Preparatory School to the Winter College on Micro and Nano Photonics for Life Sciences

4 - 8 February 2008

Introduction to biophotonics

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Introduction to Biophotonics

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Preparing this course I considered the following:

✓ The audience is non-homogeneous (from physics and/or biology)
✓ You have basic understanding of optics and biology (Preparatory lectures on cell biology, diffraction theory, optical microscopy were provided)
✓ Advanced lectures on Biophotonics topics will be presented at Winter College next week
✓ Lot of information can be found in internet
✓ Not all the information can be included in a 3 h lecture

The goal of the course is to provide you a basic introduction to Biophotonics with some history and examples and to open your appetite for this fascinating field of science.

**Motto:**

“*We should know and we will know*, David Hilbert, 1930

(expressing his disagreement with the

“*ignoramus et ignorabimus / we do not know and will not know*”

conclusion of Emil du Bois-Reymond, in his

“*On the limits of our understanding of nature*” of 1872
OUTLINE

- What is Biophotonics?

- A bit of modern science history (with “Biophotonics” in mind)

- Interaction of light with biomaterials

- Imaging and spectroscopy of biomaterials

- Various types of optical microscopy and contrast mechanisms

- Laser micro and nano surgery of biomaterials

- Optical tweezers, basics
What is Biophotonics?

Three definitions of Biophotonics among of many other:

Biophotonics is the science of generating and harnessing light (photons) to image, detect and manipulate biological materials.

1 Biophotonics is used in BIOLOGY to probe for molecular mechanisms, function and structure. It is used in MEDICINE to study tissue and blood at the macro (large-scale) and micro (very small scale) organism level to detect, diagnose and treat diseases in a way that are non-invasive to the body.

T. Husser IEEE-LEOS 2004

Interdisciplinary science studying the interaction of light with biological material – where “light” includes all forms of radiant energy whose quantum unit is the photon.

D. Matthews, Optik & Photonik June 2007 No. 2

3 The application of light and other forms of radiant energy to the life sciences.
Subject areas:
Biochemistry, Cell Biology, Developmental Biology, Ecology, Evolution and Diversity of Life, Functional and Comparative, Morphology, Genetics and Disease, Genetics and Molecular Biology, Immunology, Microbiology, Neuroscience, Plant Science, Science and Society, Structural Biology, Virology
The E-M Spectrum

“Light” Spectrum
A bit of modern science history
(with “Biophotonics” in mind)

Some important discoveries in Microscopy

- **1590** – Hans and Zacharias Janssen make the first microscope.

- **1675** – Anton van Leeuwenhoek uses a simple microscope with only one lens to look at blood, insects and many other objects. He was first to describe cells and bacteria through microscope.

- **18th century** – Several technical innovations make microscopes better and easier to handle, which leads to microscopy becoming more and more popular among scientists (e.g. Lens doublet reduces the chromatic aberrations).

- **1878** – Ernst Abbe formulates a mathematical theory correlating resolution to the wavelength of light. Abbé's formula makes calculations of maximum resolution in microscopes possible.

- **1903** – Richard Zsigmondy develops the ultramicroscope and is able to study objects below the wavelength of light.

- **1932** – Frits Zernike invents the phase-contrast microscope that allows the study of colorless and transparent biological materials.

- **1938** – Ernst Ruska develops the electron microscope. The ability to use electrons in microscopy greatly improves the resolution and greatly expands the borders of exploration.

- **1981** – Gerd Binnig and Heinrich Rohrer invent the scanning tunneling microscope that gives three-dimensional images of objects down to the atomic level.

http://nobelprize.org/
Ultramicroscopy
an example of instrumentation development, starting in 1900 ...

The sun beams fell upon a mirror S, were reflected from this into the lens L, which brought them to the focus at 6, over which was arranged a low power microscope. (total magnification about 100 diameters)

N.B.: With ordinary illumination, even with the best objectives, they were not perceptible.

1903 – Zsigmondy and Siedentopf develop the first ultramicroscope, being able to study objects well below the wavelength of light.
**Ultramicroscopy: three-dimensional visualization of neuronal networks in the whole mouse brain**

**Working principle:**
The sample is illuminated from two sides by a blue laser forming a thin sheet of light. Fluorescent light is thus emitted only from a thin optical section and collected by the objective lens. Stray light is blocked by a GFP filter and the image is projected through the tube lens onto the camera target.

(a) Surface of a whole mouse brain reconstructed from 550 optical sections. Both the GFP and autofluorescence signal are imaged.

(b) Excised whole hippocampus reconstructed from 410 optical sections. Single cell bodies are visible.

(c) 3D reconstruction of part of a whole hippocampus using 132 optical sections.

(d) 3D reconstruction of dendritic spines of CA1 pyramidal neurons obtained with a higher resolution objective (20; NA, 0.4) in a whole hippocampus (430 optical sections, deconvolved).

Major events in Cell Biology & Imaging

- Robert Hooke observes cells of a cork tree through a primitive microscope (1663)
- Leeuwenhoek discovers bacteria (1674, 1683)
- Leeuwenhoek discovers protozoa
- Koch uses aniline dyes to identify bacteria causing TB and cholera (1882)
- Kolliker describes mitochondria in muscle (1857)
- Ruska builds the first transmission electron microscope (1931)
- Sheep "cloned" (1997)
- Schleiden & Schwann propose the Cell Theory (1838)
- Golgi stains cells with silver nitrate, discovering the Golgi apparatus (1898)
- 1st commercial scanning electron microscope (1965)

Relative sizes of cells and their components:

- Small molecule
- Virus
- Bacterium
- Animal cell
- Plant cell

- Electron microscope
- Light microscope

http://www.biology.arizona.edu/
Old (1838) Schleiden & Schwann

1. The cell is the unit of structure, physiology, and organization in living things. **OK**
2. The cell retains a dual existence as a distinct entity and a building block in the construction of organisms. **OK**
3. Cells form by free-cell formation, similar to the formation of crystals (spontaneous generation). **WRONG**

Modern Cell Theory

1. All known living things are made up of cells.
2. The cell is structural & functional unit of all living things.
3. All cells come from pre-existing cells by division.
4. (Spontaneous Generation does not occur).
5. Cells contain hereditary information which is passed from cell to cell during cell division.
6. All cells are basically the same in chemical composition.
7. All energy flow (metabolism & biochemistry) of life occurs within cells.
Discoveries in the field of X rays
(Nobel Prizes – incomplete list)

1901 - W. C. Röntgen: the discovery of X-rays.

1914 - M. von Laue: the discovery of X-rays by crystals.

1915 - W. H. Bragg and W. L. Bragg: the determination of crystal structures using X-rays.

1927 - A. H. Compton: revealing the particle nature of X-rays in scattering experiments on electrons.

1936 - P. Debye: for determining molecular structures by X-ray diffraction in gases.


1962 - F. Crick, J. Watson and M. Wilkins: their discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material.

1964 - D. Crowfoot Hodgkin: the determination of the structure of penicillin and other important biochemical substances.


1988 - J. Deisenhofer, R. Huber and H. Michel: the determination of protein structures crucial to photosynthesis.

http://nobelprize.org/
Johann Friedrich Miescher identifies a weakly acidic substance of unknown function in the nuclei of human white blood cells. This substance will later be called deoxyribonucleic acid, or DNA.

Physicist Sir William Henry Bragg, and his son, Sir William Lawrence Bragg, discover that they can deduce the atomic structure of crystals from their X-ray diffraction patterns. This scientific tool will be key in helping Watson and Crick determine DNA’s structure.

Microscope studies using stains for DNA and protein show that both substances are present in chromosomes.

Franklin Griffith, a British medical officer, discovers that genetic information can be transferred from heat-killed bacteria cells to live ones. This phenomenon, called transformation, provides the first evidence that the genetic material is a heat-stable chemical.

Oswald Avery, and his colleagues Maclyn McCarty and Colin MacLeod, identify Griffith's transforming agent as DNA. However, their discovery is greeted with skepticism, in part because many scientists still believe that DNA is too simple a molecule to be the genetic material.

Erwin Chargaff, a biochemist, reports that DNA composition is species-specific; that is, that the amount of DNA and its nitrogenous bases varies from one species to another. In addition, Chargaff finds that the amount of adenine equals the amount of thymine, and the amount of guanine equals the amount of cytosine in DNA from every species.

James Watson and Francis Crick discover the molecular structure of DNA.

http://nobelprize.org/
Biophotonics has already played a central role in the most famous paper in modern biology: *J.D. Watson and F.H.C. Crick*, Nature, 25 April 1953

1953 James Watson and Francis Crick discover the molecular structure of DNA
The Nobel Prize in Physiology or Medicine 1962

"for their discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material"

Francis Harry Compton Crick

James Dewey Watson

Maurice Hugh Frederick Wilkins

1/3 of the prize
United Kingdom
MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

1/3 of the prize
USA
Harvard University, Cambridge, MA, USA

1/3 of the prize
United Kingdom and New Zealand
London University
London, United Kingdom
See/Read/Think-on their Nobel Lectures:

On the Genetic Code

The involvement of RNA in the synthesis of proteins

The molecular configuration of nucleic acids
"We wish to suggest a structure for the salt of deoxyribose nucleic acid (DNA). This structure has novel features which are of considerable biological interest ...

We wish to put forward a radically different structure for the salt of DNA.

This structure has two helical chains each coiled round the same axis."

The two ribbons symbolize the two phosphate - sugar chains, and the horizontal rods the pairs of bases holding the chains together.

The vertical line marks the fibre axis.
“The purpose of this communication is to describe, in a preliminary way, some of the experimental evidence for the poly-nucleotide chain configuration being helical, and existing in this form when in the natural state.”

Diffraction pattern of system of helices corresponding to structure of DNA.

The squares of Bessel functions are plotted about 0 on the equator and on the first, second and fifth layer lines for half of the nucleotide mass at 20 Å. A diameter and remainder distributed along a radius, the mass at a given radius being proportional to the radius. About C on the tenth layer the similar functions are plotted for an outer diameter of 12 Å.
**Life Sciences**

**Subject areas:**
- Biochemistry, Cell Biology,
- Developmental Biology, Ecology,
- Evolution and Diversity of Life,
- Functional and Comparative,
- Morphology, Genetics and Disease,
- Genetics and Molecular Biology
- Immunology, Microbiology, Neuroscience,
- Plant Science, Science and Society,
- Structural Biology, Virology

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**Light source** → **Investigated Subject** → **Detector** → **Analysis Instrumentation**
Interaction of light with biomaterials

In complex materials, any combination of interactions are possible. The exact nature of each process depends on the **physical and chemical** structure of the biomaterial.
**Light-Induced Processes**

**Tissue light interaction**

**Radiative**
- Tissue autofluorescence
- Fluorescence from various constituents of the tissue

**Nonradiative**

**Photochemical**
- Excited state reaction
- Occurs even at low optical power density

**Photoablation**
- Direct breaking of cellular structure
- Performed by high energy UV radiation

**Photodisruption**
- Shockwave generation at high pulse intensity
- Fragmentation and cutting of the tissue by mechanical force of shockwave

**Thermal**
- Light absorption converted to heat
- Can produce coagulation, vaporization, carbonization and melting

**Plasma – induced ablation**
- Induced by high intensity short pulse
- Dielectric breakdown creates ionized plasma that interacts with light to produce ablation

Prasad, Introduction to Biophotonics, John Wiley & Sons © 2003
Preparatory School to the ICTP Winter College Micro and Nano Photonics for Life Sciences, Trieste, 2008 February 4-8
Light Scattering Processes

Light Scattering

- **Elastic Scattering**
  - Incident and scattered photons are of the same frequency

  - **Rayleigh scattering**
    - Scattering by particles of size smaller than the wavelength $\lambda$ of light.
    - Scattering depends on the $\lambda^{-4}$ hence significantly more for blue than for red light.
    - Forward and backward scattering are the same.

  - **Mie scattering**
    - Scattering by particles of size comparable with $\lambda$.
    - Weaker wavelength dependence: $\lambda^{-X}$ where $0.4 < X < 0.5$.
    - Forward scattering preferred.

- **Inelastic Scattering**
  - Incident and scattered photons are of different frequencies

  - **Brillouin Scattering**
    - The difference in energy generates acoustic phonons.

  - **Raman Scattering**
    - The difference in energy generates a vibrational excitation in the molecule.
### Imaging and spectroscopy of bio-materials

<table>
<thead>
<tr>
<th>Scattering and absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence</td>
</tr>
<tr>
<td>Optical Microscopy</td>
</tr>
<tr>
<td>Transmission and Absorption</td>
</tr>
<tr>
<td>Phase contrast</td>
</tr>
<tr>
<td>Confocal</td>
</tr>
<tr>
<td>Fluorescence Correlation Spectroscopy</td>
</tr>
</tbody>
</table>

**Fluorescence Lifetime Imaging**

**Multiphoton Excitation**

**Spontaneous Raman Spectroscopy**

**Coherent Anti-Stokes Raman Scattering Microscopy (CARS)**

**Second Harmonic Generation Microscopy**

**Stimulated Emission Depletion, Image Deconvolution Microscopy**

**Nearfiled Optical Microscopy**

**Single Molecule Detection**

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*Courtesy Prof. T. Huser (slides: 25-30, 32-35, 4, 51-55, 63-64, 69, 85-88)*

*NSF Center for Biophotonics Science and Technology, University of California, Davis, USA*

Main sources for light loss in biological materials: absorption and light scattering

Tissue absorption

Water absorption

Figure courtesy Dr. Stavros Demos, LLNL

omlc.ogi.edu/spectra/wate#6E1CB
Scattering in tissue

Structures $\ll$ wavelength of light: Rayleigh scattering
Structure $\sim$ wavelength: Mie scattering
Absorption spectroscopy is a routinely used technique in the biosciences. It is widely used in bio sciences to determine concentrations by measuring optical density (OD):

- Samples in 1 cm path cells – quartz/glass; for <250 nm purge with N₂
- Proteins: Tyr/Trypt/Phe in 260 – 280 nm range $\lambda_{\text{max}}$ at ~ 280 nm
- DNA $\lambda_{\text{max}}$ at ~ 260 nm

Application for absorption spectroscopy

More order means less absorption – perfect crystals are clear
-> Single-stranded DNA absorbs more than double-stranded DNA at the same concentration

Example: Monitor denaturation/renaturation – e.g. helix-coil transition in DNA
Light microscopy enables live imaging at the cellular level

Why is light microscopy so important?

- Oldest and best understood form of microscopy
- Mostly non-invasive, non-destructive, native conditions
- Enables molecular and cellular imaging of living systems in real time
- Tissue structures are accessible over a wide range of magnifications
- Chemical characterization by infrared absorption or Raman spectroscopy possible
- Large number of contrast mechanisms developed
- Variety of techniques for subcellular - molecular imaging under development
Some relevant length scales

Various types of optical microscopy and contrast mechanisms

“Standard”
- Transmission/absorption
- Reflection
- Darkfield contrast
- Polarization contrast
- Phase contrast
- Differential Interference Contrast (DIC)
- Fluorescence microscopy

“Advanced”
- Confocal
- Total internal reflection microscopy
- Multiphoton excitation
- Raman
- Coherent Anti-Stokes Raman (CARS) Microscopy
- Second Harmonic Generation (SHG) Microscopy
- Near-field optical microscopy
- Single molecule fluorescence microscopy
- Deconvolution microscopy
Transmission/absorption microscopy of biological samples requires preparation and staining.
The most important light microscopy imaging technique: Phase contrast microscopy avoids staining
Phase contrast microscopy

Figure 9–7. Molecular Biology of the Cell, 4th Edition.

Figure 9–8 part 1 of 2. Molecular Biology of the Cell, 4th Edition.
In DIC microscopy, the spatial relationship and phase difference between ordinary and extraordinary wavefronts is governed either by the position of the objective prism (Nomarski DIC) or the relationship between the polarizer and a thin quartz retardation plate in a de Sénarmont design.

Phase contrast - DIC microscopy

HeLa cells have been cultured continuously for scientific use since they were first taken from the tumor of a woman suffering from cervical cancer in the 1950s. They have been utilized for many purposes, including the development of a polio vaccine, the pursuit of a cure for diseases such as leukemia and cancer, and the study of the cellular effects of drugs and radiation.

Polarization microscopy

http://www.microscopyu.com/articles/polarized/polarizedintro.html
Asbestos is a generic name for a group of naturally occurring mineral fibers, which have been widely used, for example, in insulating materials, brake pads and to reinforce concrete. They can be harmful to health when inhaled and it is important that their presence in the environment be easily identified. Samples are commonly screened using scanning electron microscopy and x-ray microanalysis, but polarizing microscopy provides a quicker and easier alternative that can be utilized to distinguish between asbestos and other fibers and between the major types asbestos – chrysotile, crocidolite and amosite. With the use of crossed polars it is possible to deduce the permitted vibration direction of the light as it passes through the specimen, and with the whole wave plate, a determination of the slow and fast vibration directions. Under crossed polars, chrysotile shows pale interference colors - low order whites (a). When a full wave plate is added (530-560 nanometers), the colors are transformed. Aligned Northeast-Southwest, the wave plate is additive and gives blue and yellow in the fiber (b). When aligned Northwest-Southeast (c) the plate is subtracting to give a paler yellow fiber with no blue. From this it is possible to deduce that the slow vibration direction is parallel with the long axis of the fiber.
Fluorescence

is an electronic process

The fluorescence of a single molecule is optically characterized by

- well defined absorption and emission dipole moment (polarization)
- excited state lifetime (fluorescence lifetime)
- spectral emission

Figure: W.E. Moerner and M. Orrit, *Science* 283, 1670 (1999)
Excitation / Emission spectroscopy of native fluorophores in tissue (autofluorescence)

Information about the biochemical composition of tissue can be obtained by analyzing its absorption and fluorescence spectra.
**Fluorescence microscopy**

More specific information requires **optical labeling**: Fluorescent dyes spread throughout the entire optical spectrum.

**Fluorescent probes, fluorochromes**: Molecules capable of undergoing electronic transitions that result in fluorescence.

**Fluorophore**: The structural domain or specific region of a molecule that is capable of exhibiting fluorescence.
Fluorescence microscopy
DIC and fluorescence X-ray microscopy

Scanning x-ray microscope picture of human liver cell. (a) topographic image. By varying the incoming photon energy according to the absorption edge of selected elements, and by inserting a fluorescence detector, we were able to map the distribution of some elements inside the cell.

See also the lecture on X-ray diffractive elements by D. Cojoc next week.

Fluorescence resonance energy transfer (FRET) enables measurements of molecular association/dissociation and lengths.
Fluorescent dyes can be linked to biomolecules

**Texas Red-X, succinimidylic ester**

![Chemical structure of Texas Red-X, succinimidylic ester](image)

**Linkage to antibodies enables specific targeting in cells: immunofluorescence**

![Fluorescence labeling of microtubulin](image)

Fluorescent proteins can be “fused” to other proteins and co-expressed to intrinsically label proteins in cells

Beta-can structure of the green fluorescent protein (GFP) from the jellyfish Aequorea victoria

http://www.npaci.edu/online/v4.14/gfp.html
Fluorescence microscopy

Some drawbacks:

- Small Stokes shift
- Low axial resolution
- Photobleaching
- Large Rayleigh scattering
- Use of UV optics
- Absorption – (photodamage)

Photobleaching (or fading) is when a fluorophore permanently loses the ability to fluoresce due to photon-induced chemical damage and covalent modification.

See the lectures next week for pros and cons
Total intern reflection fluorescence (TIRF) microscopy excites only a very narrow region right above the glass interface.

TIRF provides contrast of the cell surface.

http://www.olympusfluview.com/applications/tirfintro.html
Confocal fluorescence microscopy efficiently suppresses background signals and enables three-dimensional sectioning

Pinhole $\rightarrow$ High axial resolution
Examples of the value of confocal fluorescence microscopy

![Comparison of conventional fluorescence and confocal fluorescence.](image)

**Drosophila embryo**

**Pollen grain**

*Figure 9-19. Molecular Biology of the Cell, 4th Edition.*

*Figure 9-20 part 1 of 2. Molecular Biology of the Cell, 4th Edition.*
Scanning confocal fluorescence microscopy

http://www-celanphy.sci.kun.nl/jenks.htm

Doubled labeled sphere $d = 6 \, \mu m$

NIH 3T3 cells

http://www.probes.com/

bovine pulmonary artery endothelial cells
Mitochondria (in red)
Nuclei (in blue)
F-actin (in green)
Fluorescence correlation spectroscopy (FCS) measures molecular diffusion times and association/dissociation rate.
Time-correlated single photon counting (TCSPC) enables fast fluorescence lifetime measurements
Pulsed laser sources combined with TCSPC enable lifetime analysis per pixel in confocal images: fluorescence lifetime imaging

Fluorescence lifetime imaging microscopy is gaining popularity because it provides an additional contrast mechanism to distinguish fluorophores from each other or from autofluorescence background.

Data: courtesy of Prof. Costa, Lisbon

http://www.picoquant.com
2-photon fluorescence microscopy

Why 2-photon excitation?
- 2-photon excitation is a non-linear process, emission \( \sim (\text{excitation intensity})^2 \)
- No confocal pinhole necessary
- Typically requires pulsed excitation source (high peak power)

\[ P_p = 100 \text{ KW} \]

\[ \text{Photon flux} = \frac{P_p}{hv} = 1 \times 10^5 / 2.5 \times 10^{-19} \]

High photon flux!!
2-photon absorption

The transition rate for two-photon absorption is:

$$R_{ng}^{(2)} = \frac{P_{n}^{(2)}(t)}{t} = \sigma_{ng}^{(2)}(\omega) I^2$$

Where $\sigma_{ng}^{(2)}(\omega)$ is the two-photon absorption cross section:

$$\sigma_{ng}^{(2)}(\omega) = \frac{8 \pi^3}{n^2 c^2} \sum_{m} \left[ \frac{\mu_{nm} \mu_{mg}}{\hbar^2} (\omega_m - \omega) \right] \rho_f (\omega_{ng} = 2 \omega_L)$$

Expressing $\sigma_{ng}^{(2)}(\omega)$ in terms of the intensity measured in photons cm$^{-2}$ sec$^{-1}$

$$\sigma_{ng}^{(2)}(\omega) = \frac{16 \pi^2}{\Gamma n^2 c^2} \left| \frac{\mu_{nm} \mu_{mg}}{\hbar} \right|^2$$

Assume that $\mu_{nm}$ and $\mu_{mg}$ are of the order of $\varepsilon a_0 = 2.5 \times 10^{-18}$ esu

Two-photon cross sections are cited in GM units (after Maria Goeppert-Mayer who first predicted two-photon absorption as a single quantum event)

$$\sigma_{ng}^{(2)}(\omega) = 3.65 \times 10^{-50} \text{ cm}^4 \text{s photon}^{-1}$$

1 GM = $10^{-50}$ cm$^4$ sec.$^{-1}$

Multi-photon fluorescence microscopy

![Multiphoton Molecular Excitation](image)

Multi-photon fluorescence microscopy

Why multi-photon excitation?
- excite fluorescence below 450 nm: quantum dots, DNA adducts, anthracene-based dyes, protein fluorescence
- Direct excitation is limited by background (glass, lenses, filters, contaminations)
- Deep tissue penetration

![Diagram showing linear and non-linear fluorescence](image)
Multi-photon fluorescence microscopy

3-photon image of DAPI stained *C. elegans*

Living neurons

http://www.loci.wisc.edu/multiphoton/mp.html

Parc Cientific, Barcelona
Raman spectroscopy

Drawback of Fluorescence: is limited by the need to label and photobleaching
-> Raman provides molecular information

Raman spectroscopy provides
- Fingerprint spectra (molecular identity)
- Information about 3d structural changes (orientation, conformation)
- Information about intermolecular interactions
- Dynamics

Raman scattering, however, is extremely inefficient
Only 1 in $10^8$ incident photons are Raman scattered
Typical scattering cross-sections $\sim 10^{-30}$ cm$^2$ (15 orders of magnitude lower than fluorescence excitation)
Raman scattering is the interaction of photons and intrinsic molecular bonds
Confocal Raman microscopy
Example: Raman spectroscopy can distinguish between ds DNA and protein-DNA complexes.
An example for micro-Raman analysis of single cells
optically trapped cells

Real-time detection of hyperosmotic stress response in a single *Saccharomyces* yeast cell

- the cell stress response is the reaction of a living cell to ambient changes which are potentially harmful: for example, an increase in temperature, pH, saline concentration, the presence of toxins

- the basic reaction at the fermentation process is the response of the *Saccharomyces* yeast cells on the hyperosmotic stress

\[
\text{ethanol} \quad \text{C}_2\text{H}_5\text{OH} \\
\text{glycerol} \quad \text{C}_3\text{H}_8\text{O}_3
\]

+ glucose =
Real-time detection of hyperosmotic stress response in a single *Saccharomyces* yeast cell

Detection area $1 \text{ femtoL}$
Detectable concentration $100 \text{ atto}_\text{mol}$
Detectable number of molecules $10^8$

An example for micro-Raman analysis of single cells:

The biochemical changes taking place during the lag phase and G1 phase of a single
*S. cerevisiae* yeast cell

(A) The growth curve shows the phases of yeast cell population.

(B) The yeast cell cycle diagram shows the phases of the cell cycle. The abbreviations used for yeast cell cycle stand for the standard terms: 1 M, mitosis; S, synthesis; G1 and G2, gap phases. For our experiments cells that did not have a bud were chosen.

(C) Upper row shows the budding of a yeast cell and its subsequent growth while in the optical trap. Lower row shows the same cell at a moment when it is released from the optical trap

An example of combined Raman and fluorescence microscopy

Researchers studying *S. pombe* cells discovered a yet to be assigned 1602 cm\(^{-1}\) Raman band.

Huang et. al., Biochemistry, V44, 10009-10019 (2005)
Advantages and limitations of spontaneous Raman imaging

Advantages
- Minimally invasive technique
- Non-photobleaching signal for live cell studies
- Works under different conditions (temperatures and pressures)
- Chemical imaging without exogenous tags
- Works with different wavelengths

Limitations
- Fluorescence interference
- Limited spatial resolution
- Weak signal – long integration times

Raman scattering is extremely inefficient (10^{-30} \text{ cm}^2 \text{ cross sections})
1 in 10^8 incident photons are Raman scattered
Coherent Anti-Stokes Raman Scattering (CARS) microscopy

The newest member to the optical microscopy family

Two laser frequencies interact resonantly with a specific molecular vibration:

\[ \omega_{\text{vib}} = \omega_{\text{pump}} - \omega_{\text{Stokes}} \]

CARS signals are generated at wavelengths shorter than the excitation wavelengths (anti-Stokes)

\[ \omega_{\text{AS}} = 2\omega_{\text{pump}} - \omega_{\text{Stokes}} \]

Why develop CARS?

- Contrast signal based on vibrational characteristics, no need for fluorescent tagging
- CARS signal is at high frequency (lower wavelength) – no fluorescence interference
- Higher resolution
- More sensitive (stronger signals) than spontaneous Raman
- Microscopy – faster, more efficient imaging for real-time analysis
CARS microscopy setup
requires two synchronized ultrashort pulsed laser sources

Key components in a CARS setup with two tunable synchronized Lasers (5 psec, 80 MHz rep rate)

X.S. Xie et al., LNNL and Harvard
CARS microscopy: application to live cell imaging

Example 1

Lasers @ 853 nm (100 μW) and 1135 nm (100 μW)
tuned to Raman shift of 2913 cm⁻¹ C-H vibration

Unstained live bacterial cells.
Signal due to cell membranes.

Unstained live HeLa cells.
Bright spots due to mitochondria.

Tracking trajectories of organelles inside single living cells

Example 2

Raman spectroscopy for single cell cancer detection

Spontaneous Raman spectra takes 2 minutes per cell !!!

Future applications: CARS cytometry for rapid, labelless cancer cell detection and sorting

Optical trapping combined with CARS for faster spectral analysis

Trapped polystyrene bead using two CARS beams

Potential solution for faster chemical analysis of cells

Future applications: CARS *in-vivo* imaging

2845 cm⁻¹ vibration C-H lipid

*Evans et. al., PNAS, V102 16807 (2005)*
Other future directions for CARS

- Fiber based CARS for endoscopy
- CARS optical coherence tomography

See the CARS lectures by H. Rigneault next week
**Second Harmonic Generation (SHG)**

Another nonlinear optical imaging technique

**SHG: Nonlinear process in which incident light at a given frequency ($v_1$) is converted into light at twice the frequency ($v_{SHG} = 2v_1$)**

It occurs:

- only at beam focus (intense field), eliminating out-of-plane signal and enabling sub-micron spatial resolution
- when intense light interacts with matter organized on the scale of the wavelength with no inversion symmetry

To break centrosymmetry, SHG is allowed in:

- interfaces or surfaces (weak)[1]
- membranes with potentials (due to directional electrical field) [2,3]
- biophotonic crystal structures [4]

**Example: SHG microscopy in collagen**

The triple helical structure of **collagen**, the most abundant structural protein in the cornea and the entire body, satisfies this requirement

**SHG signal is uniquely sensitive to collagen structure**

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**Optical Setup**

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Example: SHG microscopy in collagen

Applications

Many pathological conditions are characterized by abnormal collagen structure

- **Cornea**: keratoconus, bullous keratopathy, trauma
- **Skin**: melanoma, Ehlers-Danlos, scarring, burns
- **Cartilage**: osteoarthritis, post-traumatic degeneration
- **Spinal column**: intervertebral disk disease
- **Liver**: fibrosis/cirrhosis
- **Bone**: osteogenesis imperfecta

Second-harmonic signal as a function of transverse position in a section of rat tail tendon obtained with a 1μm scan resolution and the 40 objective with the 100-mm collimating lens

Stimulated Emission Depletion (STED) Fluorescence Microscopy

Optical super-resolution → 20 nanometers

Working principle: Excite with a short pulse ($10^{-9}$s) Quickly follow with a second synchronised pulse (shaped so that it illuminates a ring around the sample rather than a spot) that stimulates emission.

Those molecules hit by the body of the ring are forced to dump their energy, while those that sit in the hole in the middle of the ring are allowed to fluoresce as normal. Careful tuning of the second pulse's intensity can narrow the size of the central hole to (in theory) the size of a molecule. This ring-shaped pulse effectively provides a tiny aperture for studying samples.

Observe the residual spontaneous emission by the detector.

S.W. Hell, and J. Wichmann
"Breaking the diffraction resolution limit by stimulated emission."
STED

Neurofilaments in human neuroblastoma recorded in the confocal mode and with STED after nonlinear deconvolution displaying a focal plane resolution of 20 to 30 nm

Stefan W. Hell, Far-Field Optical Nanoscopy, Review Science, 316 1153 (2007)

See the “Super-resolution” lectures by Volker Westphal
next week at the Winter College
**Nonlinear structured-illumination microscopy**

Wide-field fluorescence imaging with theoretically unlimited resolution

**Principle:** Resolution extension through the moiré effect. If an unknown sample structure (a) is multiplied by a known regular illumination pattern (b), a beat pattern (moiré fringes) will appear (c). The moiré fringes occur at the spatial difference frequencies between the pattern frequency and each spatial frequency component of the sample structure and can be coarse enough to observe through the microscope even if the original unknown pattern is unresolvable. Otherwise-unobservable sample information can be deduced from the fringes and computationally restored.

Mats G. L. Gustafsson, PNAS 102 13081 2005
Resolution extension by nonlinear structured illumination

(a) The region of frequency space that is observable by conventional microscopy
(b) An example of a sinusoidal illumination pattern.
(c) The illumination pattern has three frequency components: one at the origin (black), representing the average intensity, and two at $k1$, representing the modulation (dark gray). These are also the frequency components of the effective excitation under linear (i.e., nonsaturating) structured illumination. Under conditions of saturation, or other nonlinear effects, a theoretically infinite number of additional components appear in the effective excitation; the three lowest harmonics are shown here (light gray).
(d) Observable regions for conventional microscopy (black), linear structured illumination (dark gray), and nonlinear structured-illumination microscopy (light gray) based on those three lowest harmonics.
(e) Corresponding observable regions if the procedure is repeated with other pattern orientations.

M.G.L. Gustafsson, PNAS 102 13081 2005
Near-field scanning optical microscopy

Near-field scanning optical microscopy....
... conceived in 1928 - realized in 1984
Near-field optics relies on the production of sub-wavelength lightsources: fiber tips

Tip production: heat-and-pull technique

Angled deposition of Al coating

Electron micrographs of apertures

Optical micrograph of a NSOM tip
The strong confinement of light by near-field optics has enabled single molecule fluorescence excitation.
Logical consequence: Ultrasensitive confocal fluorescence micro-spectroscopy enables single molecule detection

**Single Molecule Detection provides**
- Fluorescence lifetime
- Triplet state lifetime
- Spectral shifts (jumps)
- Intensity fluctuations
- Orientation
- Diffusion
- Molecular distances (FRET)
- Conformational changes
- Reaction kinetics

**Single Molecule Detection is limited by:**
- no information on molecular identity
- photobleaching
- long-lived excited states limit # photons
- specific labeling necessary

Resolution: < 500 nm laterally
< 1 μm vertically
## Laser micro and nano surgery of biomaterials

### Tissue light interaction

- **Radiative**
  - **Tissue autofluorescence**
  - Fluorescence from various constituents of the tissue

- **Nonradiative**
  - **Photochemical**
    - Excited state reaction
    - Occurs even at low optical power density
    - sec-min exp
    - Low intensity
  - **Photoablation**
    - Direct breaking of cellular structure
    - Performed by high energy UV radiation
    - 10 - 100 ns pulses
  - **Photodisruption**
    - Shockwave generation at high pulse intensity
    - Fragmentation and cutting of the tissue by mechanical force of shockwave
  - **Plasma - induced ablation**
    - Induced by high intensity short pulse
    - Dielectric breakdown creates ionized plasma that interacts with light to produce ablation
    - 100 fs - 10 ns pulses
    - 10^7-10^6 Watt/cm^2
La réaction induisant l'ionisation d’un milieu exposé à une impulsion laser se divise en deux phases:

- l’absorption simultanée de plusieurs photons incidents provoque la libération d’un électron, ou électron quasi libre.

- l’ionisation en cascade. L’ionization d’impact produit deux électrons libres qui pourront être de nouveau accélérés par absorption de nouveaux photons. Le phénomène d’impact peut ainsi se reproduire en cascade pendant toute la durée de l’impulsion laser, provoquant une avalanche d’électrons libres qui forment le plasma.


Dans un milieu aqueux, l'intensité de seuil augmente de 3 ordres de grandeur lorsque la durée d'impulsion diminue de 10 ns à 100 fs. Cependant, l'énergie déposée n'est autre que la puissance multipliée par la durée d'impulsion et, par conséquent, il faut 100 fois moins d'énergie pour produire un plasma en utilisant des impulsions ultra-courtes. C'est donc autant d'énergie qui ne sera pas simultanément absorbée par le plasma.

La formation d'un plasma survient au-delà d'une énergie de seuil.


L'évolution spatiale d'un plasma en trois temps

t1 : Ionisation du milieu, formation du plasma
t2 : Expansion spatiale du plasma
t3 : Expansion maximale

Laser micro and nano surgery: cell biology applications

En biologie cellulaire et du développement, il y a deux manières d’appliquer la chirurgie au laser:

1. tire avantage des effets secondaires induits par l’expansion d’un plasma sur une échelle de quelques micromètres afin de détruire une cellule ou un groupe de cellules dans un organisme vivant en développement. Cette ablation est déjà possible en utilisant des impulsions longues de l’ordre de quelques nanosecondes et on parle alors encore de microchirurgie.

2. nanochirurgie s’adresse plus particulièrement à l’étude d’éléments subcellulaires. La destruction ciblée de structures subcellulaires ou d’organelles est ainsi possible sans affecter les parties superficielles (membranes) seulement si le procédé ablatif est parfaitement contrôlé, c’est-à-dire sans perturbation mécanique ou thermale.

Nous considérons **trois modes d'utilisation de la nanochirurgie** en biologie cellulaire, en **fonction de la cible à étudier** :

**L’ ablation** a première est la simple miniaturisation de la chirurgie classique qui consiste à éliminer une partie de la cellule tout en préservant l’imperméabilité de sa membrane et d’observer, comme en biologie du développement, les mécanismes par lesquels la cellule réagit localement ou globalement. Ainsi, il est possible de détruire spécifiquement des mitochondries, un centriole ou une neurite.

**La dissection** permet d’avoir accès à des structures cellulaires normalement masquées. On peut ainsi accéder à la membrane plasmique des cellules de plantes pour effectuer des mesures par *patch-clamp* ou y insérer des bactéries. La dissection à l’échelle intracellulaire permet également de disséquer des sous régions chromosomiques.

**L’induction** concerne plus spécifiquement l’étude de phénomènes dynamiques. L’utilisation du laser permet d’induire des modifications structurales et de changer l’équilibre dynamique de façon ciblée et non invasive, permettant l’observation du comportement hors équilibre ou du retour vers l’équilibre. Cela concerne les différents *éléments du cytosquelette*, les flux cytoplasmiques, la motilité cellulaire et la morphogenèse.

Example: Nanosurgery Cytoskeleton in Interphase Cells

A. cellule Ptk-2 transféctée de façon stable avec une construction α-tubuline-YFP. Les microtubules peuvent être sectionnés par un laser UV à impulsions (470 ps). L’irradiation à basse énergie (minimum 50 nJ) le long d’une ligne (pointillés bleus) forme un front de catastrophes artificielles, créant ainsi de nouvelles pointes plus (+) et moins (-) des microtubules présents dans le volume de dissection. Chaque microtubule réagit de manière différente.

La dépolymérisation (flèches rouges en B, C, D) de la pointe (+) est le phénomène le plus fréquent après la catastrophe et le sauvetage alors que la pointe (-) reste relativement stable.

What is an optical tweezers?

A single-beam gradient force trap obtained by tightly focusing a cw laser beam through a high NA objective - 3D trap

\[ F = Q \frac{n_m W}{c} \]

- **F** – trapping force
- **Q** – dimensionless efficiency coefficient
- **W** – power of the laser beam
- **n_m** – refractive index of the medium
- **c** – light speed

Founder:
Arthur Ashkin,
Bell Labs, USA

www.bell-labs.com/user/feature/archives/ashkin/
A. Ashkin, *et al* *Optics Letters* 11 288 1986
Sir Isaac Newton:
“For every action force, there is a corresponding reaction force which is equal in magnitude and opposite in direction”.

Ray optics explanation of optical trapping
Trapping with Gaussian beams

2D trapping with single Gaussian beam

3D trapping with counter propagating beams

Low Numerical aperture (NA < 0.8)
Long Working Distance (WD > 1 mm)
Limited resolution: \( r = 1.2\lambda/NA > 1.5\lambda \)

Particle size: \( d > \lambda \)
High index: \( n = n_p/n_m > 1 \)

3D trapping with single Gaussian beam

High NA (NA > 1)
Short WD (WD < 1 mm)
High resolution: \( r < \lambda \)

A. Ashkin et al, PRL 24 156 1970

Preparatory School to the ICTP Winter College Micro and Nano Photonics for Life Sciences,
Trieste, 2008 February 4-8
Types of particles:

- **Material:**
  Dielectric (polystyrene, silica); Metallic (gold, silver, copper), Biological (cells, macro-molecules, intracellular structures, DNA filaments), Low index (ultrasound agent contrast)
- **Size:** 5 nm – 20 μm
- **Shape:** spherical, cylindrical, arbitrary

Types of laser beams:

- **Gaussian**
- **Laguerre-Gaussian**
  LG carries also orbital angular momentum that can be transferred to the trapped particles

x-y intensity profile  
z axis propagation non diffracted beam

**Characteristics of optical traps**
### Characteristics of optical traps

Micrometer sized glass or polystyrene beads are commonly used as attachment handles of the materials under investigation.

The advantage of this approach is the clear and uniform interaction between the beads and the laser beam.

- Typical stiffness: 100 pN/micrometer
- Typical displacements: 1-500 nm
- Typical forces: 0.1-100 pN
- Measurable speeds: ~1 kHz
## Characteristics of optical traps

Comparison of forces with other techniques and biological processes:

<table>
<thead>
<tr>
<th>Technique</th>
<th>Force Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical traps</td>
<td>0.1 - 100 pN</td>
</tr>
<tr>
<td>Electric fields (electrophoresis)</td>
<td>0 - 1 pN</td>
</tr>
<tr>
<td>AFM</td>
<td>10 - 10000 pN</td>
</tr>
<tr>
<td>Kinesin step</td>
<td>3-5 pN</td>
</tr>
<tr>
<td>RNA polymerase stalling</td>
<td>15-30 pN</td>
</tr>
<tr>
<td>Virus motor stalling</td>
<td>~50 pN</td>
</tr>
<tr>
<td>DNA conformational change</td>
<td>~65 pN</td>
</tr>
<tr>
<td>Biotin-streptavidin binding</td>
<td>300-400 pN</td>
</tr>
</tbody>
</table>

Courtesy Prof. D. Petrov, ICFO, Barcelona, Spain
http://users.icfo.es/Dmitri.Petrov/Teaching/lectures.htm
The physics behind optical tweezers

Radiation pressure is the force per unit area on an object due to change in light momentum.

The light momentum of a single photon is: \(|\vec{p}| = \frac{h}{\lambda}\)

The change in momentum can be calculated by the difference in momentum flux between entering and leaving a object:

\[ \vec{F} = \left( \frac{n}{c} \right) \iint (\vec{S}_{in} - \vec{S}_{out}) \, dA \]
Some physics behind optical tweezers

A 1 mW laser beam reflecting from a mirror gives a pressure of:

$$F = 2 \left( \frac{n}{c} \right) W = 10^{-11} \text{ N}$$

The weight of a 10 um water drop is $5 \times 10^{-12} \text{ kG}$.

Gravity pulls the drop with $5 \times 10^{-11} \text{ N}$.

Hence, the gravity force and the light force are comparable.
The weight of a 10 μm water drop is $5 \times 10^{-13} \text{ kg}$.

Imagine being able to pick up and move a single cell without physically touching it. Moreover to measure mechanical properties of single cells, or making its spectroscopical studies. Such a technique could give enormous advantage for better understanding of behavior of living cells, micromachines, microfluides, colloid physics.
OPTICAL TRAP

- SELECT AND MOVE A GIVEN CELL
- REMOTELY APPLY CONTROLLED FORCES ON LIVING CELLS, INTERNAL PARTS OF CELLS, AND LARGE BIOLOGICAL MOLECULES WITHOUT OPTICAL DAMAGE
- MEASURE MECHANICAL PROPERTIES (FOR INSTANCE, ELASTICITY) OF DIFFERENT PARTS OF CELLS
- MEASURE THE FORCES GENERATED BY SINGLE MOTOR MOLECULES IN THE PICONEWTON RANGE
- USING OPTICAL SPECTROSCOPY TO MEASURE TEMPERATURE, DNA STRUCTURE, CELL VIABILITY, INTRACELLULAR pH OF A GIVEN SINGLE CELL
Pro’s and Con’s optical traps for Biophysics

Pro’s:
• Measurable forces and distances are well suited for enzyme dynamics and molecular motors
• They work in normal buffer conditions

Con’s:
Radiation damages of samples

See the lectures (5) on Optical Tweezers given by K. Dholakia, E. Di Fabrizio and D. Cojoc next week at Winter college

On line (free lectures)
http://users.icfo.es/Dmitri.Petrov/Teaching/lectures.htm
Further reading

Web sites (examples)
http://www.cbst.ucdavis.edu/
http://vlib.org/Science/Cell_Biology/index.shtml
http://omlc.bme.ogi.edu/classroom/ece532/
http://www.emc.maricopa.edu/faculty/farabee/BIOBK/BioBookTOC.html

Books

Journals (examples)
Biophotonics International (free)
Journal of Biomedical Optics
Single molecules
Acknowledgements

• Prof. J. Niemjela from ICTP for inviting me to give this lecture
• Prof. T. Huser from NSF CBST, University of California, Davis, and Prof. D. Petrov from ICFO Barcelona, for permission to use their lectures/slides
• To all the people not mentioned here, whose material about Biophotonics I could find and use
• Special thanks to Prof. G. Scoles from University of Princeton and Elettra/SISSA – Trieste, who ‘pushed’ and encouraged me constantly toward this marvelous field of science
I hope that you agree with David Hilbert:

“We should know and we will know”

(with Biophotonics in mind 😊)

Thank You for Your Patience!