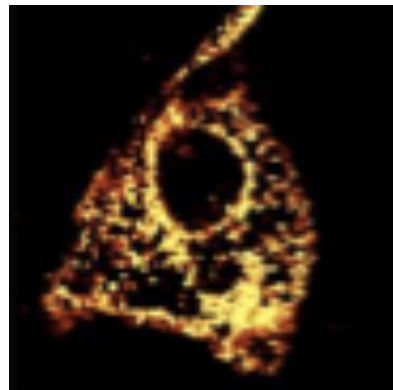
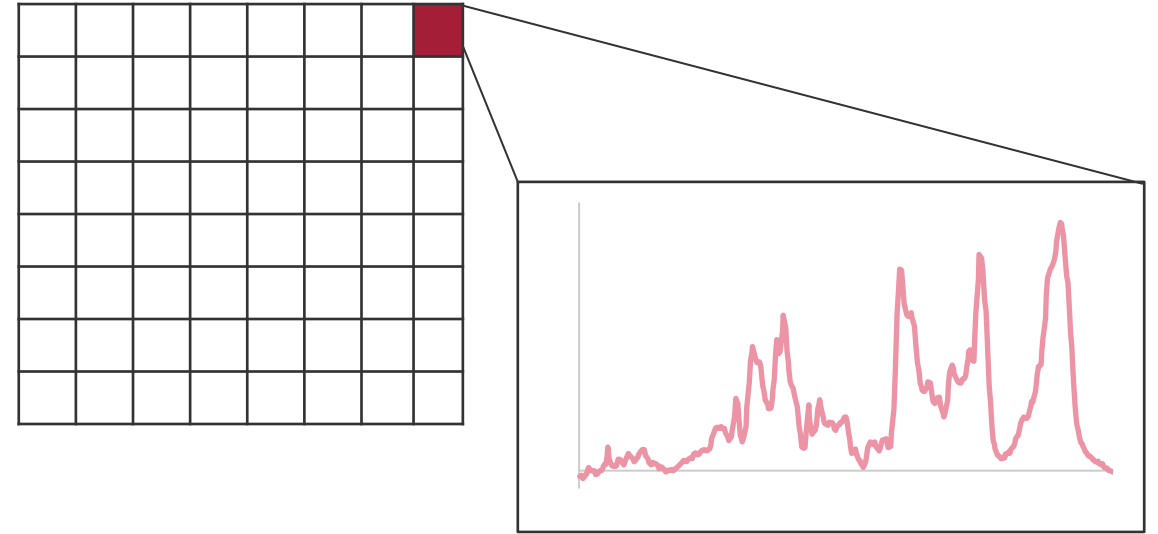
Acquisition of a spectral map

>> every pixel is assigned to a single spectrum

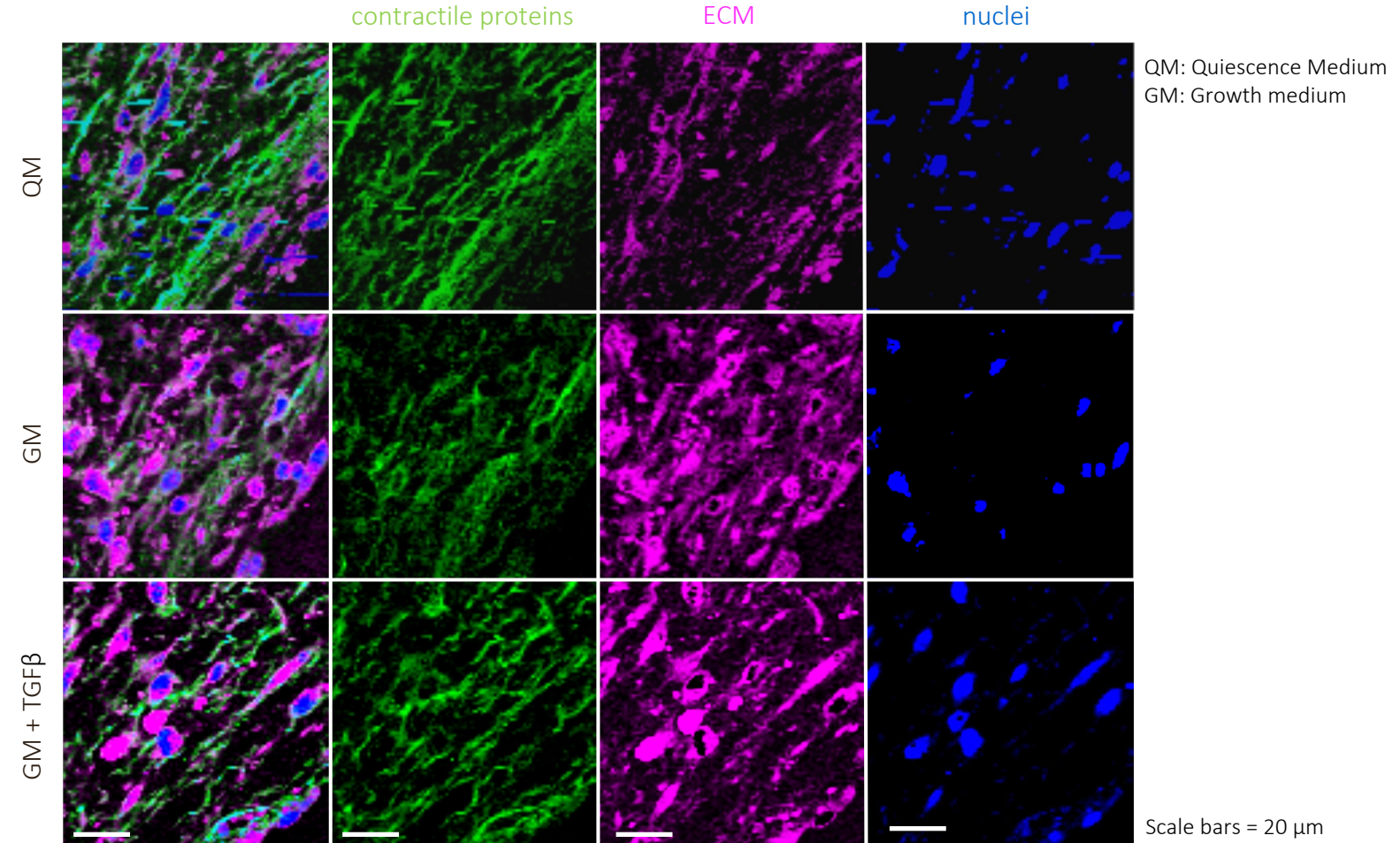
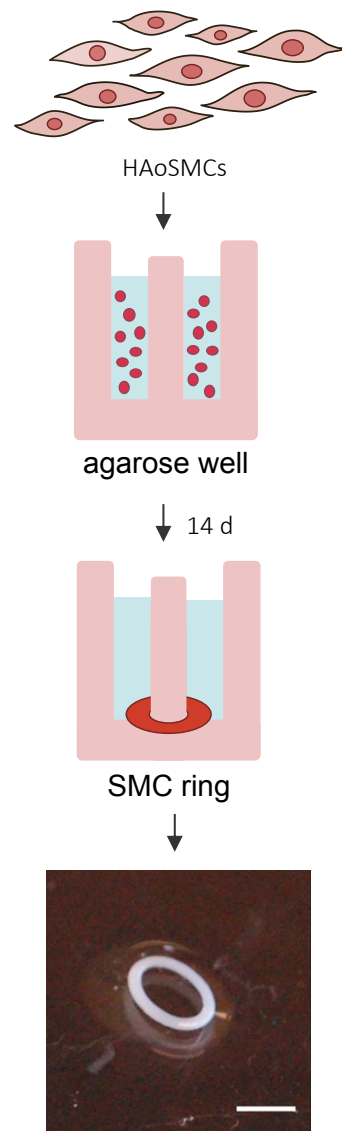
Color-coded image generation

sum filter

multivariate analysis tools (PCA, TCA)



Raman microspectroscopy – Tissue Analysis



Monitoring of ECM remodeling under different culture conditions

GM+TGF β treated SMC rings exhibit ideal tissue formation

Raman microspectroscopy - Summary

Optical detection of molecular vibrations in any sample

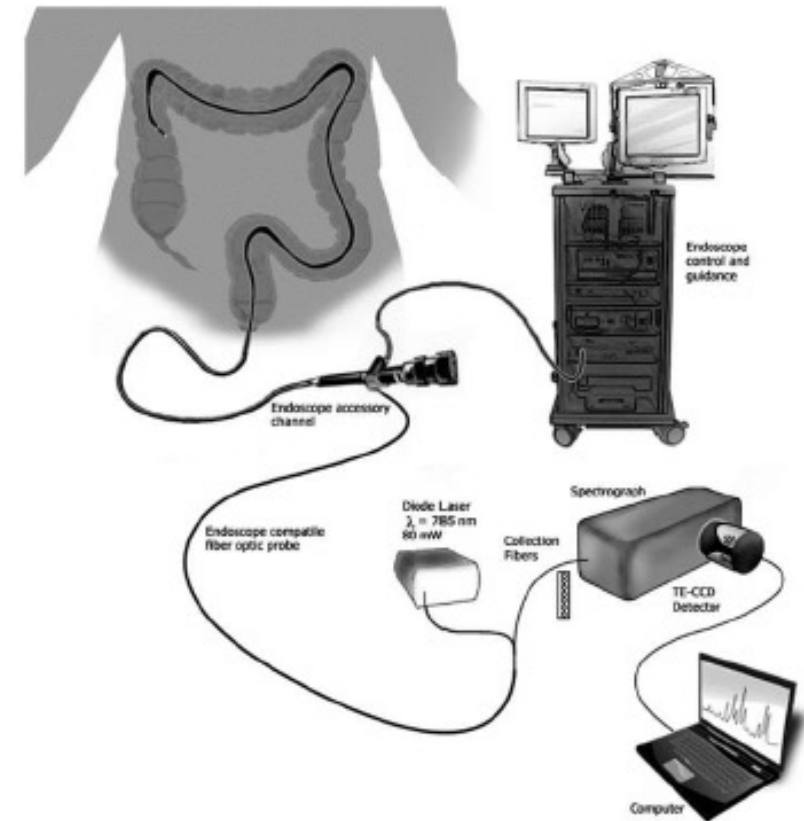
Marker-free method for cell/tissue monitoring in vitro

Biological samples result in very complex spectra

>> multivariate analysis (e.g. PCA-based methods)

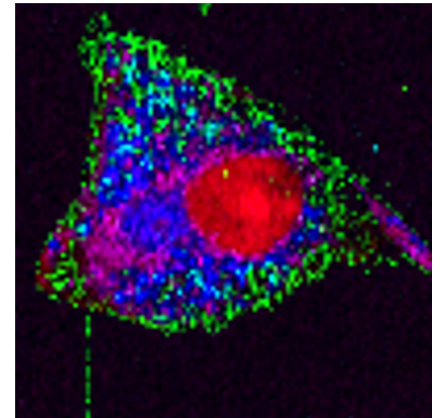
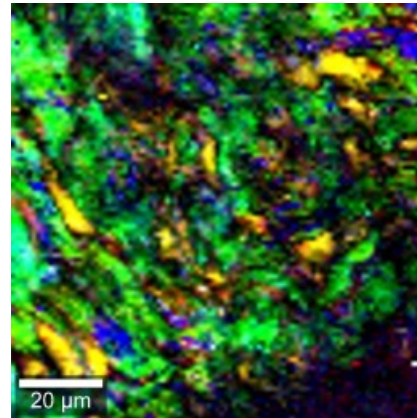
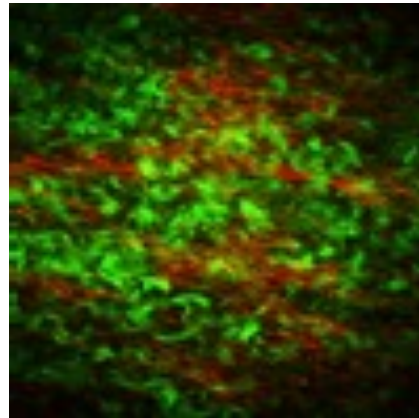
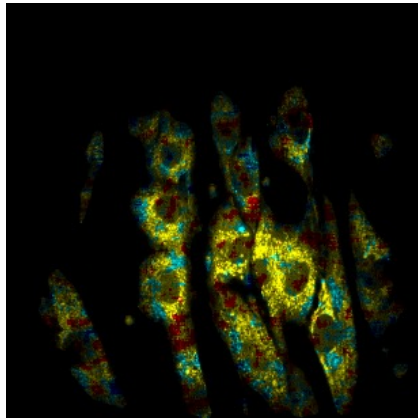
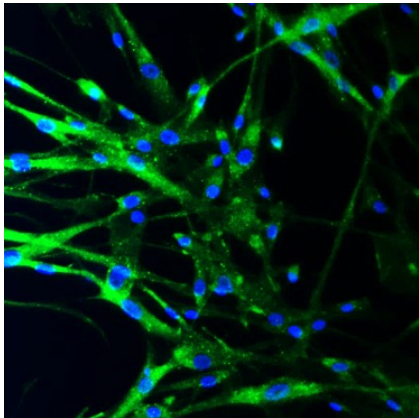
Imaging with biochemical contrast

Clinical applications



Thank you for your attention!

julia.marzi@uni-tuebingen.de
www.schenke-layland-lab.com



Literature

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Kubitscheck: Fluorescence Microscopy: From Principles to Biological Applications, Print ISBN: 9783527329229

Murphy: Fundamentals of Light Microscopy and Electronic Imaging, ISBN Print ISBN: 9780471692140

Pawley: Handbook Of Biological Confocal Microscopy, ISBN 978-0-387-45524-2

Periasamy: FLIM microscopy in medicine and biology, ISBN: 978-1420078909

Zeiss, Microscopy from the very beginning, PDF

<https://www.microscopyu.com/>