TAMPEREEN TEKNILLINEN YLIOPISTO

RIITTA MIETTINEN

MICROSCOPY, WHAT WE CAN AND WISH TO IMAGE

Master's of Science thesis

Examiner: Professor Olli Yli-Harja

Examiner and topic approved by
Faculty of Natural Sciences
9th of May, 2012

TIIVISTELMÄ

RIITTA MIETTINEN: Mikroskopia, mitä voimme ja mitä toivomme kuvantaa
TAMPEREEN TEKNILLINEN YLIOPISTO
Diplomityö, 50 sivua
Helmikuu 2016
Biotekniikan diplomi-insinöörin tutkinto-ohjelma
Pääaine: Laskennallinen systeemibiologia
Tarkastaja: Prof. Olli Yli-Harja
Avainsanat: valomikroskopia, fluoresenssimikroskopia, elektronimikroskopia, super-resoluutiomikroskopia, biologiset näytteet

Mikroskopia on aina ollut tärkeä työkalu, jonka avulla olemme lisänneet tietämystämme eliöiden toiminnallisten ilmiöiden taustalle olevista rakenteellisista osasista. Rakenteen ja toiminnan yhteyksien selvittäminen on olennaisen tärkeää, jotta voimme ymmärtää normaaleja ja patofysiologisia ilmiöitä. Toteamus: ”Rakenne ilman toimintaa on kuollut ja toiminta ilman rakennetta on haamu”, summaa tämän yhteyden tärkeyden. Tässä diplomityössä kuvataan aluksi pääperiaatteet nykypäivänä biotieteissä käytettävistä mikroskoopeista. Tämän jälkeen pohditaan mitä vaatimuksia mikroskopialle nykyään asetetaan ja mitkä saattaisivat olla tulevaisuuden odotukset. Lopullisena tavoitteena on löytää ideoita uuden mikroskooppisen kuvantamissysteemin kehittämiselle.
Ensimmäisen mikroskoopin käyttöönotosta on kulunut jo yli 400 vuotta. Kuitenkin merkittävimmät innovaatiot ja kehitysaskeleet tehtiin vasta viimeisten 50-60 vuoden aikana. Kohokohtia ovat olleet uudenlaisten fluoresenssimolekyylien kehittäminen ja innovatiivisten signaalin- ja tietojenkäsittelymenetelmien hyödyntäminen, joiden avulla valomikroskopiassa päästiin murtamaan diffraktiokynnys so. 200 nm. Superresoluutiomikroskopian avulla rakenteiden, kompleksien ja yksittäisten proteiinien tunnistaminen fluoresoivien merkkiaineiden avulla voidaan nyt toteuttaa n. 10 nm resoluutiolla. Se on tehokas instrumentti niin paikalliseen kuin ajalliseen kuvantamiseen elävissä soluissa. Rakenteiden kuvantaminen näillä tekniikoilla kuitenkin perustuu rakenteita leimaavien fluoresoivien aineiden käyttöön eikä sinällään rakenteen itsensä kuvaamiseen. Tätä voidaan pitää merkittävimpänä rajoitteena näissä tekniikoissa. Sen sijaan läpivalaisuelektronimikroskopian avulla saadaan superresoluutionkuvia (jopa 0.14 nm) rakenteesta ja sen osasista suoraan. Valitettavasti dynaamisia prosesseja elävissä soluissa on läpivalaisuelektronimikroskoopilla edelleen käytännössä mahdoton kuvantaa. Toisin sanoen valo- ja elektronimikroskopian välillä on edelleen suuri aukko. Tässä työssä tullaan ehdottamaan, että uusien mikroskooppisten systeemien kehittämistä voitaisiin suunnitella perustuvaksi toisen ja kolmannen harmonisen generoinnin pohjalle yhdistettynä linssivapaaseen tomografiseen kuvantamiseen ja/tai 4D ultranopean elektronimikroskopiaan.

ABSTRACT

MIETTINEN, RIITTA: Microscopy, what we can and wish to image
TAMPERE UNIVERSITY OF TECHNOLOGY
Master of Science thesis, 50 pages
February 2016
Master's degree program in Bioengineering
Major subject: Computational system biology
Examiner: Prof. Olli Yli-Harja
Key words: light microscopy, fluorescence microscopy, electron microscopy, super-resolution microscopy, biological specimens

Microcopy has always been an important tool to increase our knowledge about structural elements that are responsible for the functional outcome of living organisms. Unraveling the relationship between the structure and function is of crucial importance for our understanding e.g. normal and pathophysiological events. "Structure without function is dead and a function without a structure is a ghost", summarizes the importance of this relationship. In this thesis, the main principles of the microscopes used nowadays in life sciences are described followed by a discussion about the needs met by now and those that can be predicted to be also major issues in the future. The final aim is to find ideas to develop a new microscopic imaging system.
It is already 400 years since the first microscope was used. However, the major innovations and development in microscopy were done during the last 50-60 years. Highlights can be set on novel fluorescent probes and innovative methods utilizing signal processing and computation with which light microscopy has been pushed over the diffraction limit of light i.e. 200 nm. Super resolution microscopy provides detections of structures, complexes and individual proteins by fluorescent probes with a resolution around 10 nm. It is a powerful tool for both spatial and temporal imaging of living organisms. However, the major limitation is that laser beam techniques require structures to be labeled with fluorescence probes and, thus, visualizes the probe and not the structural element itself. Transmission electron microscopy, on the other hand provides super resolution (up to 0.14 nm) images about the structure and its components directly. Unfortunately, it is still practically impossible to image temporal events in living cells with electron microscope. Thus, we still have a big gap between light and electron microscopy. It is suggested in the current thesis that the microscopic systems that are based on second or third harmonic generation combined with lens free tomographic imaging and/or 4D ultrafast electron microscopy could be building blocks for a new type of microscope.

Preface

In spring 2012, when I was finalizing this thesis, an infarct attacked my brain. It took until autumn 2015, before I came back to this text. Now, everything takes hundreds of times more effort, and I have difficulties to remember what I was just doing. Anyhow, as this thesis was almost ready, I am still determined to finish and submit it. Since I do not have as much energy or capacity as earlier, I have to apologize for not referred the possible most recent publication (i.e. published in/after 2012) in the field of microcopy instrumentation. However, I do believe that this thesis will fulfill the criteria set for the Master's thesis at Tampere University of Technology.

This work is based on my ever-increasing interest towards microscopy and its usage in different applications for over 20 years in a histology laboratory. Modern histology requires knowledge not only about sample processing and staining methods, but also understanding of variety of imaging tools and digital image processing. I have been fortunate to have the possibility to broaden my knowledge in these fields at Tampere University of Technology.

Although I have used different microscopic systems including light, fluorescence, confocal and electron microscopy, I was immediately faced by the fact that microscopy is nowadays an exponentially spanning research and technology area. Labeling techniques, instruments, analytical tools, data acquisition and computation has tremendously developed and is continuously developing towards more sophisticated systems and application possibilities. This makes the review writing about microscopy extremely challenging. Therefore, it was absolutely necessary to limit the topic to microscopic systems used in life sciences and to those that have and would have potential for widespread routine use.

Microscopy should be seen as an own branch of science on which more effort, investments, resources and training should be instantly put. This will increase competence and facilitate new business activities in any technically well developed society.

I express my deepest gratitude to my supervisor Professor Olli Yli-Harja for introducing me the Computational system biology field as well as finally suggesting me to write Master thesis about microscopy. I owe my kindest thanks to my dear friend, Dr. Anett Riedel, at Liebniz Institute for Neurobiology in Magdeburg, Germany for continuous encouragement and fruitful suggestions on this thesis work. We both share the common interest in science and have faced the challenges in especially related to anatomical work for which microscopes are essential instruments.

Finally, with deep and sincere love I dedicate this work to my spouse, Arto and to our wonderful children, Hilda-Maria and Jaakko-Hermanni. I am indebted to them for their understanding, patience and tolerance to all those matters that my studies at Tampere have required during several years.

February 9, 2016  Riitta Miettinen, ramietti@gmail.com

List of figures

Abbreviations

AFM	atomic force microscope
Ar	argon
Ar-Kr	argon-krypton
CCD	charge-coupled device
CT	computer tomography
DIC	differential interference contrast
EDAX	EDX analyzer
EDX	energy dispersive X-ray detector
EELS	electron energy loss spectrometer
ESEM	environmental scanning electron microscope
ESR	electron spin resonance spectroscopy
FCS	fluorescence cross-correlation spectroscopy
FEG	field emission gun
FIB	focused ion beam
FP	focal plane
FRET	fluorescence resonance energy transfer
GFP	green fluorescence protein
GSD	ground-state depletion
He-Cd	helium-cadmium
He-Ne	helium-neon
LaB6	lanthanum hexaboride
Laser	light amplification by the stimulated emission of radiation
MRI	magnetic resonance imaging
NA	numeric aperture
NMR	nuclear magnetic resonance spectroscopy
NSOM	near-field scanning optical microscope
PACS	picture archiving and communication system
PALM/FPALM	photo-activated localization microscope/fluorescence PALM
PET	positron emission tomography
PSF	point spread function
SEM	scanning electron microscope
SHG	second harmonic generation
SPEM	saturated pattern excitation microscopy
SSIM	saturated structured-illumination microscopy
STED	stimulated emission depletion
STEM	scanning transmission electron microscope
STORM	stochastic optical reconstruction microscopy
TEM	transmission electron microscope
THG	third harmonic generation
US	ultrasound

I. Introduction

The word “microscope” originates from Greek words “micros” (small) and “skopeo” (look at). The Dutch spectacle-maker Hans Jansen and his son Zacharias are considered to be the inventors of the first microscope in 1595. However, since they are not known to have any publications about microscopic observations the merit has publicly owned by later investigators, Robert Hook and Antony van Leeuwenhoek. Hook was the one who published the first book about the microscopy in 1665. Leeuwenhoek (1632-1723) developed one of the earliest instrument followed by several simple microscopes each containing a single convex lens that could resolve details as small as 1 μm. (Rosenthal, 2009). It is interesting that for several hundred years, the light microscope, though significantly improving, was the major imaging tool to investigate biological samples. It took until 1930s, before a different type of microscope was developed and, thereby, a new magnification range was achieved in microscopy.

In 1931, Knott and Ruska invented the principle of electron microscopy implementing electrons (with wavelengths about 100.000 -fold smaller than light) as illumination source. The next revolutionary invention was done in the field of light microscopy in the 1980s, when the first laser scanning microscopes were constructed. Around the same time (1981), the development of scanning tunneling microscope by Binning and Rohrer provided the next steps for further resolution increase, i.e. sample structures at atomic level. And even though, Röntgen already in 1895 discovered the principle of imaging X-rays, it took until the 1990s that X-rays – beside their routine use in medicine – were utilized as an illumination source for microscopy (Yamamoto and Shinohara, 2002; Neu et al. 2010). The major milestones in microscopy history are summarized in Table 1.

This short historical review of the major steps in microscopy illustrates the driving force to develop new microscopes: people have always wanted to study smaller and smaller things and events. However, how far can we go in terms of resolution depends on a couple of basic physical facts.

Table 1. *Major milestones in microscopy.*

Invention of the microscope Hans and Zacharias Jansen	1595	
Diffraction limit theory Ernst Abbe	1873	
Fluorescence microscope Oskar Heimstädt	1911	
Epifluorescence microscope Philipp Ellinger and August Hirt	1929	
	1931	Transmission electron microscope Ernst Ruska and Max Knoll
Phase contrast microscopy Frits Zernike	1932	
	1938	Scanning electron microscope Manfred von Ardenne
Polarization microscopy W.J. Schmidt	1939	
	1951	X-ray spectroscopy Raymond Castaing
Differential interference contrast George Nomarski	1955	
Concept of confocal microscopy Marvin Minsky	1961	
Applications using Fluorescence recovery after photobleaching (FRAP) and Förster resonance energy transfer (FRET)	1976	
Total internal reflection fluorescence microscopy (TIRF) Daniel Axelrod	1981	
	1981	Scanning tunneling microscope Gerd Binning and Heinrich Rohrer
Realization of confocal microscopy John White et al. in UK Gerrit van Meer et al. in Germany	1987	
Two-photon microscopy Winfriend Denk and Watt W. Webb (Concept described by Maria Goeppert-Mayer, 1931)	1990	
Two-photon 4Pi microscope Stefan Hell and Ernst Stelzer	1991	
Breaking the diffraction limit: Stimulated emission depletion (STED) Stefan Hell	2000	
Breaking the diffraction limit: Photoactivated localization microscopy (PALM) Eric Betzig and Harald Hess Stochastic optical reconstruction microscopy (STROM) Xiaowei Zhuang	2006	

Already in 1873, the German pioneer in optics, Ernst Abbe, noticed that focusing of light always resulted in a blurred or diffracted spot. The size of this spot places a fundamental limit to the resolution i.e. the minimal distance for two spots to be separated as individual features (Slayter and Slayter, 1992). Mathematically, Abbe's finding is as follows. In the focal plane (XY dimension, equation 1), the resolution for microscope is determined by $d_{xy} = \frac{\lambda}{2 \cdot n \cdot sin\alpha}$ *(1)*

and in z-axis (2),

$$d_z = \frac{2 \cdot \lambda}{n \cdot sin^2 \alpha} \qquad (2)$$

where λ is the wavelength of the light and nsinα is numeric aperture (NA) of the lens (Slayter and Slayter, 1992).
In three-dimensional space, the spot is commonly represented by the point spread function (PSF). Due to diffraction and PSP a round profile is seen as an airy disk pattern in XY-plane and as an elongated, ellipsoid structure in z-dimension. (Slayter and Slayter, 1992; Fernandez-Suarez and Ting, 2008; Hell 2009.) Thus, a point represented as a single pixel in an ideal image, is seen to be something other than a single pixel in the image gained from this point. Mathematically, image formation can be described by a convolution equation (3)

$$g = h * f \qquad (3)$$

where h is PSF and f is the source of light, the object.

We can see from the equations (1 and 2) that resolution is highly dependent on the NA of the lens. The aperture angle (sinα) can be 90° at most to give maximum sinα value 1.0; however, the technical limit is 0.7. Choosing a high refractive index medium between the sample and the lens can further increase NA. (Slayter and Slayter, 1992.) Most of the oil-immersion lenses have NA around 1.4. The resolution can be further improved by selecting the minimum wavelength possible. However, for biological samples this is undesirable because low wavelength light has high energy and, thus, entails a greater risk for cell damage. Thus, in practice, the resolution limit still remains around 200 nm laterally and 500 nm axially or approximately the full width at half-maximum of the PSF even with confocal and multiphoton fluorescence microscopes. Therefore, other types of approaches have been implemented. Those include e.g. 1) using two opposing lenses to image the sample from two directions (I5M and 4Pi), 2) manipulating the emitted signal (stimulated emission depletion, STED), 3) stochastically switching the fluorescing molecules on and off followed by reconstruction made from multiple excitation cycles (stochastic optical reconstruction microscopy, STORM). With these new systems that require usage of fluorescing molecules, resolution has been pushed even down to less than 10 nm. (Hell, 2003; Fernandez-Suarez and Ting, 2008; de Souza, 2009; Schmolze et al. 2011, Aquino et al. 2011.) Thus, the diffraction barrier has been overcome by these techniques.
As introduced above, electrons can be used as an illumination source because they actually behave like light. When they are accelerated in vacuum, they travel in straight lines and the electron paths can be manipulated by electrical and magnetic fields in a similar manner as glass lenses influence light beam in optical microscopes. Most importantly the wavelength is remarkably smaller (e.g. 0.0037 nm at an acceleration

voltage of 100 kV) than that of light and enables to achieve resolving power of even 0.05 nm. (Slayter and Slayter, 1992.) However, like light microscopes also electron microscopes have limitations which leave a big gap between structural and - nowadays more important -, functional, analyses. In other words, we still need to find ways to bridge electron and current light microscopy.

This thesis will describe the main principles of the microscopes used nowadays. In order to make the thesis compact and focused the work concentrates on the microscopes that can be implemented in life sciences. The selection is based on the fact that biological and material science samples differ in respect of 1) the question settled for the analyses, 2) the sensitivity of the samples to the exposing light or electron beam and 3) the way in which the samples are prepared for investigation. Nevertheless, many of the microscopes described here are suitable also for material sciences. However, the reader should be aware that they may not be the first choice of investigating materials.

The types of microscopes and their general relations presented in the current thesis are shown in the Figure 1. The description of these systems is followed by a discussion about the needs met by now and those that can be predicted to be also major issues in the future microscopy. The microscopic systems that sufficiently fulfill these requirements are then studied in more details. Finally, some technical solutions that can serve as a primer for the development of an improved microscopic system are presented at the end of the thesis work.

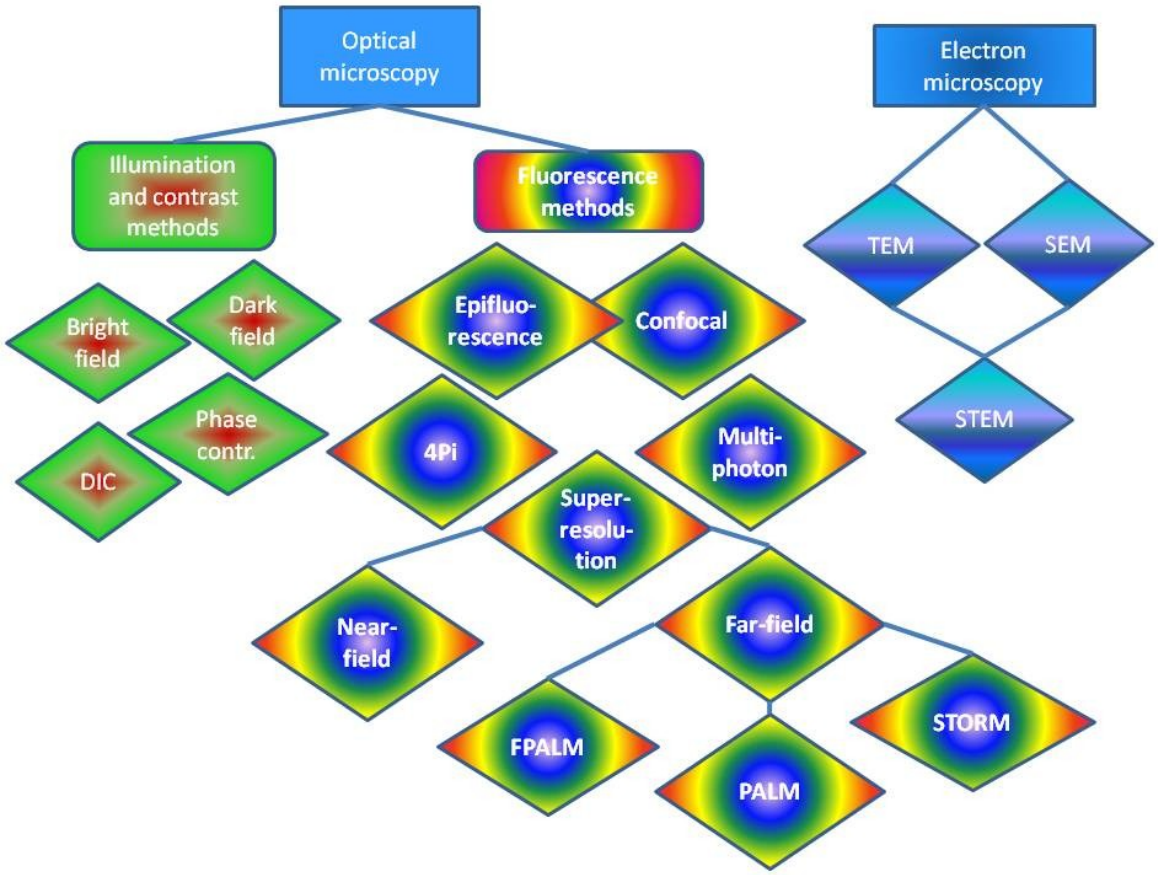

Figure 1. *The types and relations of the microscopes presented in the current thesis. It should be noted that the microscopic systems can be classified into subgroups in many different ways. This figure illustrates the ideology how the classification is done in the current thesis work.*

II. Today's microscopes

1. Conventional light microscopy: Illumination and contrast methods

The light microscope is an optical microscope that uses visible light to detect small objects in a specimen. The basic parts of it are the light source (usually a tungsten filament light bulb), field and aperture diaphragms to eliminate scattering of light, a condenser lens to focus light onto the specimen, a stage to hold and move the specimen at right angel to the microscopic axis, knobs for moving the specimen in horizontal plane, knobs for changing the distance (i.e. focusing) between the objective and the specimen, objectives to magnify the object and oculars for viewing and further magnifying the sample (Slayter and Slayter, 1992), (Fig 2).
The image formation in conventional light microscopy is based on different illumination and contrast induced by microscope settings and/or accessories in the light path. Nowadays, there are basically four different types of illumination/contrast methods that are utilized in conventional light microscopes: 1) brightfield, 2) darkfield, 3) phase contrast and 4) differential interference contrast microscopy.

1.1. Brightfield

The standard light microscope is a classical brightfield microscope (Fig. 2A). Discrimination of structures in the light path is based on the fact that distinct pigments or stains in the sample absorb light differentially and that the eye perceives the color complementary to the absorbed one. Resolution and contrast are gained by objective and ocular lenses, but also by properly setting a condenser lens into the light path to maximize light beam intensity within the region under investigation (so called Koehler illumination). (Slayter and Slayter, 1992.) The conventional brightfield microscope is most suitable for viewing experimentally stained or naturally occurring color pigments in the specimens of interest. If the specimen contains natural pigments it can be viewed also when it is alive. However, if the sample has to be stained for investigation, it usually needs to be fixed and treated with different chemicals. This results in a loss of viability of the structures. To overcome this problem, specimens can be viewed under the darkfield, phase contrast or interference contrast.

Figure 2. *Photographs illustrating different parts and accessories of optical microscopes. A) A light microscope having a camera lucida drawing tube (f) to facilitate drawing of structures seen in the microscope field of view. B) A light microscope having also fluorescence attachment (j) and a recorder for microscope stage position (l). C) A fully computer controlled light microscope. Abbreviations: a, field diaphragm; b, condenser with aperture diaphragm; c; coarse and fine stage movement knobs; d, sample stage; e, objectives in revolving nosepiece; f, camera lucida drawing tube; g, oculars; h, coarse and fine focus adjustment knobs; i, light bulb; j, mercury lamp; k, power supply for mercury lamp; l, stage coordinator recorder; m, ocular to aid view focusing for camera; n, film camera; o, joystick to move stage in xy-coordinates; p, focusing knob; q, computer controlled motorized stage; r, Lucivid containing microdisplay to view computer display via oculars; s, CCD camera. Different parts of the microscope are indicated in those photographs where they are best visible.*

1.2. Darkfield

In the darkfield setup, an annular aperture (opaque disk) is placed under the condenser lens of the microscope (Fig. 3). This blocks the direct light to the specimen, but lets the light from the edges of the condenser lens pass through. As the light passes the specimen in an angle, the central parts that are in the viewing field will scatter. This scattered/reflected light from different components of the sample is perceived as a bright, white color against a dark almost black background. (Slayter and Slayter, 1992.) Darkfield is useful for low contrast or unstained specimens, dust, liquid specimens (cell and bacteria in a culture) and e.g. to enhance the contrast of small thin fibers that have been stained. The advantage of dark field is that it shows the structures through the whole specimen (i.e. the entire z-dimension) equally bright – whether they are in focus or not (Fig. 4). It is even better than classical epifluorescence microscope in this respect (own experience). Furthermore, as the structures can be illuminated without a stain, living samples can be studied. In fact, viability and motility of bacteria, parasites or spermatozoon are well appreciated with darkfield setting.

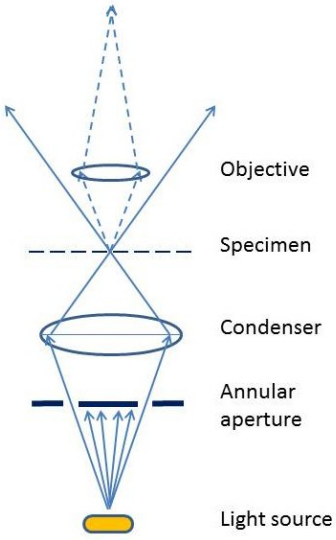

Figure 3. *Image formation in the darkfield microscope (see text for details).*

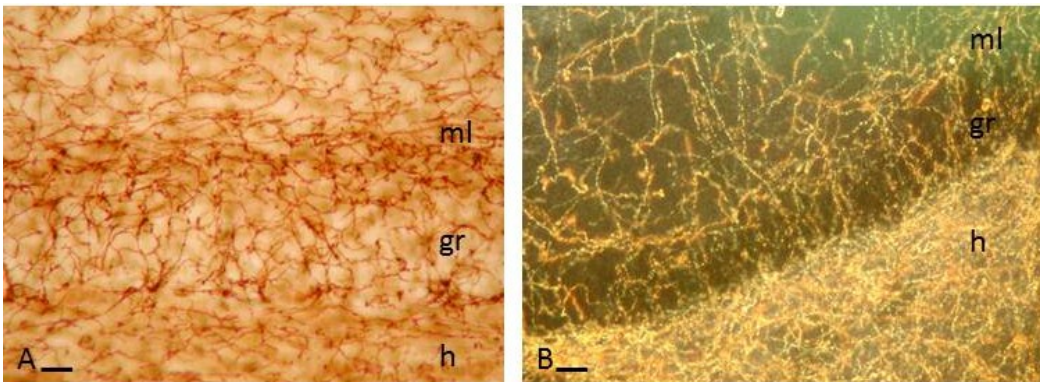

Figure 4. *The difference in the brightfield (A) and darkfield (B) microscopic images. Acetyl cholinesterase histochemistry shows an intensive network of fibers in 50 µm thick*

hippocampal sections. Notice that with the darkfield illumination more fibers in the hilus (h) and fibers in the deeper parts of the section (especially appreciable in the molecular layer (ml) and granule cell layer (gr)) are visible, while in the brightfield illumination only the fibers that are in the focal plane can be clearly seen. Scales, 10 µm

1.3. Phase contrast

The other possibility to study unstained, living cells is phase contrast microscopy. The principle is based on the fact that distinct organelles have different refractive indices. Since the structures bend light differentially, they can be distinguished from each other. A structure having a high refractive index bends light more than that having low refractive index. The refracted/diffracted light waves are retarded in about a quarter of wavelength while the undeviated light passing directly through is not affected. Due to the retardation of light, some waves are out-of-phase compared to others i.e. are lagging behind. However, the λ/4 shift is not visible. In order to make the difference visible to human eye, a phase plate is set into the optical path. (Slayter and Slayter, 1992.) By this method, the direct light is "speeded up" by a quarter of wavelength so that the direct and diffracted light has half a wavelength difference at the eyepiece level (Zernike's method, Zernike, 1955). The destructive interference results in darker details seen against light background in the specimen. If the phase plate is constructed in a way that it slows down the direct light, the details in the sample are bright and the background is dark, respectively. (Slayter and Slayter, 1992.)

1.4. Differential interference contrast

Phase contrast microscopy requires thin specimens. Furthermore, it suffers from halo artifacts. These limitations are overcome by differential interference contrast (DIC) microscopy. This system produces relief-like images of unstained cells and tissues (Fig. 5). This is accomplished by locating two Wollaston prisms in the light path. In a typical setup, a polarizer is placed underneath the condenser and an analyzer (also a polarizer) above the objective. A modified Wollaston or Nomarski prism is placed in the condenser turret assembly (different for distinct objectives to accommodate the focal length and aperture size of them). One prism is located in the nosepiece. (Allen et al, 1969, Lasslett, 2006.)

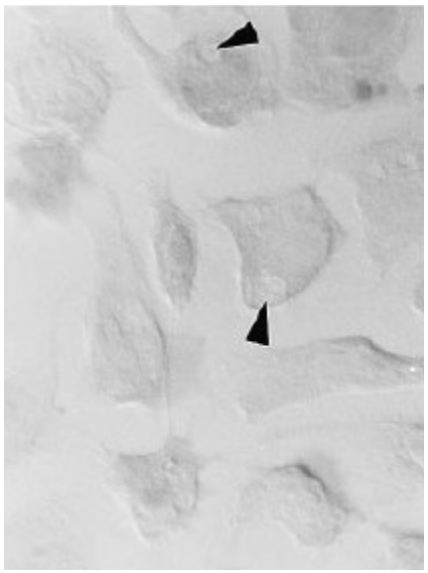

Figure 5. *CasKi cells photographed using DIC setup. Note that the cells become visible even though they are unstained. The large vacuoles inside the cytoplasm can be clearly distinguished (arrowheads) (from the study published by Lappalainen and Miettinen et al. 1997).*

In DIC microscope, the polarized light enters to the Wollaston prism that separates it into two rays of polarized light (ordinary and extraordinary rays) that are perpendicular to each other. The condenser focuses the paths to the specimen. The paths are separated by a very small difference, about 200 nm from each other. Due to the difference of the refractive index of different components of the sample, the optical path length of light passing the neighboring points will become different i.e. the phase will change. After travelling through the objective, the light rays enter the second Wollaston prism where they are combined into one polarized ray. Finally, the light enters the analyzer that permits elliptically polarized light to form the image, but cuts out all the remaining light. This combination leads to interference, which is seen either darkening or lightening depending on the optical path difference. (Slayter and Slayter, 1992; Lasslett, 2006.)

1.5. Image capturing

Parallel to oculars, the microscope can have a CCD camera to capture the microscopic image that is then transferred to the computer monitor (Fig. 2C). By that, simple viewing and archiving as well as quantitative analyses and measurements of the specimen are possible. Images can be collected also on a film camera (Fig. 2B), though this is nowadays less frequent approach. Earlier, when camera systems applied to microscope were less common or even nonexisting, documentation of the microscopic view was done by drawing the structures on paper via camera lucida drawing tube (Fig. 2A) (see e.g. drawings made by the father of modern neuroanatomy, Santiago Ramon y Cajal (DeFelipe and Jones, 1988). Nowadays, drawing can be implemented by the aid of more sofisticated computer-connected drawing tubes (Fig. 2C) or by using image

processing softwares that enables automatic detection and tracing structures of interest in the specimen (see e.g. FilamentTracer by Bitplane, http://www.bitplane.com/go/products/filamenttracer).

2. Fluorescence microscopy

As stated in the introduction one way to improve the resolution in light microscopy is to use shorter wavelength light. This was behind the idea of e.g. August Köhler (1904) and Oskar Heimstädt (1911) to construct a microscope that utilizes ultraviolet light. However, it took decades to realize the possibilities of fluorescence microscopy. The most critical steps have been the development of the technique of secondary fluorescence by using exogenously fluorescent chemicals and their binding properties with different structural elements, and later the development of immunofluorescence technique for labeling antibodies. The later invention made by Albert Coons in early 1940s is a cornerstone for modern cell biology. (Rusk, 2009; van Ooji, 2009.) With the development of new super resolution microscopes it has revolutionized research in life sciences during last two decades.

2.1. Excitation and emission, interaction of light and fluorochromes

Fluorescence microscopy utilizes 1) the ability of specific dyes (fluorochromes) to bind and release energy (photoluminiscence), and 2) the fact that the released energy is detectable and, thus, exploitable as a signal. When an electron of an atom absorbs energy, it is elevated into a higher energy state. When this energy is released, a lower energy photon is emitted while the electron returns to its original ground state. (Hauglund et al. 2002; Spring 2003; Lichtman and Conchello, 2005.) Electron excitation can be produced e.g. by a laser (see chapter 2.3.2) or arc-discharge lamps.
The relationship between the energy of the electromagnetic wave and the wavelength (Fig. 6) is expressed by an equation (4)

$$E = hv = \frac{hc}{\lambda} \qquad (4)$$

where E is the energy, h is Planck's constant, v is velocity, c is the velocity and λ the wavelength of light. From this equation it is appreciable that the shorter the wavelength of the electromagnetic wave is (such as ultraviolet light, X-rays and gamma waves), the higher energy it has.

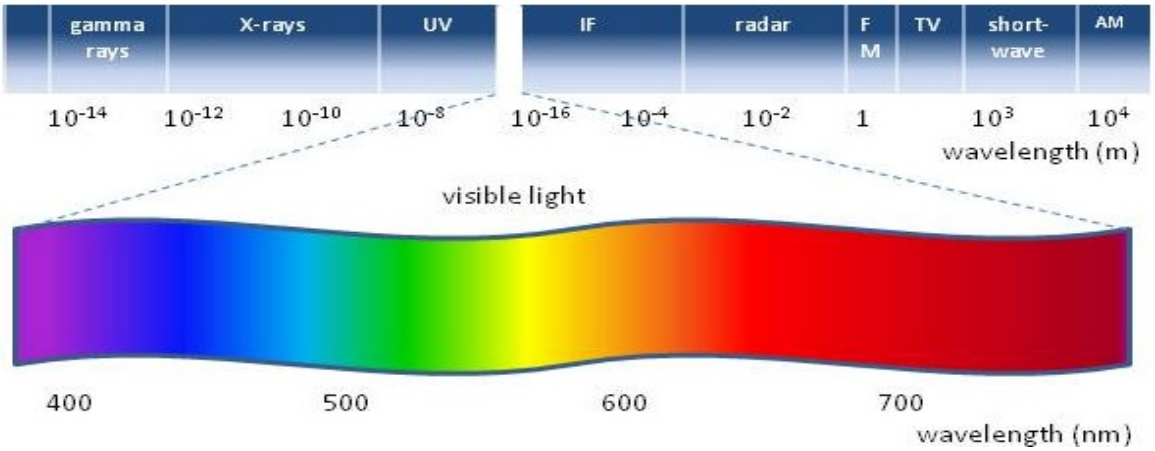

Figure 6. *The spectrum of visible light in relation to other electromagnetic wavelengths. Modified from http://www.yorku.ca/eye/spectru.htm, 28.11.2011*

In fluorescence microcopy, this means that when a fluorochrome is excited by e.g. green light (around 520 nm) the emitted light (that has lower energy) is red (around 700 nm). Each fluorochrome has its own characteristic excitation and emission spectra with characteristic maxima and band widths, respectively (Fig. 7). The difference between the positions of the excitation and emission maxima is called a Stokes-shift, when the emitted photon has lower energy than the absorbed photon. When the emitted light has higher energy than the absorbed photon, this is called anti-Stokes-shift (see below upconverting phosphors). (Spring 2003; Lichtman and Conchello, 2005.) Nowadays, there is a large variety of fluorochromes commercially available, basically due to the increased demand of them in microscopic applications utilizing laser beam (see e.g. Molecular Probes®).

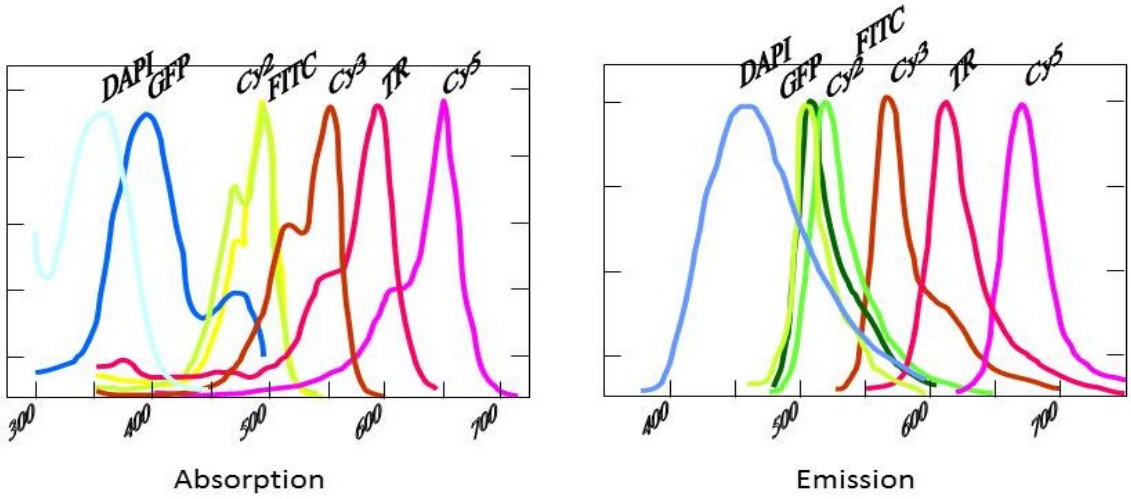

Figure 7. *Absorption and emission spectra of some commonly used fluorochromes. Note for example a large Stokes-shift of DAPI from 350 nm to 450 nm.*

A unique type of photoluminescence is the so-called photon upconversion in which lower-energy (longer wavelength) excitation is converted into higher-energy emission (shorter wavelength) (Soukka et al. 2008). Inorganic rare earth-doped crystals (upconverting phosphors) have the upconversion property. They have been used in

several biomedical imaging applications basically due to easiness of their usage. Since these upconverting phosphors have large anti-Stokes shifts scattered excitation light can be easily separated from the emission. Furthermore, detection instruments are inexpensive since they need only a high power laser diode and standard filters and a photomultiplier (Soukka et al, 2008).

Due to the development of high-resolution microscopy (see chapter 2.4.) multiple new techniques are developed to investigate protein-protein interaction, concentration and motility. Fluorescence resonance energy transfer (FRET) is a typical example of technique to image protein-protein interactions at high resolution. When energy from an exited donor is transferred to an acceptor, FRET decreases the donor fluorescence and increases the acceptor fluorescence. FRET occurs only when the donor and acceptor are very close (within nanometers) to each other. Using different sensors, Ca2+ dynamics and activity of enzymes can be monitored. (Rouy et al 2008.) Fluorescence cross-correlation spectroscopy (FCS), on the other hand, uses confocal setup to analyze concentration and motility of low number of particles that induce fluorescence fluctuation (Bacia et al. 2006, Tian et al. 2011). Fluctuation is recorded as a function of time and it is statistically analyzed by autocorrelation analysis. Both FRET and FCS are utilized in several microfluidics applications (Gambin and Deniz, 2010; Tian et al. 2011). As these techniques are based primarily on characteristics of the fluorescing molecules on the imaging system as such, they are mentioned here only as fluorescence based research techniques, not as imaging system per se. They anyhow demonstrate the broad application possibilities in fluorescence techniques.

2.2. Epifluorescence microscope

The conventional fluorescence microscope is based on epifluorescence i.e. the excitation light is exposed to the sample and emission is collected via the same objective. The components of the conventional fluorescence microscope are similar to those in the classical light microscope except that the microscope needs a specific light source that emits fluorescing light and a set of extra equipment for the adjustment and control of light wavelengths (Fig. 2B and 2C). The typical arc-discharge lamp in an epifluorescence microscope is a mercury lamp (Petty 2007). The path of the fluorescing light is controlled with filters, reflectors and aperture lenses. To excite the fluorochrome of interest in a sample, the suitable wavelength is selected from the fluorescing light by appropriate filter setup. The emitted light is collected to the detector or viewed by the eyepiece oculars. Like in the classical microscope and in confocal microscope, the microscope can be either upright or inverted microscope. (Spring 2003.) In the first option, the objective turret is located above the sample and in the latter the turret is located underneath the sample. The practical difference between these two options is that the latter is more suitable to view cell culture plates as the cells are grown on the bottom of the plate. Thus, when using an inverted microscope, the objective can be placed nearer to the specimen. As the working distance of the objectives, especially

objective with high NA, is very narrow, this fact needs to be taken into consideration when viewing samples in cell culture plates. Since inverted microscopes are more common when using fluorescence techniques, this type microscope in brought up here.

2.3. Confocal microscope

In a conventional fluorescence microscope, the entire field of view, also along the z-axis is illuminated and excited. The emission is also collected from the same parts, thus the resulting image also includes the information that does not originate from the focal plane. The out-of-focus light is disturbing the main information and the signal to noise ratio can be poor, especially if the sample contains a lot of background signal. This problem is overcome by the confocal microscope. (Conchello and Lichtman, 2005.)
The principle of confocal microscope was patented already in 1957 by Marvin Minsky (Minsky, 1988). In this microscope the problem of out-of-focus light is solved by using pinholes (Schmolze et al. 2011). The pinhole in front of the detector cuts out the fluorescent emission coming from neighboring focal planes. To improve the point excitation of the specimen, another pinhole is set in front of the laser light so that only a "narrow" beam of light is sent to the specimen. (Fig. 8). (Conchello and Lichtman, 2005.) Confocal actually means "having the same focus or foci" (Morris, 1992).

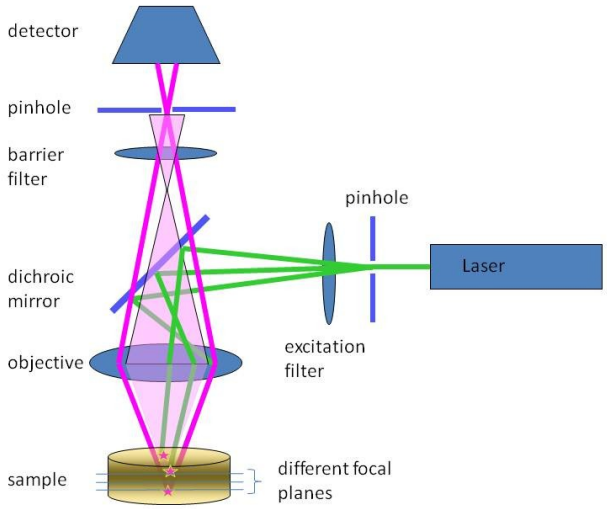

Figure 8. *Schematic drawing of the basic principle of the confocal microscope.*

2.3.1. Light source, the laser

The source of illumination in confocal microscope (as well as in super resolution microscopes described later) is a laser (= **L**ight **A**mplification by the **S**timulated **E**mission of **R**adiation). The lasers are used in confocal microscopes because they can provide single wavelength light as well as very bright light at its wavelength (Gratton and vandeVen, 2006). The unique properties of lasers such as the high degree of

monochromacy, small divergence angle, high brightness and high degree of spatial and temporal coherence make them ideal light source for confocal microscopes. Lasers can be divided into subcategories in multiple different ways. For microscopic applications, selection of the laser type is affected primarily by the mode of operation, peak power strength and wavelength lines required for investigation. On the basis of the mode of operation, lasers can be divided into continuous wave and pulsed lasers. According to the gain medium, lasers are divided into gas, dye and solid-state laser. The solid-state lasers include e.g. semiconductor lasers. A special class of laser setup is the fiber laser that is an optical fiber doped with some rare- earth elements. (Gratton and vandeVen, 2006.) As there is nowadays ever-increasing type of lasers produced only the most frequently used laser types are presented below.

Frequently used laser types.

Argon (Ar) was the first (single gas) laser type used in confocal microscopes: it has strong excitation lines at 488 nm and 514 nm. The 488 nm light excites e.g. FITC, its derivatives and green fluorescence protein (GFP) that is broadly used in gene expression studies in living cells. Originally, 514 nm light was used to excite rhodamine. However the 514 nm excitation could not be applied in double labeling experiments since this line also excites FITC, even better than rhodamine. This illustrates the major problem in fluorescence labeling studies i.e. the crosstalk of either excitation or emission of the fluorescing markers (Brown 2007).

Later on, mixed gas lasers were produces. Argon-Krypton (Ar-Kr) laser provides blue, yellow-green and red excitation lights. This laser is extensively used, since it provides broad spectral coverage for multi-color labeling studies (Fig. 15). Furthermore, the laser light lines (488, 514, 647 nm) are far from each other thus it gives better possibilities to avoid signal overlap. Helium-Neon (He-Ne) laser has in certain cases replaced Ar-Kr laser since their lifetime is much longer (Zucker and Price, 2001). In addition to having emissions in green (543 nm), yellow (594 nm) and orange (612 nm), new He-Ne lasers have emission also at near infrared region (1523 nm). Helium-Cadmium (He-Cd) laser has near UV range excitation. Therefore, it is commonly used in several biological applications utilizing probes such as Hoescht, DAPI, calcium probes, Indo-1 and Fura-2, AMCA that are excited in the UV range. (Gratton and vandeVen, 2006.) (Fig. 9).

There are also a number of other types of lasers that have become common in microscope setup. These include alkali (cesium and rubidium) vapor and dye (neodymium-yttrium aluminum garnet and neodymium-yttrium lithium fluoride) lasers which can be implemented into confocal microscope. Especially due to their low cost and compact design they have potential in several applications. Limitation of continuous wave lasers are that they are not enough powerful even for biological imaging and especially when high resolution is required. Therefore, pulsed-lasers having high peak laser power have been developed also to answer to the needs in life sciences. In the first 2-photon laser scanning microscope, colliding-pulse mode-locked dye laser was used. Nowadays, titanium-sapphire lasers have replaced them as they can produce 100 fs

pulses with 100x higher power. They cover 660 or 700 to 1100 nm and are ideal light source for 2- and multiphoton imaging. (Gratton and vandeVen, 2006.)

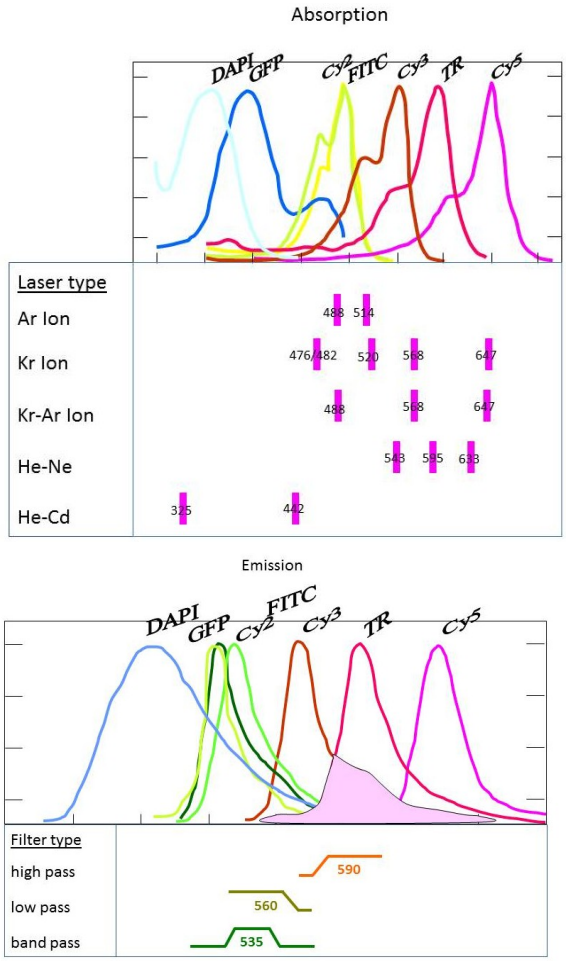

Figure 9. *Absorption and emission spectra of commonly used fluorochromes viewed in relation to the lasers and some typical filter types. Different laser types provide different laser light lines from which suitable line has to be selected with filter setup. Filters are used also to select the correct wavelength from the emission as shown in the right side illustration.*

2.3.2. Light path and scanning the specimen

The light path in the confocal microscope is presented in figure 8. The pinhole selects a ray from the coherent light beam coming from the laser. The dichroic mirror directs and the objective lens focuses the light to the specimen. The special properties of the dichroic mirror allow separation of two light paths, excitation and emission, with the same mirror (sometimes also called a beam splitter). The light coming from the laser is reflected by the surface of the dichroic mirror, whereas the fluorescence emission coming out from the specimen passes through the mirror to the detector. The image is usually captured by a CCD camera like in a Nipkow disc-installed system (see below). In systems that utilize the line scanning procedure, also photomultiplier tubes are used. However, the main configuration setup depends on the purpose of the analyses, required

resolution, speed of detection etc. The other parts of the confocal microscope are comparable to the conventional light or fluorescence microscopes. However, special attention has to put on the objectives and their properties (NA, working distance etc.) in order facilitate accuracy in fluorescence signal detection in thick specimens that are commonly used in confocal microscopy. Furthermore, as scanning of the specimen is controlled by the computer program, a motorized stage is a prerequisite for the microscope setup. (Conchello and Lichtman, 2005; Claxton et al, 2006.)

There are different scanning methods that are employed in confocal imaging. In principle, scanning is done either moving the stage or the beam. The beam movement is enabled by pairs of mirrors that scan over the specimen in xy-directions line by line. This procedure is called line scanning. In addition to this there is an option where both the stage and the beam are stationary. This system - instead of single spot of light – utilizes a spinning disk (Nipkow disc, Nakano, 2002). In this procedure, the laser light itself is not moving instead the disc having holes of equidistant is spinning illuminating each point in the sample only for a short time, but many spots simultaneously (Fig. 10). (Minsky, 1988; Claxton et al 2006..

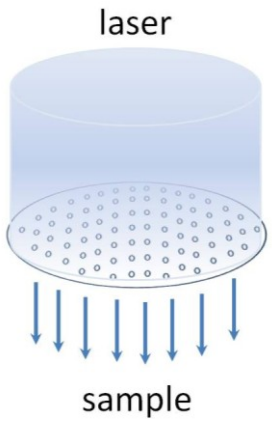

Figure 10. *Nipkow disk*

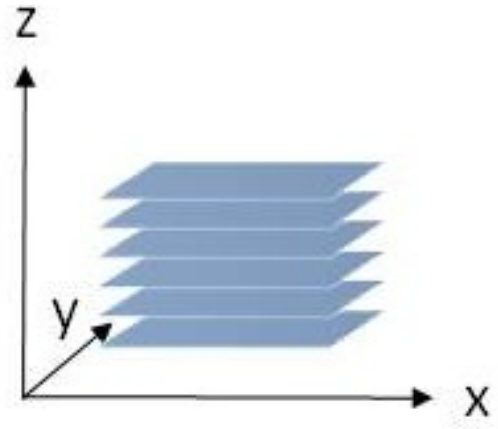

Figure 11. *Optical sections obtained by confocal microscope.*

When the focus (i.e. the scanning depth) is changed, accurate point sampling is done in 3D-space. Via this approach, confocal microscope is able to generate so called optical slides/sections from the specimen. By changing the depth of scanned level in the specimen, 3D data packages are collected (Fig. 11). These data packages can then be manipulated, rotated, used for animations etc. given much more information about the structural configuration of the specimen. (Conchello and Lichtman, 2005; Claxton et al 2006.) Furthermore, by elimination of out-of focus light and point measuring, confocal microscope provides higher resolution so that the structures can be detected and analyzed (especially with super-resolution microscopes) at the same size scale (i.e. nanoscale) as what can be obtained by conventional electron microscopes. However, similar ultrastructural details are not achieved as in the electron microscopic level. For

confocal microscopy samples need to be labeled with photoactivatable or photoswitchable fluorescent probes or heavy metals that have refractive properties. The latter option is, used mainly in material sciences, but can be applied to life sciences to a certain extent.

2.4. Advanced microscopic systems utilizing laser beam

2.4.1. Two- photon and multi-photon microscopes

Ordinary confocal systems have several limitations. First, the fluorophore is excited once with photons comprising the full energy. Then, fluorophores outside the focal plane are also excited. This out-of-focus emission is cut out (by pinhole), but it is also lost for further imaging (bleaching). Thus, a lot of energy is not efficiently used. Two-photon microscopy, which uses a pulsed infrared laser, now overcomes these limitations to a certain degree. The principle is that the fluorophore is excited with two photons comprising half of the energy instead of a single full-energy photon. Although the amount of energy is finally the same, the absorption is confined to a smaller volume in the focal plane and each spot of the sample is exposed only to a minimal laser light. Thus, photobleaching is restricted to a small area in the sample at a certain time point or frame. The other advantage is that the infrared light that is used in 2-photon microscopy penetrates deeper into the sample than the visible or UV light. This results in a significant improvement especially for live cell or even live organism imaging purposes. Multi-photon microscopy is a further development of two-photon microscopy. The disadvantage of these methods is high price of the pulsed lasers, the complexity of the system as well as its poor axial resolution (about 500 µm). (Schmolze et al, 2011.)

2.4.2. 4-Pi.

The double-confocal scanning microscope was patented in 1990 by Stefan Hell (Hell 1990). This system has later been referred to as 4Pi microscope. It uses two opposing high NA lenses on different sides of the sample that is between two coverslips (Fig. 12). The counter-propagating spherical wavefronts of the excitation light fields are coherently summed at the common focal plane. Spherical wavefronts of the emitted fluorescence light are also coherently summed at the detector. These operations sharpen the spot along the optical axis and yield three- to sevenfold resolution increase. (Gugel et al. 2004, Hell 2009.) Later, the 4-Pi principle has been implemented into several super resolution systems described in the next chapter e.g. single marker switching microscopy. These systems have been improved to better meet the requirements in non-invasive 3D multicolor imaging in nanoscale range given localization precision of less than10 nm (Aquino et al 2011).

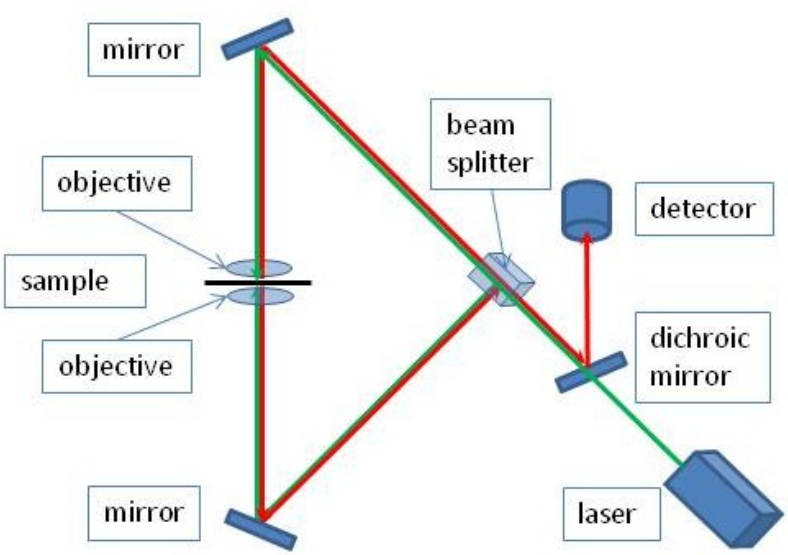

Figure 12. *Schematic drawing of the basic principle of the 4Pi microscope. Excitation light originating from the laser is split into two beams by beam splitter and focused by objective lenses to the specimen. Both objectives collect the fluorescence that is recombined by beam splitter and then directed to the detector. (Modified from Hell, 2009 and Gugel et al. 2004.)*

2.4.3. Super-resolution microscopy

Concomitant development of fluorescence markers, high power computer systems, imaging techniques and principles as well as implementation of computation processes has enabled beginning of a new era in the microscopic imaging. In microscopy history this is often referred to as "Breaking the diffraction limit" –milestone. These techniques are divided in two basic approaches: 1) near-field and 2) far-field imaging techniques.

Near-field super-resolution imaging

In near-field scanning optical microscope (NSOM), lenses are replaced by using only a small NA objective and near-field imaging. The samples are placed very close to the aperture so that the light cannot diffract. With this system, 20-120 nm lateral resolution is obtained. However, disadvantage of this system is that it is not suitable for intracellular imaging. Furthermore, keeping the sample in constant distance from the aperture is challenging and results in longer image acquisition times. (Fernandez-Suarez and Ting, 2008.)

Far-field super-resolution imaging

The far-field super-resolution techniques use lenses. Thus, the samples can be placed more distant from the optics. To overcome the diffraction limit, the systems take in use of the transition of the fluorophore between two molecular states (i.e. dark and bright state). By switching the fluorophores on and off, objects that are located 200 µm apart or nearer, can be distinguished. For example, stimulated emission depletion (STED), ground-state depletion (GSD), saturated pattern excitation microscopy (SPEM), and

saturated structured-illumination microscopy (SSIM) and its combination with I^5M (I^5S) give super-resolution by narrowing PSF of an image ensemble from several fluorophores. Photoactivated localization microscopy (PALM/FPALM) and stochastic optical reconstruction microscopy (STORM) count on the fact that if sufficient numbers of photons can be collected, a single emitter (i.e. single molecule) can be detected with high accuracy. (Fernandez-Suarez and Ting, 2008; Hell 2009.)

The list of systems utilizing modifications and combination of hard- and software setups as well as different properties of fluorophores has tremendously increased during recent decade. However, since the difference between the systems is based largely on the properties (molecular transitions) of the fluorescent markers used to label the structures of interest and not necessarily on the systems' technical configuration as such, only a few examples are presented here in more details.

STED. In STED, two laser beams are used one after each other. The first is the actual excitation beam and the second is red-shifted pulse called the STED beam. When excited fluorophores are exposed to the STED beam they almost immediately return to ground state. To go over the diffraction limit, the STED beam is modified so that it has a zero intensity point at the focal center and highest intensity at circumference. Thus, when the two pulses are superimposed, only the molecules that are close to zero retain fluorescing and maximal emission is detected there (Fig. 13). (Fernandez-Suarez and Ting, 2008; Schmolze et al. 2011.)

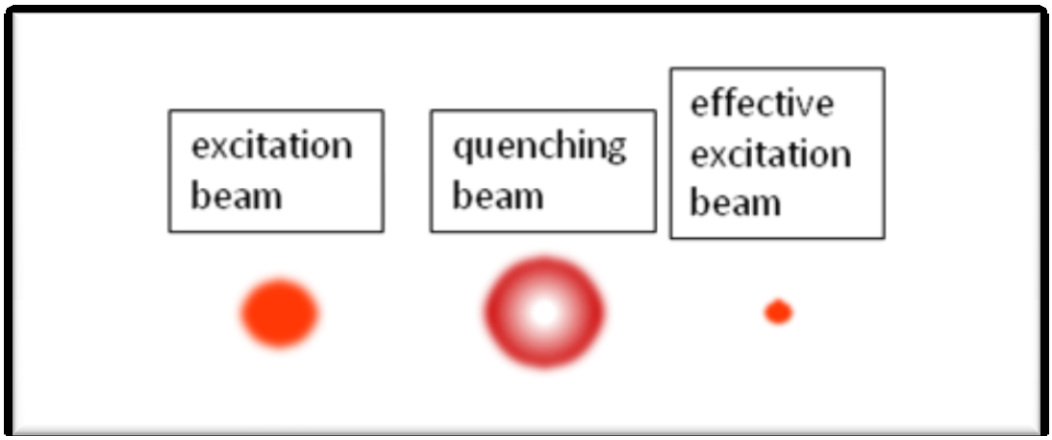

Figure 13. *Principle of stimulated emission depletion. An initial excitation beam is followed by a second beam that quenches the fluorescence of the first beam in donut shaped pattern. The effective excitation beam is thus smaller than the original. (Modified form Fernandez-Suarez and Ting, 2008 and Schmolze et al. 2011)*

PALM, FPALM and STORM. With these systems single molecules can be localized even with 1 nm accuracy, if enough photons can be collected and there is no similarly emitting molecule within 200 nm. However, this is usually hindered by the dense labeling. To overcome this problem, fluorescence molecules are sequentially activated and deactivated with different wavelengths. Switching the molecules on and off is done stochastically. In each imaging cycle, most molecules remain dark while only a few are

stochastically switched on and imaged. When the process is repeated multiple times, super-resolution images can be reconstructed. (Juette et. al 2008, Fernandez-Suarez and Ting, 2008.)

With STORM, 20-30 nm lateral and 50 nm axial resolutions has been obtained. With FPALM imaging the corresponding values are 30 nm and 75 nm (Juette et al. 2008). This is achieved by biplane FPALM in which two axially separated object planes are detected simultaneously. In this system, a 50:50 beam splitter divides the focused light into transmitted and reflected paths. These beams form images on different regions of the detector. These images represent object planes that are about 350 nm further away (transmitted path) and closer (reflected) to the objective than the original object plane. (Juette et al 2008.) (Fig. 14).

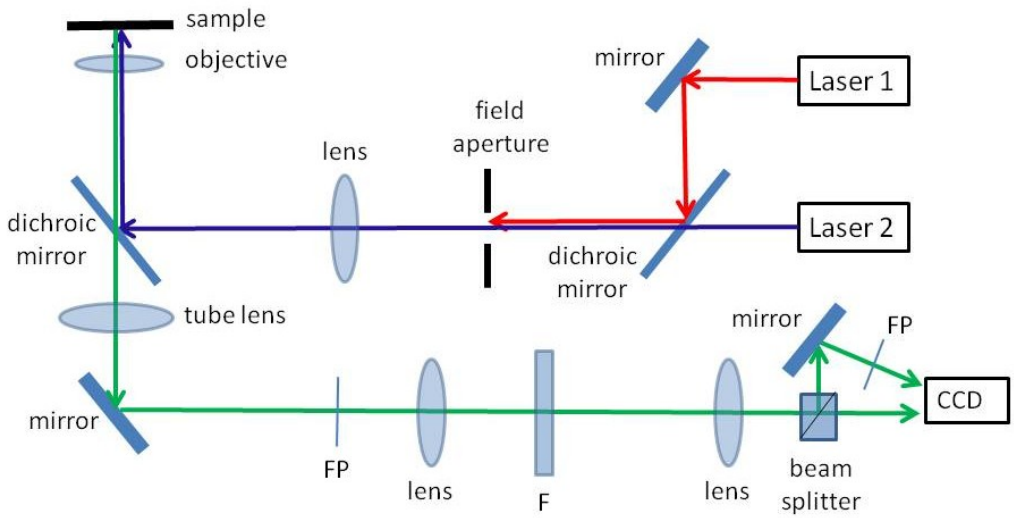

Figure 14. *FPALM setup demonstrating an advanced microscope utilizing two lasers to activate and deactivate fluorochromes and facilitating detection of two object planes by signal division before entrance to CCD camera. The beams originating from two lasers are combined by a dichroic beam splitter and they can be switched on and off by electrical shutter. The beam is directed to the sample where from the fluorescence is collected by the same objective and then transmitted through the dichroic mirror and focused by a tube-lens into an intermediate focal plane (FP). The fluorescence goes through two achromatic lenses between which there are filters (F) to reduce scattered laser light. CCD camera can be moved nearer to the tube lens. This moves the corresponding object plane deeper into the sample. The fluorescence is divided into two paths by a 50:50 beam splitter situated in the front of the camera. The transmitted light path goes directly to the CCD camera. The reflected light forming a second image on CCD corresponds to a second object plane within the sample. As a result, signal from two recorded regions are combined into 3D data stacks in one high sensitive camera. (Modified from Juette et al 2008).*

3. Electron microscopy

There are, in principle, two major types of electron microscope, transmission electron microscope (TEM) and scanning electron microscope (SEM). The difference between these two systems is that in TEM, the electrons pass through the specimen and the transmitted electrons are detected with the detecting system (eye from the fluorescence plate or the digital camera). In SEM, electrons that are scattered from the surface of the specimens are detected by a digital camera or other detectors after electron amplification. (Slayter and Slayter, 1992.) Otherwise, the basic structural components of operating systems are, in principle, similar.

3.1. Electron gun

The central component of the electron microscope is the electron source. The electron gun comprises a filament, a Wehnelt cylinder and an anode (Fig. 15). The classical electron source is a tungsten filament, but nowadays, systems use often either LaB6 or field emission gun (FEG) electron source due to longer lifetime and intensity of the beam. The hairpin-shaped tungsten filament is heated up to about 2700°C. When high positive potential difference is applied between the filament (=cathode) and the anode, electrons extracted from the electron cloud around the filament are accelerated towards the anode. The role of the Wehnelt cylinder is to push the electrons into finely focused beam. While traveling through the column, the electron beam is condensed by the condenser lens so that is composed of a parallel beam at the level of the specimen. The higher the accelerating voltage, the higher is the speed of the electrons, and the thicker the sections for the analyses can be. Compared to tungsten and LaB6 filaments, where the electrons are extracted by thermal electron emission, in FEG they are extracted by the electric field. (Slayter and Slayter, 1992; Voutou et al. 2008.)

To obtain very high resolutions, acceleration voltage and the current have to be extremely stable. This requires sophisticated electronic circuits. Earlier models of electron microscopes were controlled manually via multiple knobs in the panel. Nowadays, the control, monitoring and recording the operation system is done by fast and powerful PCs. This has enabled usage of special techniques with the same setup and control. Furthermore, when the PC is attached to the network, backups, service, image archiving, managing, processing and consulting with several workstations connected to the network is made possible (Bárcena and Koster 2009; Lawson, 2010).

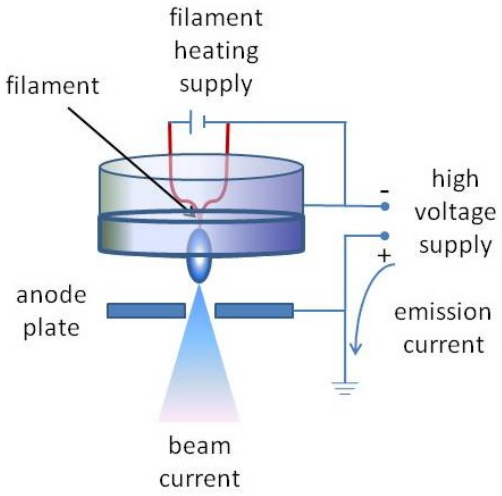

Figure 15. *Schematic drawing of a tungsten filament-Wehnelt assembly. (Modified from http://www4.nau.edu/microanalysis/Microprobe-SEM/Instrumentation.html).*

3.2. Path of the electron beam and image capturing

Electrons behave like light only under vacuum. In order to achieve this, the whole column has to be evacuated. The system consists of several airlocks and pumps in order to maximize safety and control over the vacuum in the column. How high the vacuum should be, depends on the system itself (electron source, energy requirements etc.) and the purpose for which the system is used (routine pathological examination vs. high resolution applications/elemental analyses). The residual pressure in the microscope is around 2.5 x 10^{-5} Pa (comp. normal air pressure 1 x 10^{5} Pa). (Slayter and Slayter, 1992.)
The electron beam is controlled by electromagnetic lenses via its travel towards the specimen (condenser lenses) and after it has passed the specimen (objective lenses, projection and magnifying lenses) (Fig. 16). An electromagnetic lens is composed of electrical coils through which electrical current is passed to create electromagnetic field between the pole pieces. Via varying the current, the magnification can be varied. To stop the scattered electrons that are not useful for image formation, are arrested from the path by different apertures. (Slayter and Slayter, 1992.)
The image formed by the electron beam can be viewed from the fluorescence screen directly through a large window in the projection chamber. To record the image, two options are available. 1) Electrons are used to expose a traditional film or 2) the image is captured by a digital camera. Nowadays, the second option is more common also when sample is just investigated by viewing it from the monitor (i.e. not directly from the fluorescence screen). (Fan and Ellisman, 2000.)

3.3. Electron-specimen interaction

When electrons meet the specimen, the following events take place (Lepistö, 2005; Voutou et al. 2008; Müller et al. 2008)

1) Transmission though or absorption into the specimen, usually due to the specimen thickness. This results in amplitude contrast in the image.
2) Scattering from the specimen, depends on the composition of the specimen. This results in phase contrast.
3) Electron scattering from crystalline specimen results in diffraction contrast that is typical for different crystals.
4) Some electrons are backscattered.
5) Secondary electrons which are emitted from the specimen produce SEM image.
6) X-rays which are emitted when impinging electrons hit the specimen, the energy and wavelength of these X-rays are related to the elemental composition of the specimen and can thus be used for elemental analyses.
7) Catholuminance, specimen emits photons (or light).
8) Energy loss of electrons when they pass through the specimen can be used for elemental analyses by electron loss spectrometer (EELS).

The first two contribute to formation of an image from biological samples in normal TEM. For crystalline structures (mostly material sciences), phase contrast and diffraction are the most important factors in image formation. To detect the remaining 5 electron interaction with the specimen requires additional accessories attached to the microscope.

3.4. Transmission electron microscope

TEM can be compared with a slide projector. In a slide projector, the light passes through lenses and establishes a parallel beam before it enters to the slide through which it is projected to the screen. Similarly, in TEM the electron beam is made parallel by magnetic lenses. The electrons penetrate through the sample and are detected by the fluorescence screen that emits light when the electrons hit to it. The contrast between the structural elements are formed by the fact that the specimen contains different substances that either let the electrons to pass through or stop them. The first option results in light and the latter dark areas (electron dense) on the fluorescence screen. (Slayter and Slayter, 1992; Voutou et al. 2008.)

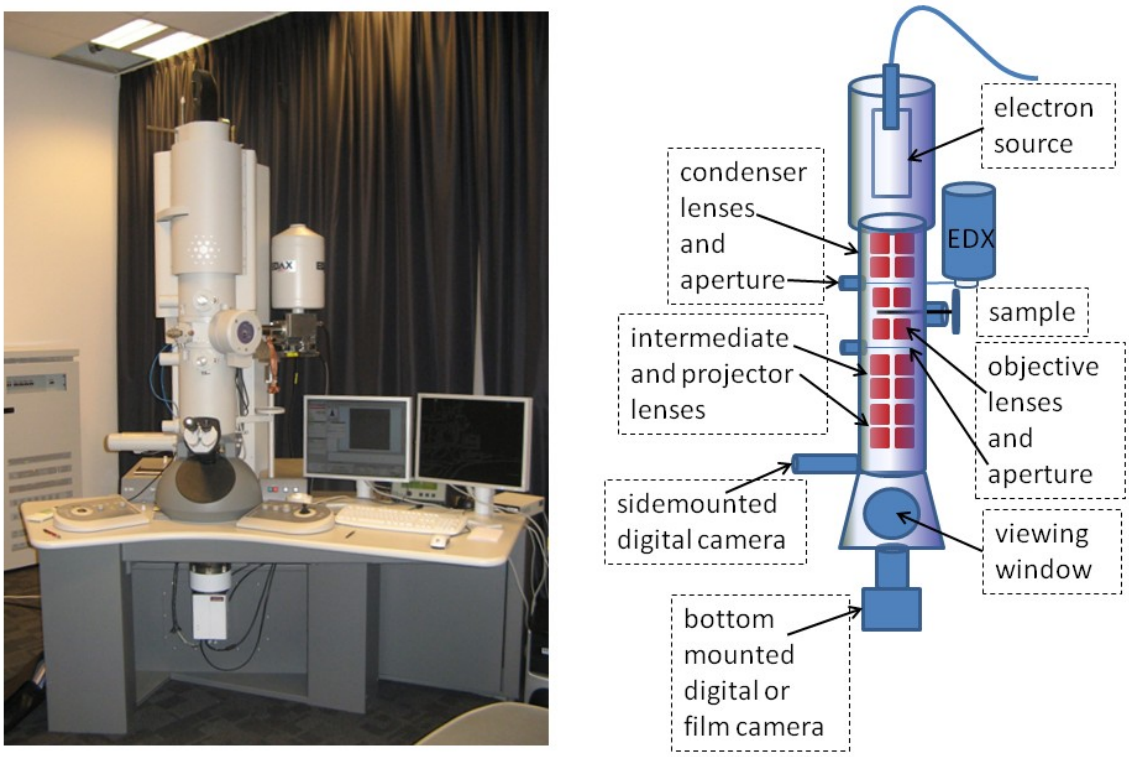

Figure 16. *A typical transmission electron microscope used in life sciences. This TEM (Tecnai G2 20, FEI) has also an EDX analyzer (EDAX) for elemental analyses. Diagram on right shows the basic components of a transmission electron microscope.*

3.5. Scanning electron microscope

In order to enable electrons to pass through the specimen, the specimen has to be made very thin (usually around 50-80 nm thick) for TEM applications. However, not all specimens can be made thin. Furthermore, there are situations when the surface structure is in the main interest. For this purpose, a scanning electron microscope (SEM) was developed.

The main components (electron gun, anode, condenser lens etc.) in SEM are basically similar to those in TEM. The main differences compared to TEM, is that the beam is moving i.e. scanning over the specimen thus it is not stable/static like in TEM. The beam movement is obtained by a scanning coil beneath the magnetic lenses. Furthermore, the accelerating voltages used in SEM are much lower (0.2-30 kV) than in TEM. Specimens can be relatively large and do not necessarily need complex preparation methods. Furthermore, SEM is collecting information from the specimen using the last five electron-specimen interaction events listed in the previous chapter. These include secondary electrons, backscattered electrons, and absorbed electrons to the specimen, X-rays or photons emitted from the specimen after the electron has impinged to it. The resolution in optimal conditions is usually around 1 nm, while in TEM it is 1/10 of it. (Slayter and Slayter, 1992; Voutou et al. 2008; Schatten 2011.)

In general, for the focus of the current work, SEM is not the main system to be considered. However, the combination of SEM and TEM (i.e. STEM, Fig. 17) would raise possibilities that allow studying of structural details in parallel to elemental information about the specimen. In this way, the backscattered and secondary electrons that are useless otherwise in TEM, are now recorded with the scanning apparatus thus given elemental information added to structural information. (Slayter and Slayter, 1992; Stolojan 2008; Müller et al. 2008.)

Figure 17. *A Scanning Transmission Electron Microscope (STEM) providing both TEM and SEM images together with EDX and EELS analyses (HD-2300, HITACHI)*

3.6. Useful techniques and accessories for electron microscopy

3.6.1. Cryotechnique

The processing of routine EM samples includes fixation with glutaraldehyde and/or paraformaldehyde followed by a second fixative, osmium tetroxide. Osmium tetroxide is a lipophilic agent that oxidizes unsaturated bonds on fatty acids and thus produces contrast for different parts of the cell. After fixation, samples are dehydrated in organic solvents and embedded in plastic resin that is polymerized either by heating up to 60ºC

or exposure to UV-light. Ultrathin sections cut with an ultramicrotome are stained with uranyl acetate and lead citrate to further enhance the contrast of cellular structures. It can be understood that after such an extensive treatment the cells are very stabile for EM analyses, but they are also entirely dead and may even contain some distortion of the structure due to the processing. (Dykstra and Reuss 2003; Pilhofer et al, 2010; Schatten 2011.)

In order to get structural information that is relevant to the native state of the cell, the specimens have to be prepared so that structural changes do not take place either during processing or during imaging. At electron microscopic research, two factors have to be specifically taken into consideration: 1) samples must be in vitreous state and 2) low electron doses should be used (Subramanian, 2005, Leis et al. 2009). These two factors are fulfilled by cryo-electron microscopy that has revolutionized analysis of macromolecular structure at electron microscopic level. This technique was first described by Christensen over four decades ago (1971) and being increasingly adapted during recent years.

The main idea behind cryo-electron microscopy is that the samples are studied at low temperatures (100-113K). Low temperatures lead to very low sublimation rate of the ice and when low doses of electrons (10-20 e/Å^2) are used, beam damage to the specimen is minimized. For cryo-electron microscopic investigation, samples are embedded in vitreous ice on holey carbon grid. Vitreous ice is performed by rapidly cooling the samples below 273K. This, so called cryo-fixation is provoked either by high-pressure freezing (~2100 bars), slam freezing (against cold plate) or plunge freezing technique. During this snap freezing step, water molecules stop moving before they have formed hydrogen bonds needed to create crystalline ice. This results in formation of an amorphous ice in which water remains in a water-like arrangement without destruction of the sample with ice crystals. As vitreous ice is a structure-less medium, details of the sample can be studied in more natural environment without chemical fixation, embedding or staining with contrast agents, metals etc. (compare resin infiltrated samples in routine TEM). (Rupreht and Nield, 2001, Leis et al. 2009, Pilhofer et al 2010.) Interestingly, the cells can even survive during cryo-electron microscopy process (Erk et al. 1998).

3.6.2. Energy Dispersive X-ray Detector (EDX)

When an electron hits an atom it can remove an electron from its orbit. As a result, the atom contains also the energy that was required to remove the electron from it. In other words, the atom is in an excited state. When the missing electron is replaced by an electron from some other orbit or from the surrounding the transfer results in release of energy as an X-ray quantum (in the case of outer to inner orbital transitions) or a light quantum (in the case of free electron to outer orbit transfers). The energy of these X-rays depends on the elemental composition of the specimen. Energy dispersive X-ray detector (EDX) is developed to detect these energies. The peaks in the spectrum indicate

the elements and their concentration in the specimen. With this system very small quantities (10^{-12} g or less) can be detected. However, the detection sensitivity and resolution depends on the qualification (electron beam efficacy, intensity and beam size) of the electron microscope itself. This system can be used both in TEM and SEM where, in addition, to structural details it provides information about elemental composition of the specimen. (Slayter and Slayter, 1992; Voutou et al. 2008.)

3.6.3. Electron Energy Loss Spectrometer (EELS)

When electrons travel through the specimen they lose energy. The loss of energy can be measured with EELS and it is dependent on the elemental composition of the specimen. A practical difference between these EDX and EELS is sensitivity. EDX that is relatively easy to use and it is working best for the heavier elements. EELS, in turn requires more training, but can give more information, e.g. the forms of the same element than EDX. Furthermore, it works efficiently at lower atomic numbers. (Slayter and Slayter, 1992; Voutou et al. 2008.)
While EDX is located on the upper side of the specimen, EELS is below the sample, under the projection chamber of TEM. However, Zeiss has launched a new system that has an Omega-type monochromator and corrected Omega energy filter. This carries two advantages. 1) The monochromator preserves the spot size and allows imaging with maximum brightness improving contrast and resolution. 2) After passing through the specimen the electrons pass through the corrected OMEGA energy filter. By this approach, the energy spread of the source is not interfering the spectrum thus given better resolution (even 0.14 nm). This yields high resolution EELS spectroscopy. (http://www.zeiss.com/C1256E4600307C70/EmbedTitelIntern/EFTEMBrochure/$File/EFTEM_lq.pdf).

3.6.4. 3D reconstruction at SEM

TEM provides information about the internal structure of the cells and organelles. SEM instead is commonly used to provide structural information of surfaces. However, during recent years, new types of approaches are developed that facilitate SEM to be used for 3D structure investigation. According to one approach, the samples are embedded in resin and then put into a chamber that contains sectioning microtome as well as the imaging system. After every cut, the surface of the block is photographed. By this approach image series usually having interval of about 70 nm (i.e. the section thickness) is achieved. (Denk and Horstmann, 2004.) In another approach, the sectioning is done by using a focused ion beam (FIB). Imaging is done by using a dual–beam microscope from the sectioned specimen. This yields image series with about 100 nm intervals. The benefit of the latter approach is that it can be applied to both resin embedded and vitrified specimens. (Gestmann and Subramaniam, 2004.)

III. Discussion

1. Microscopy: requirements at present and possible future expectations

1.1. Live or fixed cell imaging

When we consider a microscope meant for life science purposes, the primary question is whether the microscope is made suitable for live cell imaging or whether it is sufficient to study fixed material containing immobile dead cells. Imaging is going nowadays more and more towards capturing different events taking place in living cells. Real-time molecular and biodynamic studies tell much more about the physiology of the normal and pathological tissue. At the moment, we know e.g. genes responsible for certain diseases, and the structural changes caused by that gene. However, the precise disease mechanism at cellular level is still implicitly speculated on the basis of findings obtained by these two entities. Therefore, it is important to evaluate the systems from the viewpoint how well they are suited for investigating live cells and events and fill this gap. Anyhow, this type of approach does not make a microscope ineligible for dead cell imaging. The reverse approach, however, would lead to narrower viewpoint i.e. the microscopes that are suitable for dead cell imaging, do not necessarily carry features that live cell imaging requires.

1.2. From high resolution to 3D structure

When it is decided to design a microscope for live cell imaging, the straightforward fact is that the system has to be made for high resolution purposes in order to get the best out from the system. This means that the system has to be able to detect smallest particles possible. At this point, first question is: “What is the smallest particle we can image?”
Atoms are the basic building blocks of everything. They carry a nucleus containing positively charged protons (mass 1.67 x 10^{-27}kg, spin ½) and neutrons (mass 1.67 x 10^{-27}kg, spin ½) that do not carry charge. The nucleus is circled by negatively charged electrons (mass 9.11 x 10^{-31}kg, spin ½). Both protons and neutrons are formed by even smaller elementary particles, up- and down-quarks. An up-quark can transform to a down-quark by absorbing or emitting a W boson (a weak force elementary particle), and vice versa. (Young and Freedman, 2004.)This mechanism is behind the process of radioactive beta decay that is used in medical imaging, positron emission tomography (PET). The spin property of the elementary particles have been used in many applications such as nuclear magnetic resonance (NMR) spectroscopy, electron spin

resonance (ESR) spectroscopy and magnetic resonance imaging (MRI) which all are used in medicine. (Jan, 2005.)

The atoms are the building units of the molecules that can be formed by a single chemical element or different elements establishing covalent bonds between each other. The size of molecules varies tremendously from less than an Å (0.1 nm) to several dozen Å. The smallest molecules cannot be seen by naked eye, but the macromolecules, the polymers that are composed of repeating structural units, can reach the detection limit of the eye. (Zumdahl, 2005.)

Now, during the post-genomic era, it has become evident that the structure, localization, and function of the gene product i.e. the proteins has to be investigated more properly. In order to understand how protein complexes execute biological functions, we need to know the structure, chemical composition, conformational changes and exact location of these large, fragile constructions in their natural environment "at work". For example, one of the key issues in drug development has been the lack of high resolution information of the protein complexes, which would allow protein-based approach in drug design. This would be important additional approach to ligand-based approaches. (Singh et al. 2006; Favia, 2011.) Imaging tools facilitate more accurate molecule modeling and are essential tools in studying different molecules. However, the future microscopes should be able to answer to the question not only at the molecular level, but also at the atomic level in living (and behaving) organism.

In addition to the high resolution information it is necessary to get an overall structural view of the molecule in its natural environment. This means that information about the conformation and possible conformational changes associated with the molecule needs to be seen and imaged in three dimensions. Furthermore, how these 3D-structures are situated in the entire cell and/or body gives much more information than just seeing 2D image or a molecule without a visible reference space. In addition, e.g. understanding TEM images in which structures are represented in different grey levels in a section of a few tens of nanometers in thickness is commonly found very challenging. TEM-tomography providing 3D images with multicolor profiles is welcome solution for this problem. However, speed and usability of the software packages, acquisition and registration of image series and reconstruction of tomographic 3D images still need to be improved to make it every day routine.

1.3. Detection of labeled/unlabeled structures and particles

The main focus in life sciences is on biopolymers such as nucleic acid and proteins via which the cells execute their biological functions. At this point we have a major bottleneck which would be one of ground issues to be resolved in the future. The molecules can be visualized even at atom level with the atomic force microscope (AFM) that gives resolution down to 0.25 nm. However, this microscope gives primarily information about the surface features. Since the biological events take place inside the closed structures, cells, the surface information has a limited value. Nevertheless, as

unlike the scanning electron microscope, AFM does not need vacuum, this microscope has some potential for live cell imaging. (Allen et al. 2003; Müller et al. 2011.)
In order to get information about the molecular events and interactions in the living cells, the molecules have to be labeled with marker/markers to make them visible/detectable in the imaging system. Thus, the crucial question is what are our possibilities to visualize the molecules without labeling them with additional markers and still know what molecule it is? Would this be the main requirement for the future microscope? Anyhow, we need tools to analyze internal cellular components while they are "at work" i.e. forming, disassembling and interacting with other components and at the same time know what is what without perturbing them with exogenous labels.

1.4. High throughput screening

Our understanding and knowledge about molecular composition, structure, function, interaction with other molecules in normal and pathological situations have increased tremendously during recent decades. In medicine, it is essential to know what the molecular abnormalities are in different diseases. By knowing them it is possible to design new target-specific drugs or look for new indication areas for previously discovered drugs that could have therapeutic effects for the patient carrying a different disease.
Computer modeling has greatly facilitated the design of new ligands, the molecules that can modulate, increase or decrease the activity of the naturally occurring molecules (Singh et al. 2006; Favia, 2011). All of this has set a great demand for systems that can perform hundreds and thousands tests simultaneously. These systems need to use robotics in sample and chemical handling, require sensitive and fast detectors for the analyses as well as sophisticated software for data processing and storage.
What are still required are the nanoscale sensors that could transmit multiple signals on the same channels at the same time. In addition, sensor technology should be extended so that the large variety of analytes could be measured in user-friendly platforms in reliable and reproducible way. All of these should produce biologically meaningful results. (Connelly and Sharp, 2009.) Therefore, it is important to integrate experimental and computational research and view the things from molecules through cellular processes and interactions to a living organism having its behavior. Since cells have different roles in different tissues their structure and function may vary considerably from tissue to tissue. Therefore, it is of crucial importance to collect and compare results from different studies to try to make consensus of the different events taken place in that particular tissue. This leads to massive amount of data to be stored as well as being available online for other users. Furthermore, in order to make results comparable from experiment to experiment, all the steps in the experiment and analyses have to be traceable and repeatable. This requires setting of standardization and validation of the methods at universal level. The standardization and validation methods as well as the results from experiments performed already have to be available for every user in

biological databases. Actually the quality controlling step in the workflow could be done using these databases in database management system. In other words, the microscope unit should be fully integrated to the network.

1.5. Usability

The main issue concerning the usability of the microscopes has largely been on ergonomic aspects. Long term work in an unusual position to look through the oculars with limited possibility to move while changing and focusing the view of interest with extended arms having bend wrists has coursed common problem among microscope users i.e. pain in the neck and wrists, headache and tiredness of the eyes (Sillanpää and Nyberg, 2010). However, general interest on occupational health aspects during last two decades has provoked systematic work to improve microscope ergonomics by the manufacturers. Interestingly, ergonomics has been improved by relatively little manipulations of the hardware configuration such as extended and flexible eye tube, lowering the specimen movement controlling knobs as well as providing external armrests (Carr and Davidson, 2000; Sillanpää and Nyberg, 2010). Furthermore, microscopic investigation has moved more for viewing the sample on the computer screen instead of viewing the sample all the time through oculars that also irritates eyes with bright light.

At the moment, the focus on usability of the microscopes should be set more on the usability of the instrument and software. While the microscopes are developed more sensitive and sophisticated they have become more complex in operational point of view. At the end, the usage needs expert knowledge and trained specialist i.e. man power though at the same time the systems should be for high throughput screening purposes. Thus, these issues bring important usability challenges that need to be taken care when designing novel instruments.

2. Current technical solutions that sufficiently meet the requirements

All microscopes presented in the current thesis are suitable for live cell imaging. However, they still have some specific limitations in what, at which state and dimension the structure and events can be imaged. In this chapter, the microscopy systems that meet most of the requirements set in the previous chapter are brought up. The limitations that they still have are also discussed.

2.1. Laser beam microscopy as a tool for high resolution imaging in living cells

The confocal microscope can visualize live events with high resolution providing 3D-data. It collects information from well-defined optical levels rather than from the entire specimen at once. Therefore, it produces depth-selective images. It eliminates stray light, which can be a big problem in the conventional epifluorescence microscope where out of focus light obscures structures of interest. This increases detection sensitivity, contrast and clarity of the images. Thus, this microscopic system is in a way slicing the cells optically and thus enabling analyses of small intracellular details. Confocal microscopy is a sort of noninvasive method by which the details can be observed in their own environment instead of physically sectioning the samples. (Claxton et al. 2006; Schmolze et al 2011.)

In addition to improve detection and production of 3D images, far-field optical microscopes provide non-invasive access to interior of the living cells with high sensitivity and resolution. STED gives 20 nm lateral and 45 nm axial resolutions. (see rev. Fernandez-Suarez and Ting, 2008.) Unfortunately, the instrument is still relatively complicated which limits its widespread use. However, STED system with continuous wave beams implemented in conventional confocal set-up might give a solution for this problem. When using PALM, FPALM and STORM, single molecules can be localized even with 1 nm accuracy. This is achieved if enough photons can be collected, and there is no similarly emitting molecule in 200 nm or nearer. However, this ability to localize molecules in 1 nm accuracy does not directly apply to similar resolution range. With STORM, spatial resolution is 20-30 nm in lateral and 50 nm in axial dimension. With FPALM imaging, the corresponding values are 30 nm and 75 nm. (Hell et al. 2009; Juette et al. 2008, Fernandez-Suarez and Ting, 2008.) On the other hand, Aquino and colleagues (2011) recently suggested by using combination of 4Pi and PALM or STORM techniques potential resolution of 5.4-6.6 nm in axial and 8.3.-22.3 nm in lateral direction in a 650 nm thickness. This clearly indicates necessity of combination of techniques in order to improve resolution further. Figure 18 summarizes the resolutions gained so far with different imaging system utilizing single imaging technique.

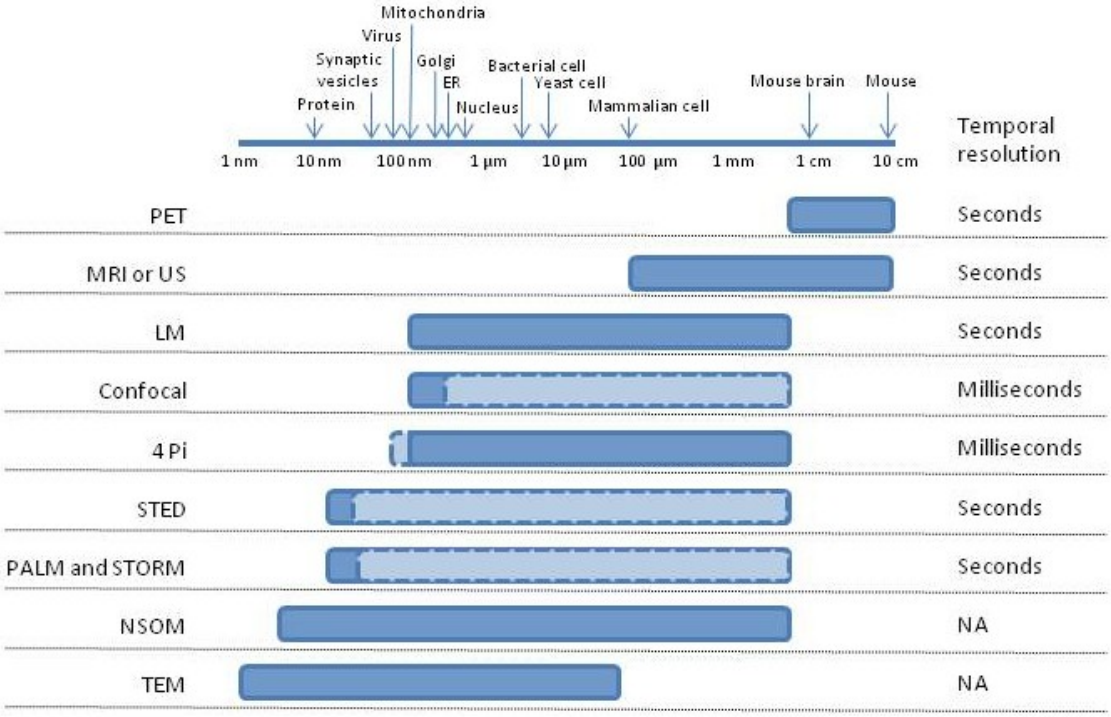

Figure 18. *Comparison of lateral (blue bar with solid line) and axial resolution (light blue bar with dashed line) and temporal resolution of different microscopes. PET, MRI and ultrasound (US) are presented as reference. Modified from Fernandez-Suarez and Ting, 2008. Note that combination of 4Pi and PALM or STORM has recently been shown (Aquino et al 2011) to give resolution potential of at 5.4-6.6 nm in axial and 8.3.-22.3 nm in lateral direction in 650 nm thick optical layer (not marked into the figure).*

The aperture of two opposing lenses have provided nearly isotropic 3D resolution by improving axial resolution and modulation or switching of the fluorescence of adjacent markers have enabled to overcome the diffraction limit. (Hell et al, 2009.) To improve resolution, one can manipulate the probes used for investigation. Furthermore, number of photons detected from each fluorophore has to be high enough, but not overlapping in order to distinguish them from the background and the neighboring fluorophores. When viewing dynamic processes, the motility of the fluorophores should be slow enough to facilitate image acquisition without blurring. (Henriques et al 2011.) Furthermore, there are still some factors that need to be taken into consideration when using, in general, systems that rest on fluorescence. It is well known that high intensity light or light with short wavelength cause photobleaching and/or phototoxicity. In addition to minimize these effects by adding reducing and oxidizing agents into the buffers used in the experiment (Henriques et al. 2011), these effects can be diminished also by the technology design. In confocal microscopy, usage of spinning disks has decreased phototoxicity and photobleaching caused by the excitation light (Nakano, 2002; Petty 2007). 2-photon microscopy using pulsed laser beam have decreased these drawbacks further. Manipulation of also the pulse duration and frequency has been helpful in decreasing photobleaching of the fluorophores. This increases accuracy,

sensitivity and resolution of the detection. In addition, when using infrared light it is possible to get better penetration properties with low phototoxicity effect (Schmolze et al 2011). At the same time when hardware/software setup has been improved, the labels to study the events associated with the molecules of interest have also developed considerably. Especially usage of fluorescent semiconductor nanocrystals (quantum dots) and upconverting phosphors have made studies of the location and activity of individual cells within a living organism possible with high accuracy and less phototoxicity. Thus, methods have been improving, but researchers still have to be aware of the above facts in order to get reliable, accurate and artifact free data when using super high sensitive methods. Furthermore, there are still challenges with hardware itself (stage drift) as well as with software (repeated measurements and computation increases risks and aggravate computational artifacts). One way to minimize false interpretations is to use several different methods to study the same thing. The findings from different studies should support each other and enable to make a sensible and concise data.

2.2. Transmission electron microscopy visualizes structures directly

TEM allows visualization of the microstructure of the cell, pathological changes occurring in them, exact localization of the molecules and 3D structure of the protein complexes at a resolution that falls between that of atomic structures defined by X-ray crystallography and more global patterns revealed by the light microscopy (Müller et al. 2008). Thus, it sounds like a promising tool to fulfill the requirements described in the beginning of this chapter. Together with the development of image data collection and 3D reconstruction, this field of research will be expanding and most exciting advanced techniques are yet to come.

Laser beam microscopes provide accurate images of very small structures at work. However, the molecules have to be labeled as these systems are based on detecting fluorescence and not exactly visualizing the structural component itself. TEM on the other hand has a great advantage over the fluorescence based systems that it directly shows structural features (Leslie 2011). After viewing samples at the electron microscope, no speculation about the structural element remains as “Seeing is believing.” On the other hand, to study what type of molecules structure of interest contains, the molecules need to be identified by labeling them with cytochemical and/or immunocytochemical methods also for TEM imaging. The advantage over the fluorescence based systems is that at TEM the label is seen to be attached directly onto a structural component that can be identified according to ultrastructural features (Fig. 19).

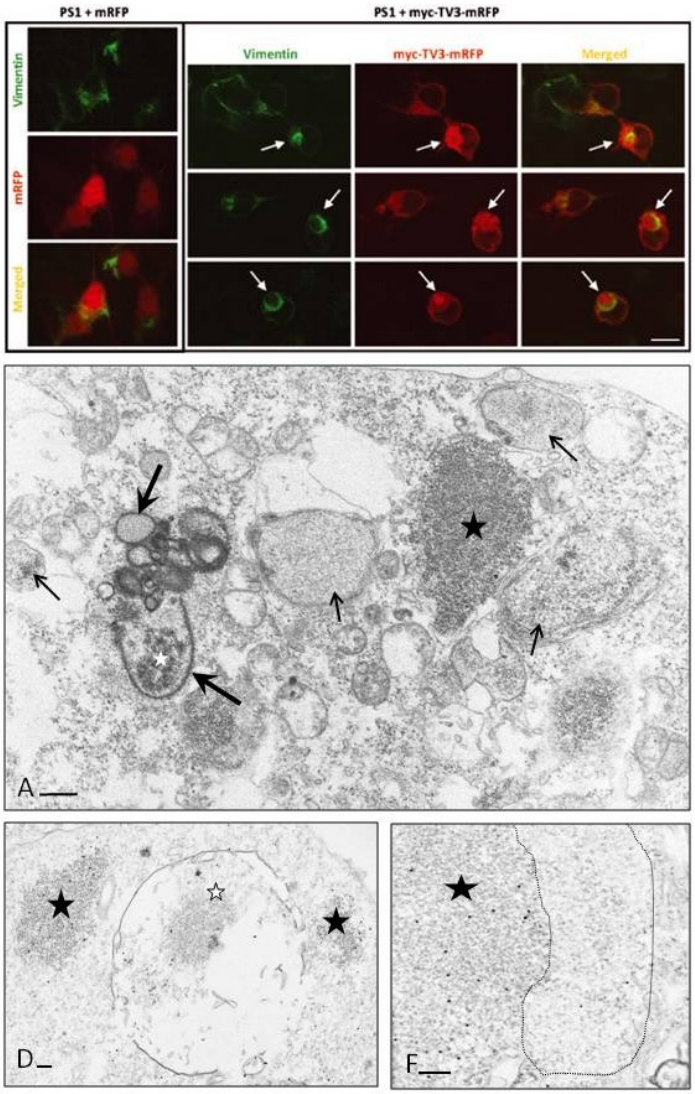

Figure 19. *Differences of confocal and electron microscopic images to localize the structures of interest. B) Confocal microscopic images. Vimentin (green) localizes intracellular and close to the plasma membrane in cells cotransfected with PS1 and mRFP control plasmid (left panel). In cells cotransfected with PS1 and myc-TV3-mRFP, vimentin redistributes to and forms an envelope around the aggresome, as indicated by a green ring surrounding the red TV3-containing aggresome core (right panel). Scale 10 µm. A, D, F) Transmission electron micrographs. A) Photoconversion of mRFP results in electron-dense deposit formation that is located in between the double membranes forming autophagic vacuoles (large arrows). Aggresomes are either freely dispersed in the cytoplasm (black star) or inside the forming autophagosome (white star). Scale 500 nm. D) Gold particles are concentrated in the aggregates in myc-immunostained sections. Aggregates in the cytoplasm gather stronger immunoreactvity than that inside the vacuole (white star). F) This aggresome has close contact with the neighboring interfilament bundle (dashed line around). Scale in D and F: 200 nm. (Published in Viswanathan and Miettinen et al. 2011).*

2.3. Possibilities to image living cells in 3D by using electron microscopy

The example presented in figure 1 demonstrates well the advantages of the electron microscopic investigation when compared to confocal microscopy. However, in this example, the material had gone through extensive series of treatment with fixatives, solvents and contrasting agents. In other words, the cells viewed at TEM were immobile dead cells. To overcome this problem cryo-TEM technology should be used. This technology is a promising tool to study living cells though it still has some limitations when compared to fluorescence based microscopy as well as routine EM (Pilhofer et al. 2010). Cryo-TEM applications, although they are advertised as a tool to investigate live cells, require cells to be immobile in order to facilitate investigation under electron beam. For cryo-TEM the cells are immobilized by freezing (Leis et al. 2009). After this procedure the cells can be considered to be alive, since they are not fixed with any chemical fixative that denatures proteins. Furthermore, it has been shown that after cryo-TEM cells can be grown again after they have thawed (Erk et al. 1998). Nevertheless, while imaging, it is not possible to study momentarily temporal events with TEM on biological tissue. The new 4D ultrafast electron microscopes utilizing single-electron stroboscopic imaging are opening new avenues to study dynamic processes at electron microscopic level (Lobastov et al., 2005; Ortelan and Zewail, 2011). However, these systems are not yet fully applicable in biological living sample. Future will show how well they fill the gap between electron microscopic and fluorescence based imaging techniques.

The other limitation with cryo-EM is that cryo sections are poor in contrast. Biomolecules are mainly composed of light atoms that do not capture electrons like the stain particles in traditional EM-sections. To increase contrast, high electron irradiation dose is required. However, this would destroy unfixed biological molecules. It has been estimated that around 50-200 $e^-/Å^2$ as a total dose can be used for collecting an image series before structural damage takes place. (A total dose for resin embedded specimens can be 1000 $e^-/Å^2$ or more (Subramanian 2005)). This is not enough to acquire sharp images of atoms or even secondary structure of proteins. As a result the domains and rough morphology of complexes are identified.

For conventional SEM, the samples need to be dry, clean, vacuum compatible and electrically conductive. This limits usage of SEM in biological samples that needs to be alive or at least unfixed. To overcome these limitations, an environmental scanning electron microscope (ESEM) was developed in the mid eighties. It allows imaging specimens that are in natural state, i.e. wet and uncoated. The major advantage of this instrument is that it allows studying specimens in varying temperature, pressure and gas composition. The resolution is, however, in the range of that obtained by SEM and thus does not provide major improvement for the currently available TEM methods. (Kimseng and Meissel, 2001; Danilatos 1991.)

To perform optimal cryo-EM, the TEM should use accelerating voltage at least 200-400 keV with FEG. This would yield higher coherence and brightness of the beam. Furthermore, as in these systems electrons have higher energy the interaction with the specimen remains lower as they can pass through the specimen faster. An additional electron filter that can separate electrons by their energy is very useful equipment to discard the electron that have lost energy due to this interaction and are finally adding only noise to the image. For keeping the specimen in vitrified state, cryogenic conditions (-196ºC), a cryoholder or an integrated liquid nitrogen cooled stage has to be used. The digital camera and stage controlling unit need to be computer controlled in order to automatically collect image series during tilting the specimen. The cameras are usually nowadays 4 to 16 megapixel cameras that must be highly sensitive to electrons. (Koning and Koster, 2009.) All of these requirements increase the costs in TEM analyses in which already the basic instrument is costly and needs specialists to take care, make specimens and instruct new users for the instrument thus limiting more widespread use of these systems.

Like in other imaging modalities, tomography is a powerful tool to gain more information about the structure also at the electron microscopic level. When it is applied to routine EM-samples it can work also in combination with cryo technique. Image series at EM level are collected while tilting the specimen holder. Depending on the sample thickness, tilting axes can be ±70º and the series is collected with e.g. 1-2º increment. There are some practical difficulties since the goniometer is never absolutely perfect and some drift will appear. Thus, the beam and the image have to be shifted and the focus corrected after each image exposure. Single images in a tilt series is then merged together to form a 3D-reconstruction of the sample. (Bárcena and Koster 2009; Leis et al. 2009; Pilhofer et al 2010.) Like in other imaging modalities this requires multiple image processing and registration steps. There are nowadays, both commercially and freely available software packages to acquire image series and make the tomograms. These software packages allow several tomograms to be produced in a same day. This enables screening of several samples and cells that is a prerequisite for any research, these days.

Electron tomography, in general, is filling the gap between the x-ray crystallography and optical microscopy. With the aid of development of automation technologies for processing samples, collecting and segmentation and mining specific features in images, the technology will open new perspectives and make vital contribution for our understanding of biological events at nanoscale level. Electron tomography is comparable to medical imaging modalities, computer tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) etc. that provide 3D-views at the macroscopic level (Subramaniam, 2005; Bárcena and Koster 2009). Electron tomography does it at the subcellular level.

2.4. From nanoscale measurements to network

Recent development of image cytometry has filled the gap between flow cytometry that provides quantitative data of large populations of cells and fluorescence microscopy that gives qualitative, morphological data of cells, but usually only in low throughput range (Kim et al 2010). Silicon microelectronics has made computation faster, cheaper, more accessible and powerful. Microfluidics chips, miniaturized sensors and analytical devices are possible to be generated so that real-time measurements at the level of single cells or even cellular compartment become feasible. Chips require only little space and maintenance. For example, cell culture chips can hold individual cells in hundreds of small champers. In those miniature vials, high number of different chemicals, treatments or disease related proteins etc. can be tested simultaneously. (Connelly and Sharp, 2009.) These types of high-throughput studies are not only possible in light microscopy range, but are addressed to automated preparation, analyses, data collection, 3D-reconstruction etc. of the electron microscopic specimens as well (Lyumkis et al. 2010).

Digitization of images and its adoption in different imaging modalities has increased the speed of data collection, analyses and sharing it with other researchers. It has also enabled analysis in a controlled manner thus increasing also quality and reproducibility. The dramatic increase in network speed and economical transmission of data has made communication between researchers possible much easier and faster. Furthermore, high-powered computers and large-capacity of data storage devices are nowadays affordable. All of these have been the building blocks for today's picture archiving and communication system (PACS). The PACS system is supposed to include at least image display, data archiving and data management components. PACS server is the key element in entire PACS system. It receives the images from various imaging modalities, manages data, is the interface between other registration systems, sends the images to PACS workstations, controls the image storage archives, controls the interfaces between PACS workstation and other devices and distributes the images. (Jan, 2005.) Thus, this could be used as a framework to build architecture for integrating microscopic investigation, analyses, controlling and sharing the data among researchers and different forums needing and utilizing the data. An example of such system is a common tool that is being developed by the World Wide Protein Databank in conjunction with EM DataBank for depositing and achieving cryo-EM-derived macromolecule structural data (Lawson, 2010). The future plans involve also inclusion of validation tools and criteria for assessment of map and fitted coordinate models.

3. Technical solutions offered for the future

3.1. SHG and THG provide label-free microscopy

In addition to fluorescence emission that is an incoherent process, coherent nonlinear molecular spectroscopy also generates a large variety of optical signals. Among these techniques that include Raman scattering, two-photon absorption, pump-probe spectroscopy, second and third Harmonic Generation (SHG and THG) microscopy could be a solution for imaging cells and tissue without prior labeling the structural elements (Min et al. 2011). These techniques are based on producing contrast by detecting variations in specimen's ability to generate second or third harmonic light from the incident light. In these microscopes, two (in SHG) or three (in THG) simultaneously incoming photons are transformed into one emitted photon with exactly half (in SHG) or one third (in THG) the wavelength of light entering into the specimen. (Rehberg et al, 2011.) The light source is commonly a titanium-sapphire laser providing high peak power. In addition to the fact that these systems do not require labeling of the tissue, relatively deep structures (up to almost 600 µm deep) can be investigated without inducing phototoxic damage. Even though these microscopes have already been used to study living cells and tissue with great details (Nuriya et al 2005, Rehberg et al 2011), these systems still needs to be improved to reach similar resolution as super resolution fluorescence microscope or transmission electron microscope. Furthermore, major worry with these systems is that the material under investigation has to have a specific molecular orientation. If the harmophoric structure is not in suitable orientation to the incoming laser beam and if the optical conditions in surrounding sample are unfavorable to provide contrast, the strength of SHG and THG signals can be weak or even absent. (Rehberg et al 2011.) As a result some structures or parts of them may not become visible. However, these systems have great potentials in label-free live cell/tissue imaging if resolution is improved. The problem with non-existing structures could be resolved by imaging the object in different orientation. The 3D-scanning instruments, such as MRI or PET could be used as model to design a miniature MRI utilizing SHG or THG paradigm. One possibility is provided by the lens free optical tomography (see next chapter) or otherwise viewing the sample from different angles either rotating the light source or the sample under the objective (Fig. 20).

3.2. Lens free optical tomographic microscope as a prototype for future microscope

Lensless wide-field fluorescent imaging has been presented during recent years especially applied to high-throughput imaging cytometry, rare cell analysis and microarray research (Coskun et al 2010). Recently, Ikizman and colleagues (2011)

introduced a lens free optical tomographic microscope that has many advantages over the current approaches and that could also resolve many of the issues raised up in the above discussion. First of all, the system is very small thus, representing different approach than seen during the history of microscopy i.e. making imaging systems larger and more complex. The system is small enough to fit onto an opto-electronic chip thus providing great potentials for miniaturizing tools in microscopy for life-sciences. The other major invention in this system is that no lenses are used in imaging. To image the cellular structures is based on the fact that the organic structures are partially transparent and the cell outlines and subcellular structures can be visualized by the shadow that forms after shining the sample by the light. The holograms are captured by digital sensor array thus providing larger field of view (15-96 mm^3) and depth of field when compared to lenses that focuses only on a narrow area of a sample. This lens-free system is envisioned to be a component of a lab-on-a-chip platform. It could be e.g. under a microfluidic chip facilitating automated high-throughput studies and simultaneous analyses of samples. 3D-imaging is performed by rotating the light source around the sample in multiple angles along two orthogonal axes (i.e. dual axes tomography, Fig. 20A). (Isikman et al, 2011.) Unfortunately this system gives only 1 μm lateral and 3 μm axial resolutions at the moment. If the field of view is increased, resolution lateral resolution is reduced further i.e. 1.2-1.8 μm laterally and 3-6 μm axially. This is much worse than resolution (20-120 nm) that is obtained by near-field scanning optical microscope (NSOM) in which lenses are replaced by using only small aperture and near-field imaging (Fernandez-Suarez and Ting, 2008). However, the general idea is intriguing providing tool for live cell spatial and temporal imaging with speed, high-throughput, low cost and simple approach. This system when adapted into other microscopic system such as SHG/THG or even ultrafast 4D electron microscopy that gives atomic level resolution could constitute as an initiative for future microscope.

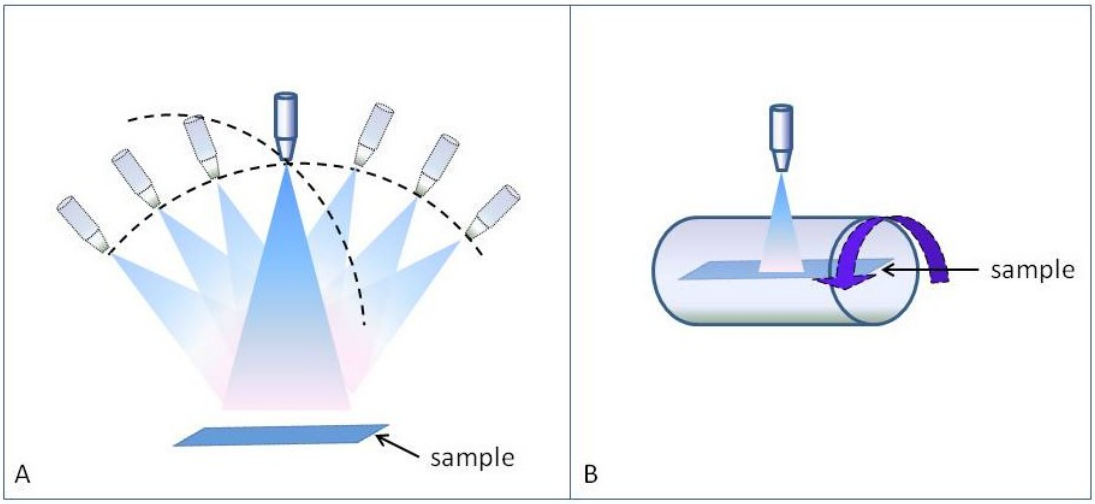

Figure 20. *Two options for tomographic imaging at microscopic level. A) The lens free optical tomographic system suggested by Ikisman and colleagues (2011). The light source is rotated around the specimen in multiple angles. B) Another option could be by rotating the sample itself, the light source being stationary. In both cases, the detector could be beneath the sample or the signal could be collected with the same objective used to illuminate the sample. Both options could be applied for SHG or THG measurements to get signal from structural components of varying orientations.*

3.3. Microscopic analyses integrated to the network

Whatever the imaging modality is the data produced by this system should be available for other researchers as well. This enables fast utilization of the research information in practical applications as well as further development. A schematic drawing about the workflow and the network in which the microscope unit could be integrated is provided in figure 21 below.

The main idea is to be able to collect and compare results from different studies in order to make consensus what is actually happening at cellular level and what it would be at organism level. Quality controlling step would help to make experiments standardized and validated. This facilitates repetition and further tests also by other researchers. Communication with other researchers and databases should be flexible and fast. The data should be easy to access and use. In other words the microscope unit should be fully integrated to the network. However, one key issue in this type of network is to take care of data privacy and security. The design of security system depends especially on the material used in the experiment (note confidentially concerning protected and private information). As setting the security system includes multiple different issues such as confidentiality, data integrity, availability to authorized persons, accountability, traceability etc. and all of them are unique for setting and the data handled in the network, they are not discussed here in more detail. Here, only the general idea and the major components that can be integrated to the network associated to microscopic work is presented.

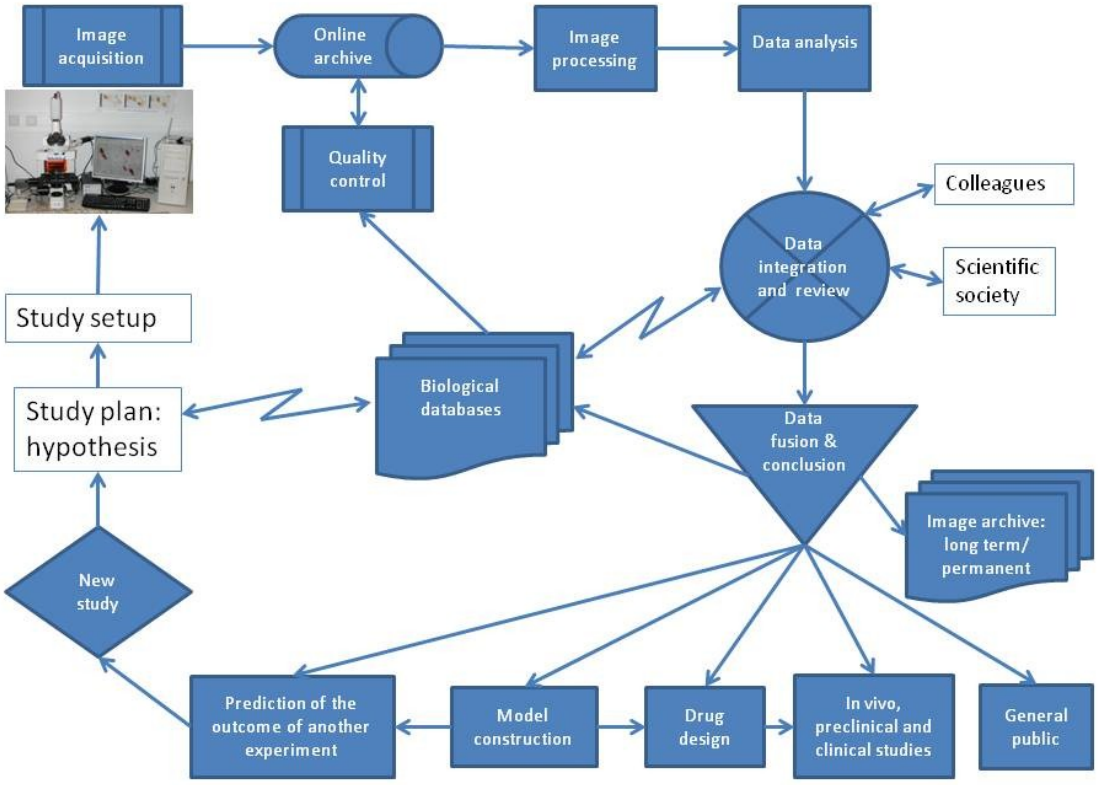

Figure 21. *Schematic drawing of microscope unit integrated into the network.*

Conclusions

Fluorescence far-field optical microscopy has revolutionized visualization of the molecular events in living cells. It holds key role in the current research in life sciences even though electron and scanning probe microscopy can provide higher resolution. As overall in research, not a single system is sufficient to give the full answer, but investigation has to be done by using different approaches. Also in microscopy, different microscopes are still required to get the full picture of the problem in interest. Especially helpful are e.g. correlative light and electron microscopic approaches (Miettinen and Freund, 1992; Plitzko et al. 2009). However, when carefully going through and collecting the best features from the current microscopic systems, new approaches can be settled for new technologies. The researchers are actively looking for new solutions to get the best features of super-resolution microscopy and electron microscopy into the same instrument. Now there are two different approaches: 1) merging super-resolution microscopy with electron microscopy and 2) integration cryo-electron microscopy with fluorescence microscopy (Leslie, 2011). These approaches are in line with the main idea of the current thesis i.e. to implement more electron microscopy to the studies in life sciences.

As it is obvious from the above presentation, microscopy nowadays is not anymore simple viewing the specimens through oculars, but a branch of science that requires multidisciplinary knowledge and skills to understand the outcome of the analyses. It is not anymore sufficient to master the sample processing and staining methods, but understanding of variety of fluorescence tagging techniques, imaging tools, digital image processing, computation, data management etc. are required. The operator needs broad knowledge not only in anatomy, cell biology, but also physiology, physics, chemistry, signal processing and computer science, in general. All of this should also be adapted and used in a high throughput range. Actually, at the end we would like to have a system that would run the experiment using variety of parameters (hopefully set by the system itself), analyze the material, give the ready results, compare them with existing databases, make the illustrations and finally write the publication, and further on give suggestions to the next experiment. This self-iterating process would need a sort of “perpetual motion machine” that was a dream already in the Middle Ages, but now it should be applied to microscopic research.

"Student: Dr. Einstein, Aren't these the same questions as last year's [physics] final exam?
Dr. Einstein: Yes; But this year the answers are different."
— Albert Einstein —

References

Allen, R.D., David, G.B. & Nomarski, G. 1969. The Zeiss-Nomarski differential interference equipment for transmitted-light microscopy. *Zeitschrift für Wissenschaftliche Mikroskopie und Mikroskopische Technik* 69, pp. 193-221.

Allen, S., Rigby-Singleton, S.M., Harris, H., Davies, M.C. & O'Shea, P. 2003. Measuring and visualizing single molecular interactions in biology. Biochem Soc Trans Oct 31, 5, pp. 1052-1057.

Aquino, D., Schönle, A., Geisler, C., Middendorff, C.V., Wurm, C.A., Okamura, Y., Lang, T., Hell, S.W., Egner, A. 2011. Two-color nanoscopy of three-dimensional volumes by 4Pi detection of stochastically switched fluorophores. Nat Methods Apr 8, 4, pp. 353-359.

Bacia, K., Kim, S.A. & Schwille, P. 2006. Fluorescence cross-correlation spectroscopy in living cells. Nat Methods Feb 3, 2, pp. 83-89.

Bárcena, M. & Koster, A.J. 2009. Electron tomography in life science. Semin Cell Dev Biol Oct 20, 8, pp. 920-930.

Brown, C.M. 2007. Fluorescence microscopy--avoiding the pitfalls. J Cell Sci May 15, 120(Pt 10), pp. 1703-1705.

Carr, K.E. & Davidson, M.W. 2000. Basic microscope ergonomics. [Cited 25.4.2012]. Available: http://www.microscopyu.com/articles/ergonomics/ergointro.html.

Christensen, A.K.1971. Frozen thin sections of fresh tissue for electron microscopy, with a description of pancreas and liver. J Cell Biol Dec 51, 3, pp. 772-804.

Conchello, J.-A. & Lichtman, J.W. 2005. Optical sectioning microscopy. Nature Methods 2, pp. 920 – 931.

Claxton, N.S., Fellers, T.J. & Davidson, M.W. 2006. Microscopy, Confocal. In Encyclopedia of Medical Devices and Instrumentation. John Wiley & Sons Inc. see also Laser scanning confocal microscopy. http://www.olympusconfocal.com /theory/LSCMIntro.pdf, 2.1.2012

Connelly, T. & Sharp, P. 2009. A new biology for the 21st century. Committee of A new biology for the 21st century: Ensuring the United States leads the coming biology revolution. National Research Council. The National Academic Press, Washington D.C. [internet publication] 112 p., [Cited 11.11.2011]. Available: http://www.nap.edu/catalog/12764.htlm.

Coskun, A.F., Sencan, I., Su, T.W. & Ozcan, A. 2010. Lensless wide-field fluorescent imaging on a chip using compressive decoding of sparse objects. Opt Express May 10, 18(10), pp. 10510-10523.

Danilatos, G.D. 1991. Review and outline of environmental SEM at present. J Microsc 162, pp. 391-402.

DeFelipe, J. & Jones, E.G. 1992. Cajal on the cerebral cortex. An annotated translation of the complete writings. Oxford University Press, New York, pp. 5-6.

Denk, W. & Horstmann, H. 2004. Serial block-face scanning electron microscopy to reconstruct three-dimensional tissue nanostructure. PLoS Biol Nov 2, 11, p. 329.

De Souza, N. 2009. Milestone 21. Light microscopy at the limit. In Milestones in light microscopy. Nat Cell Biol Oct 11, 10, p. 1165. S22.

Dykstra, M.J. & Reuss, L.E. 2003. Theory, Techniques, and troubleshooting. In Biological Electron microscopy. 2nd Ed. Kluwer Academic/Plenum Publishers, New York, USA, 73 p.

Erk, I., Nicolas, G., Caroff, A. & Lepault, J. 1998. Electron microscopy of frozen biological objects: a study using cryosectioning and cryosubstitution. J Microsc Mar 189, 3, pp. 236-248.

Fan, G.Y. & Ellisman, M.H. 2000. Digital imaging in transmission electron microscopy. J Microsc Oct; 200, 1, pp. 1-13.

Favia, A.D. 2011. Theoretical and computational approaches to ligand-based drug discovery. Front Biosci Jan 1, 16, pp.1276-1290.

FilamentTracer by Bitplane. [Cited 4.1.2012]. Available: http://www.bitplane.com/go/products/filamenttracer.

Fernández-Suárez, M. & Ting, A.Y. 2008. Fluorescent probes for super-resolution imaging in living cells. Nat Rev Mol Cell Biol Dec 9, 12, pp. 929-943.

Gambin, Y. & Deniz, A.A. 2010. Multicolor single-molecule FRET to explore protein folding and binding. Mol Biosyst Sep 6, 9, pp. 1540-1547.

Gugel, H., Bewersdorf, J., Jakobs, S., Engelhardt, J., Storz, R. & Hell, S.W. 2004. Cooperative 4Pi excitation and detection yields sevenfold sharper optical sections in live-cell microscopy. Biophys J Dec 87, 6, pp. 4146-4152.

Gratton, E. & vandeVen, M.J. 2006. Laser Sources for Confocal Microscopy. *Handbook of Biological Confocal Microscopy*, third edition, ed. James B. Pawley, Springer Science Business Media, New York, 2006. p. 80-125

Hauglund, R. P., Gregory, J., Spence, M. T. Z., Johnson, I. & Miller, E. 2002. Introduction to fluorescence techniques. In: Handbook of Fluorescent Probes and Research Products. Molecular Probes, Inc., Eugene, Oregon, pp. 1-6.

Hell, S.W. Double confocal microscope. European Patent 0491289 (1990).

Hell, S.W. 2009. Microscopy and its focal switch. Nat Methods Jan 6, 1, pp. 24-32.

Hell, S.W., Schmidt, R. & Egner, A. 2009. Diffraction-unlimited three-dimentional optical nanoscopy with opposing lenses. Nature Photonics June 29, pp. 381-387.

Hell, S.W. 2003. Towards fluorescence nanoscopy. Nature Biotechnology 21, 11, pp. 1347-1355.

Henriques, R., Griffiths, C., Hesper R.E & Mhlanga, M.M. 2011. PALM and STORM: unlocking live-cell super-resolution. Biopolymers May 95, 5, pp. 322-331.

Isikman, S.O., Bishara, W., Mavandadi, S., Yu, F.W., Feng, S., Lau, R. & Ozcan, A. 2011. Lens-free optical tomographic microscope with a large imaging volume on a chip. PNAS 108, 18, pp. 7296-7301.

Jan, J. 2005. Medical Image Processing, Reconstruction and Restoration. Concepts and Methods
CRC Press. Taylor & Francis Group, Boca Raton, 710 p.

Juette., M.F., Gould, T.J., Lessard, M.D., Mlodzianoski, M.J., Nagpure, B.S., Bennett, B.T., Hess, S.T. & Bewersdorf, J. 2008. Three-dimensional sub-100 nm resolution fluorescence microscopy of thick samples. Nat Methods Jun 5, 6, pp. 527-529.

Rosenthal C. 2009. Milestone 1. The beginning. In: Milestones in light microscopy. Nat Cell Biol Oct 11, 10, p. 1165. S6

Kimseng, K. & Meissel, M. Short overview about ESEM, Environmental electron microscope. [Cited 12.1.2012]. Available: http://www.calce.umd.edu/TSFA/ESEM.pdf.

Koning, R.I. & Koster, A.J. 2009. Cryo-electron tomography in biology and medicine. Ann Anat Nov 191, 5, pp. 427-445.

Lappalainen, K., Miettinen, R., Kellokoski, J., Jääskeläinen, I. & Syrjänen, S. 1997. Intracellular distribution of oligonucleotides delivered by cationic liposomes: light and electron microscopic study. J Histochem Cytochem Feb 45, 2, pp. 265-274.

Lasslett, A. 2006. Principles and applications of differential interference contrast light microscopy. Microscopy and Analysis 20, pp. 9-11.

Lawson C.L. 2010. Chapter four - Unified data resource for cryo-EM. 2010. Methods Enzymol 483, pp. 73-90.

Leis, A., Rockel, B., Andrees, L. & Baumeister, W. 2009. Visualizing cells at the nanoscale. Trends Biochem Sci Feb 34, 2, pp. 60-70.

Lepistö T. (2005) 2803000 Materiaalitieteen fysikaaliset tutkimusmentelmät – luentomoniste 1 ja 2 (vedos 090904). Tampereen teknillinen yliopisto

Leslie, M. 2011. Two microscopes are better than one. In Focus. J Cell Biol 3, p. 3.

Lichtman, J.W. & Conchello, J.-A. 2005. Fluorescence microscopy Nature Methods 2, pp. 910 – 919.

Lobastov, V.A., Srinivasan, R. & Zewail, A.H. 2005. Four-dimentional ultrafast electron microscopy. PNAS 102, 20, pp. 7069-7073.

Lyumkis, D., Moeller, A., Cheng, A., Herold, A., Hou, E., Irving, C., Jacovetty, E.L., Lau, P.W., Mulder, A.M., Pulokas, J., Quispe, J.D., Voss, N.R., Potter, C.S. & Carragher, B. 2010. Automation in single-particle electron microscopy connecting the pieces. Methods Enzymol 483, pp. 291-338.

Miettinen, R. & Freund, T.F. 1992. Neuropeptide Y-containing interneurons in the hippocampus receive synaptic input from median raphe and GABAergic septal afferents. Neuropeptides Jul 22, 3, pp. 185-193.

Min, W., Freudiger, C.W., Lu, S. & Xie, X.S. 2011. Coherent nonlinear optical imaging: beyond fluorescence microscopy. Annu Rev Phys Chem May 62, pp. 507-530.

Minsky, M. 1988. Memoir on inventing the confocal scanning microscope. Scanning 10, pp. 128-138.

Molecular Probes. [Cited 31.1.2012]. Available: http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Analysis/Cellular-Imaging/Fluorescence-Microscopy-and-Immunofluorescence-IF.html.

Morris, C.G. 1992. Ed. *Academic Press dictionary of science and technology*. Academic Press Ltd, London, 2432 p.

Müller, S.A., Aebi, U. & Engel, A. 2008. What transmission electron microscopes can visualize now and in the future. J Struct Biol Sep 163, 3, p. 235

Müller, S.A., Müller, D.J. & Engel, A. 2011. Assessing the structure and function of single biomolecules with scanning transmission electron and atomic force microscopes. Micron Feb 42, 2, pp. 186-195.

Nakano, A. 2002. Spinning-disk confocal microscopy -- a cutting-edge tool for imaging of membrane traffic. Cell Struct Funct Oct 27, 5, pp. 349-355.

Neu, T.R., Manz, B., Volke, F., Dynes, J.J., Hitchcock, A.P. & Lawrence, J.R. 2010. Advanced imaging techniques for assessment of structure, composition and function in biofilm systems. FEMS Microbiol Ecol. 2010 Apr 72, 1, pp. 1-21.

Ortelan, O. & Zewail, A.H. 2011. 4D Scanning transmission ultrafast electron microscopy: single particle imaging and spectroscopy. J Am Chem 133, 28, pp. 10732-10735.

Petty, H. R. 2007. Fluorescence microscopy: established and emerging methods, experimental strategies, and application in immunology. Microscopy Research and Technique 70, pp. 687-709.

Pilhofer, M., Ladinsky, M.S., McDowall, A.W. & Jensen, G.J. 2010. Bacterial TEM: new insights from cryo-microscopy. Methods Cell Biol 96, pp. 21-45.

Plitzko, J.M., Rigort, A. & Leis, A. 2009. Correlative cryo-light microscopy and cryo-electron tomography: from cellular territories to molecular landscapes. Curr Opin Biotechnol Feb 20, 1, pp. 83-89.

Rehberg, M., Krombach, F., Pohl, U. & Dietzel, S. 2011. Label-free 3D visualization of cellular and tissue structures in intact muscle with second and third harmonic generation microscopy. PLOS One 6, 11, e28237.

Rusk, N. 2009. Milestone 4. The fluorescence microscope. In: Milestones in light microscopy. Nat Cell Biol Oct 11, 10, p. 1165. S8

Schatten, H. 2011. Low voltage high-resolution SEM (LVHRSEM) for biological structural and molecular analysis. Micron Feb 42, 2, pp. 175-185.

Schmolze, D.B., Standley, C., Fogarty, K.E. & Fischer, A.H. 2011. Advances in microscopy techniques. Arch Pathol Lab Med Feb 135, 2, pp. 255-63.

Singh, N., Chevé, G., Ferguson, D.M. & McCurdy, C.R. 2006. A combined ligand-based and target-based drug design approach for G-protein coupled receptors: application to salvinorin A, a selective kappa opioid receptor agonist. J Comput Aided Mol Des Jul-Aug 20, 7-8, pp. 471-493.

Sillanpää, J. & Nyberg, M. 2010. The ergonomics of microscope work. In: Microscopy: Science, Technology, Applications and Education. Eds. Méndez-Vilas A and Diaz J. FORMATEX pp. 1533-1538.

Slayter and Slayter. Light and electron microscopy. Cambridge University Press, New York. 1992

Soukka, T., Rantanen, T. & Kuningas, K. 2008. Photon upconversion in homogeneous fluorescence-based bioanalytical assays. Ann N Y Acad Sci 1130, 188-200.

Spring, K. R. 2003. Fluorescence microscopy. In: Encyclopedia of Optical Engineering, Marcel Dekker, New York, New York, pages 548-555 (2003).

Stolojan, V. 2008. Scanning transmission electron microscopy. A tool for biology and materials sciences. Microscopy and Analysis 22, 4, pp. 15-18.

Subramanian, S. 2005. Bridging the imaging gap: visualizing cellular architecture with electron tomography. Curr Opin Microbiol June 8, 3, pp. 316-322.

Van Ooji, C. 2009. Milestone 7. Recipe for fluorescent antibodies. In: Milestones in light microscopy. Nat Cell Biol Oct 11, 10, p. 1165. S10-11

Viswanathan, J., Haapasalo, A., Böttcher, C., Miettinen, R., Kurkinen, K.M., Lu, A., Thomas, A., Maynard, C.J., Romano, D., Hyman, B.T., Berezovska, O., Bertram, L., Soininen, H., Dantuma, N.P., Tanzi, R.E. & Hiltunen, M. 2011. Alzheimer's disease-associated ubiquilin-1 regulates presenilin-1 accumulation and aggresome formation. Traffic Mar 12, 3, pp. 330-348.

Voutou, B. & Stefanali, E.-C. Electron microscopy: The basics. Physics of Advantced Materials Winter School. [Cited 28.2.2012]. Available: http://www.mansic.eu/documents/PAM1/Giannakopoulos1.pdf.

Tian, Y., Martinez, M.M. & Pappas, D. 2011. Fluorescence correlation spectroscopy: a review of biochemical and microfluidic applications. Appl Spectrosc Apr 65, 4, pp. 115A-124A.

Yamamoto, Y.& Shinohara, K. 2002. Application of X-ray microscopy in analysis of living hydrated cells. Anat Rec Oct 15, 269(5), pp. 217-223.

Young, H.D. & Freedman, R.A. 2004. Particle physics and cosmology. In: University Physics 11th Ed. Pearson, Addision Wesley, San Francisco. 2004 pp.1668-1710.

Zernike, F. 1955. How I discovered phase contrast. Science Mar 11, 121(3141), pp. 345-349.

Zucker, R.M. & Price, O.T. 2001. Evaluation of confocal microscopy system performance. Cytometry 44, pp. 273-294.

Zumdahl, S.S. 2005. Atoms, Molecules, and Ions. In: Chemical Principals. 5th Ed. Houghton Mifflin Company, Boston, USA. Total pages pp.14-44.