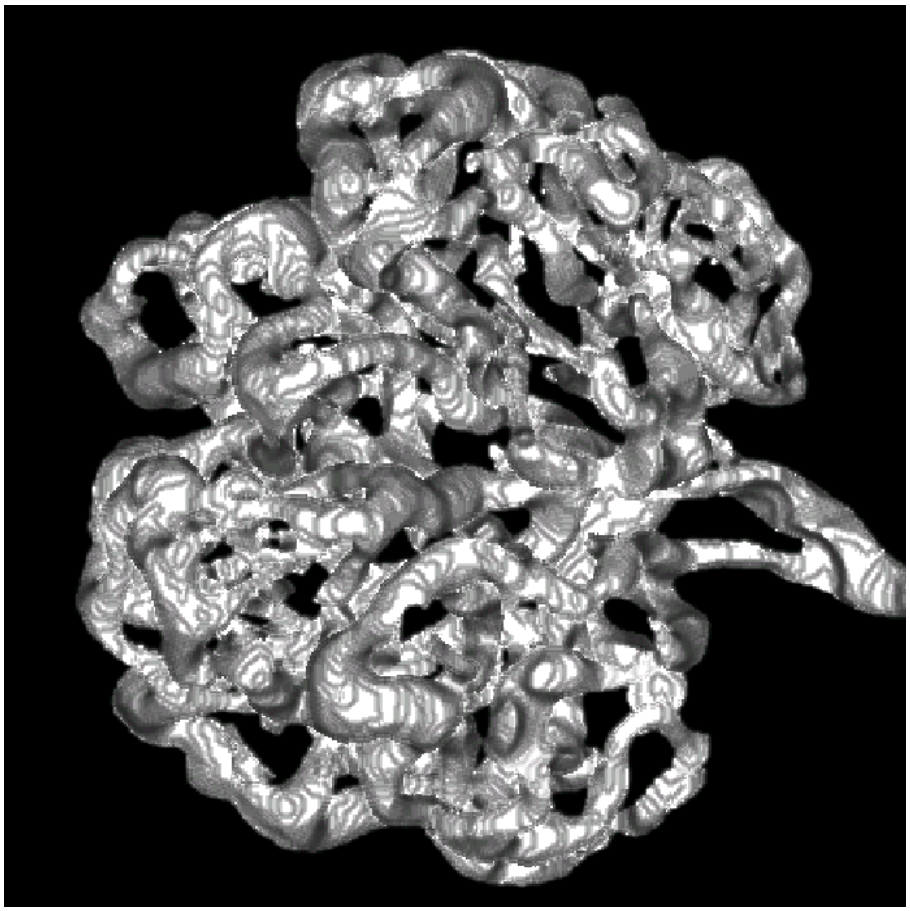
Biomedical & X-ray Physics



Three-dimensional reconstruction of the capillary network in a glomerulus (part of kidney) from rat. Image courtesy of Hjalmar Brismar.

Confocal Microscopy

Laboratory instructions for course SK2500/01, Physics of Biomedical Microscopy

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updated

Önfelt, B, 2013

Important notes:

You should study sections 1-3 of these instructions carefully before the lab session starts!

(It is also recommended that you browse through the rest to familiarize yourself with the contents.)

Preparatory exercises 1-3 must be solved, and written solutions should be presented when you arrive to do the lab.

If you are not properly prepared, you will not be able to carry out the lab exercises.

Bring a copy of these lab instructions with you to lab sessions 3 & 4.

(If you want to keep any of the microscope images you produce, you should bring USB memory stick with you to lab session 4)

1. Introduction

Figure 1 shows a schematic diagram of a confocal microscope. Rather than illuminating an extended region of the specimen, as is done in conventional microscopy, only a single specimen point is illuminated at a time. A laser is commonly used as light source because it provides an intense beam of well-collimated light. The size of the illuminated spot is determined by diffraction effects (and aberrations) in the optics, and is typically on the order of 0.5 μm. Reflected or fluorescent light from the illuminated specimen spot is collected by the objective and focused onto a small aperture. Light passing through this aperture will reach the detector, which is usually a photomultiplier tube (PMT). See ref. 1 for a description of PMTs. (The reference list is included at the end of these instructions, before the appendices). Because only a single specimen point is illuminated, the confocal arrangement does not by itself produce an image of the specimen. It is therefore necessary to include a scanning arrangement in all confocal systems.

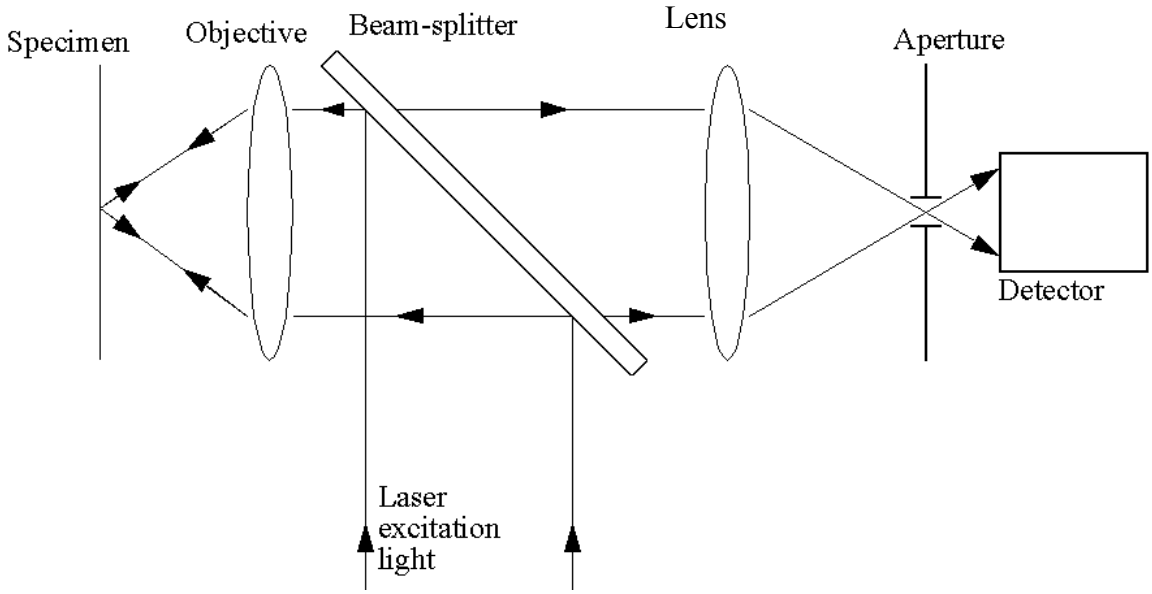


Fig. 1. The confocal principle.

Scanning can be performed in two different ways - either the specimen is moved and the laser beam is kept stationary or vice versa. Many early confocal microscopes used the specimen scanning method, whereas today beam scanning dominates strongly. However, both methods have certain advantages and disadvantages. The specimen scanning method gives uniform imaging properties over the entire image area since off-axis aberrations and vignetting will not be present. But specimen scanning requires precise movements of the specimen at high speed which can be difficult to achieve, especially for heavy specimens. Beam scanning, on the other hand, is much less demanding concerning mechanical precision since the scanning mechanism can be placed on the image side of the microscope objective, thus acting on a magnified image of the specimen. Also, specimen size and weight is of no concern when beam scanning is used. The disadvantage with beam scanning is that the imaging properties will not be uniform over the image area due to off-axis aberrations and vignetting.

Confocal microscopy is superior to conventional microscopy for two reasons. First, it produces images with higher resolution. Second, it is possible to record the three-dimensional

(3-D) structure of a specimen. The improvement in resolution is 36% under ideal conditions (infinitely small aperture and no aberrations) and is, in most cases, not large enough to justify the higher cost of a confocal microscope. On the other hand, the 3-D imaging properties make a confocal microscope ideal for studying thick biological specimens, e.g. nerve tissue, which are very difficult to study with a conventional microscope.

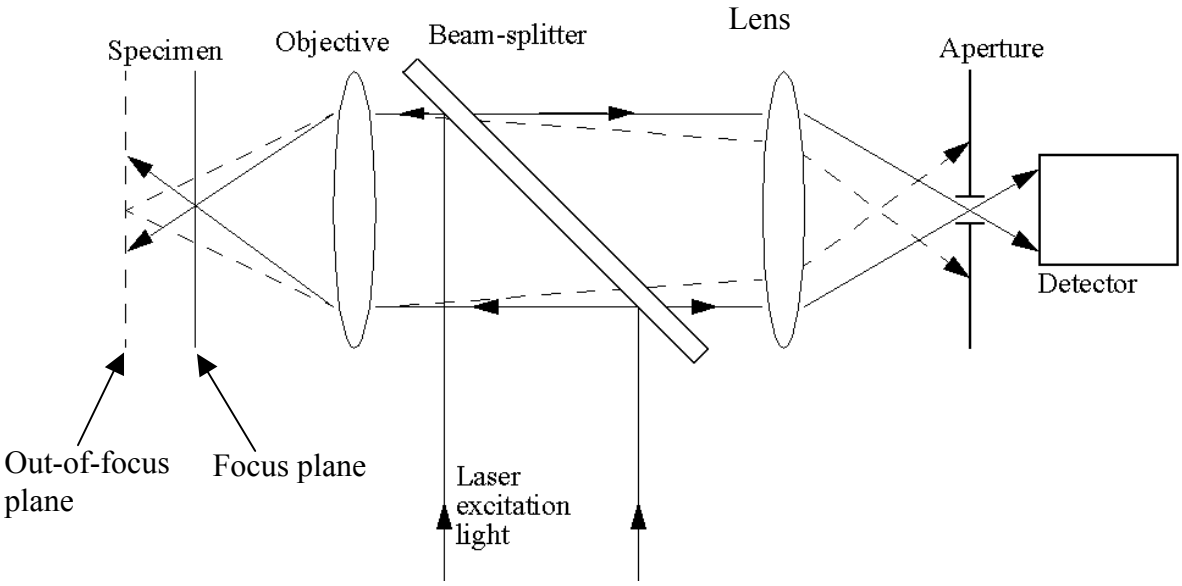


Fig. 2. Illustration of optical sectioning with a confocal microscope. Only light emanating from the focus plane of the microscope will be transmitted through the small aperture.

The mechanism behind the 3-D imaging capability of confocal microscopy is illustrated in Fig. 2. Light from specimen parts in focus (solid line) can pass through the tiny aperture and reach the detector, whereas light from out-of-focus specimen parts is mostly blocked. The effect of this is that only light from a very thin specimen layer is detected. This property of confocal microscopy is called "optical sectioning", and opens the possibility to study very thin (less than 1 μm) sections of biological specimens. By recording a number of such optical sections, and change focus of the microscope slightly between individual sections, it is possible to obtain a "stack" of images representing the 3-D specimen structure. This has several advantages compared with the traditional method of mechanically sectioning the specimen:

- It is much faster.
- The recorded sections will be perfectly aligned, simplifying 3-D reconstruction.
- Living specimens can be studied.

To perform such optical sectioning, it is necessary that the specimen is semi-transparent, so that light can penetrate the uppermost layers to illuminate those below. This is the case for most biological specimens, and volumes with a depth of several hundred μm are routinely studied with confocal microscopy. Most specimens studied with confocal microscopy are labeled with fluorescent substances which emit light in the green, yellow and red parts of the spectrum (see section 3). To excite fluorescence in such substances, blue or green laser wavelengths are commonly used. In some cases ultraviolet (UV) excitation is used, but this is difficult because of the demands it puts on the microscope optics as well as the high cost of the laser equipment needed. Also, many biological samples are not as transparent for UV-

radiation as they are for visible light.
When testing the optical sectioning performance for objectives it is much more convenient to use a reflecting specimen than a fluorescent one. A flat, surface-reflecting mirror is often used for this purpose. In ref. 2 the full-width-half-maximum (FWHM) depth response function for an infinitely thin fluorescent layer recorded by (a perfect) confocal microscope is given by eq. 18. When recording a reflecting specimen, the FWHM will be reduced by about 35 % (the constant 8.5 in eq. 18 is replaced by 5.6).

► **Preparatory exercise 1:** Calculate the FWHM depth response when scanning a reflecting specimen with objectives 10/0.3 (dry), 20/0.5 (dry) and 40/1.3 (oil immersion). λ = 488 nm. n_{oil} = 1.52

The FWHM results in prep. exercise 1 are valid only if the optics are free of aberrations, and the detector aperture is infinitely small. The influence of aperture size is discussed in ref. 2 (sect. 2.3).

► **Preparatory exercise 2:** Calculate the maximum detector aperture diameter that can be used when scanning a reflecting specimen with objectives 20/0.5 (dry) and 40/1.3 (oil immersion). The FWHM depth response must not be more than 10% larger than for an infinitely small aperture. λ = 488 nm.

More information on the imaging properties of confocal microscopy is found in ref. 2

Confocal microscopy is a rather old invention - it was described by Marvin Minsky in a US patent issued in 1961 (he originally got the idea in the mid-fifties). Development was then rather slow and sporadic over the next fifteen to twenty years, but in the last couple of decades interest in this field has increased dramatically. The rapid development of computer equipment has put the computing power necessary to utilize the data from a confocal microscope within reach of all scientific laboratories. Also, developments in laser technology have resulted in lasers better suited as light sources in confocal microscopy.

2. The Zeiss LSM 5 Pascal confocal microscope

The confocal microscope used in this laboratory exercise is a Zeiss LSM 5 Pascal with an inverted microscope, Fig. 3. Inverted microscopes are often used in biomedical laboratories because they have certain advantages compared with upright (= normal) microscopes. For example, an inverted microscope is easier to use if you want to study cells growing on the bottom surface of a liquid-filled dish (cells are often cultured in this way). It is also easier to view the specimen while applying electrodes to measure electrical signals, or in other ways manipulate the specimen, if an inverted rather than an upright microscope is used. On the other hand, it is more difficult to see how close the objective is to the specimen when focusing an inverted microscope and it is more difficult to work with immersion liquids.

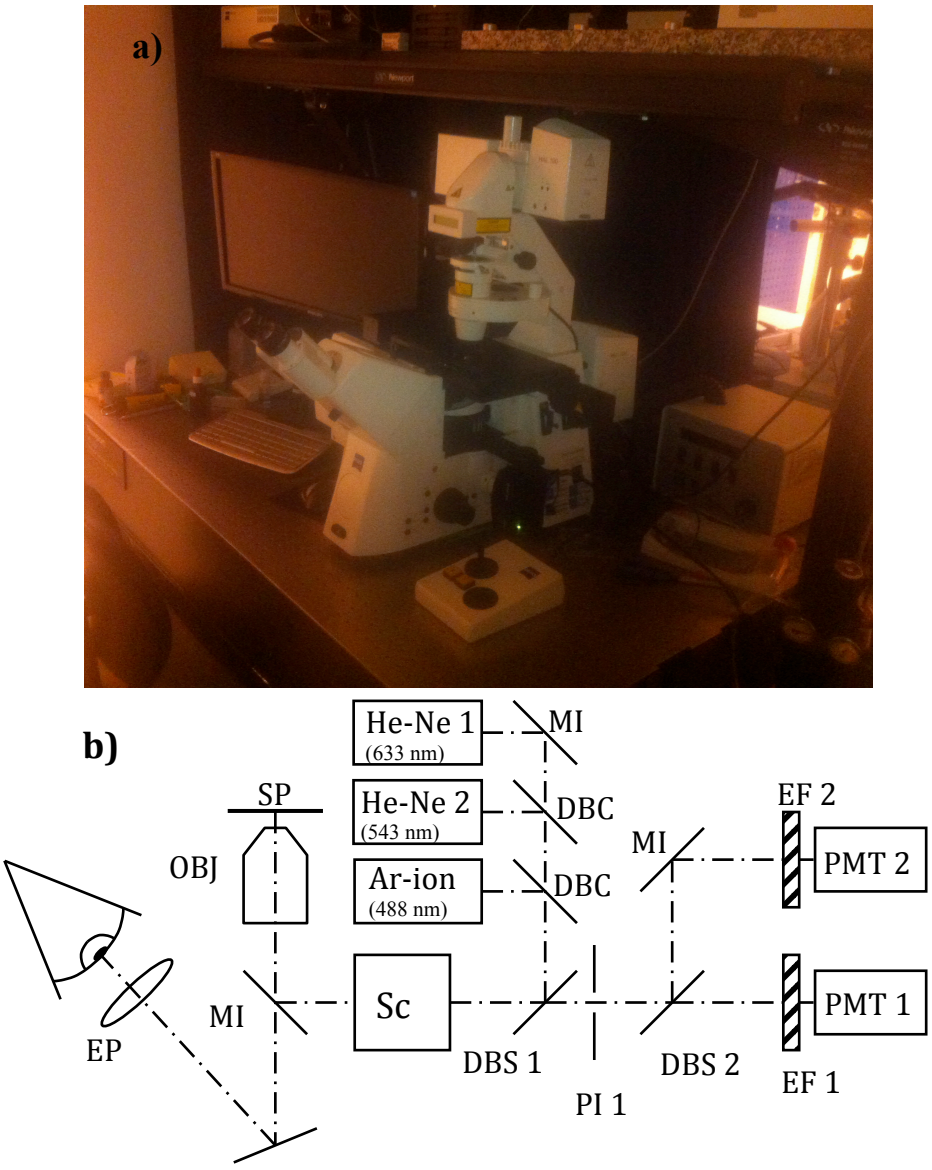


Fig. 3. Zeiss LSM 5 Pascal confocal microscope. a) Photograph of the instrument. b) Simplified schematic diagram. EP = Eyepiece, MI = Mirror, DBC = Dichroic beam combiner, DBS = Dichroic beam splitter, OBJ = Objective, SP = Specimen, Sc = Galvo-scanners, PI = Pinhole (aperture), EF = Emission filter and PMT = Photomultiplier tube. See text for details.

LSM 5 Pascal is equipped with three different lasers: one air-cooled argon ion laser, and two helium-neon lasers. It is possible to get three different illumination wavelengths, 488 nm (Ar-ion), 543 nm (He-Ne) and 633 nm (He-Ne). Depending on the type of specimen, the most suitable wavelength is chosen. Most functions are computer-controlled, for example choice of laser wavelength, microscope objective and optical filters. Referring to Fig. 3b, a brief description of the function will be given. (For details concerning the practical operation, see Appendix A)

The operator can use the microscope as an ordinary inverted microscope with halogen or mercury-lamp illumination of the specimen (illumination details have been omitted in Fig. 3b). In this mode the mirror closest to the objective is removed for transillumination, or replaced with a suitable dichroic beam splitter for epi-fluorescence. In laser-scanning mode, the desired laser is lit and the light passes through a scanning unit with two galvo-scanners that deflect the light in two perpendicular directions (the scanning principle is illustrated in Fig. 19 of ref. 2). The laser light is then reflected via a mirror toward the microscope objective, which focuses the light to a small spot in the specimen. Due to the galvo-scanners, this spot will move back and forth in a plane perpendicular to the optical axis. Fluorescent (or reflected) light from the specimen travels along the same path as the laser light, but in the opposite direction. It will consequently be “descanned” by the galvo-scanners and form a stationary beam when it reaches the dichroic beam splitter DBS 1. The light from the specimen is (at least partially) transmitted by DBS 1, and reaches one of the detectors (PMT1 or 2). The detector signals are analog-to-digital (A/D) converted and stored in the computer. Two detectors are included in the instrument so that two fluorophores with different colors can be detected simultaneously (DBS 2 reflects one color and transmits the other). This is an advantage in biomedical applications where multiple-labeled specimens are often used. Different fluorophores that bind specifically to different types of structures in the specimen can then be used. Immuno-fluorescence, where the fluorophore molecules are attached to different antibodies, is one way of achieving such selective binding.

As can be seen from Fig. 3b there is a large number of dichroic beam splitters and filters in the instrument. The spectral transmission and reflection properties of these, as well as the laser wavelength, must be chosen carefully to get good images. The choice depends on the fluorophores used in a particular application. A very large number of fluorophores, with different properties, exist. This is illustrated and further discussed in ref. 2 (Fig. 30 and associated text).

3. Fluorophores, beam splitter and filters

The laws of quantum mechanics govern, that in a molecule, only certain discrete energy levels are possible. These energy levels are composed of electronic states, which are split up into closely spaced vibrational (and even rotational) sub-levels (Fig. 4). A molecule in an excited electronic state may get rid of its excess energy by emitting a photon. For many molecules, the energy gaps between certain electronic states correspond to energies for photons in the visible range. It is important to note, that a molecule may only absorb light if the photon energy is equal to the energy needed to transfer the molecule between two energy states, and that emitted light may only have certain discrete wavelengths.

Fluorescence

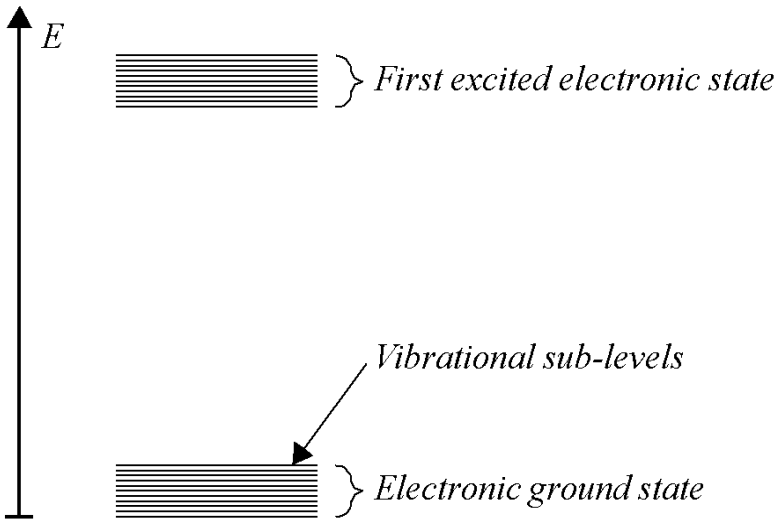


Fig. 4. Energy diagram for a typical molecule. The electronic states are split up into vibrational sub-levels.

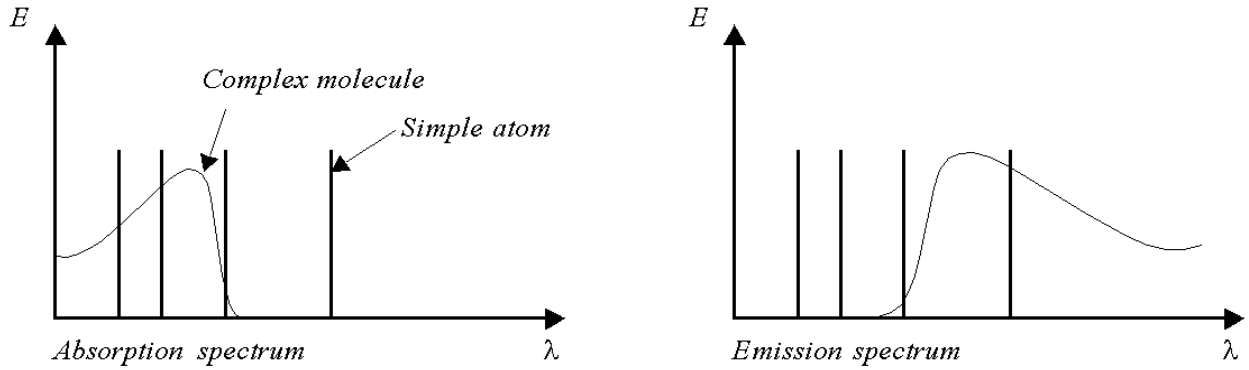


Fig. 5. Typical absorption and emission spectra for simple atoms and complex molecules. The absorption spectra show the ability to absorb incoming light, while the emission spectra show the composition of the emitted light.

Fluorophores are very large organic molecules with a complex structure. The number of energy levels is so large, that a continuous range of wavelengths may be absorbed and emitted (Fig. 5). When an incoming photon is absorbed by the fluorescent medium, the fluorophore molecule is transferred to an excited electronic state. The molecule is likely to be transferred to a higher vibrational sub-level, Fig. 6. From there it is rapidly transferred to the lowest vibrational sub-level within the excited state. After a certain time, the molecule emits a fluorescent photon, and returns to the electronic ground state. The mean lifetime in the excited state is called the fluorescence lifetime. This is often denoted τ, and is typically 1-5 ns. After emitting a photon, the molecule is likely to be in an upper vibrational sub-level of the electronic ground state. From there, it will relax to lower vibrational sub-levels. It is important to note, that the fluorescent photon has no knowledge of the exciting photon. Therefore, the wavelength of the fluorescent light does not depend on the wavelength of the exciting light.

An absorption spectrum shows the ability of a molecule to absorb light of different wavelengths. An emission spectrum shows how the emitted light is distributed over different wavelengths. The absorption spectrum for a typical fluorophore comes to a rather distinct end towards longer wavelengths (lower photon energies). When the energies of the exciting photons are not high enough to transfer the molecule from the electronic ground state to the first excited electronic state, absorption is no longer possible. The absorption spectrum often has a long tail towards shorter wavelengths (higher energies). For the emission spectrum, on the other hand, it is the other way around. Emission of photons with higher energies than the exciting light is unlikely to occur. The emission spectrum often has a long tail towards longer wavelengths. Therefore, the emission spectrum often resembles the mirror image of the absorption spectrum.

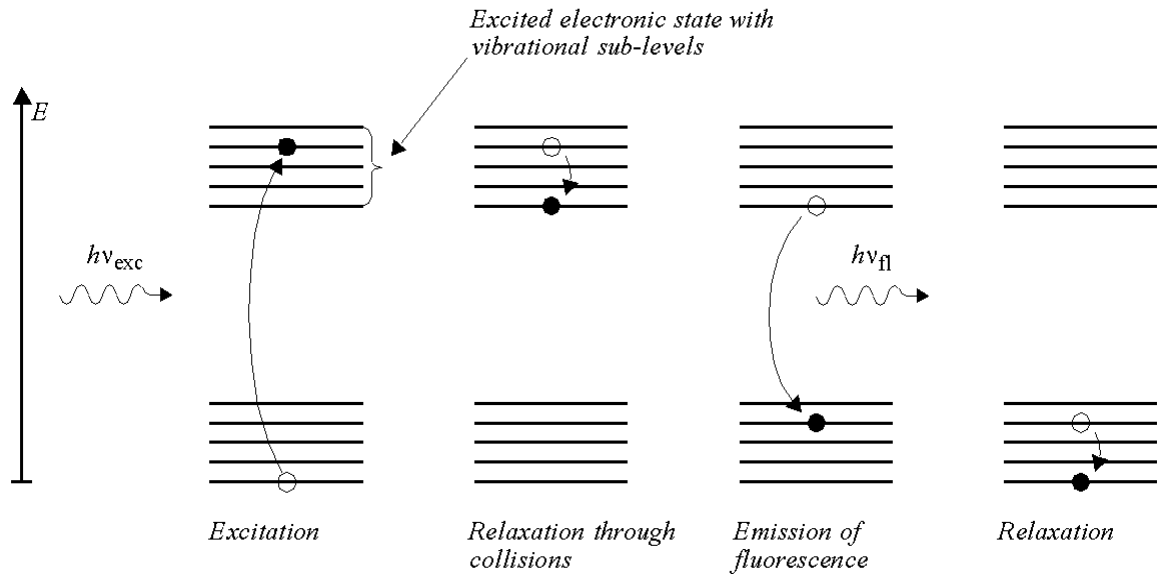


Fig. 6. Fluorescence. A molecule absorbs an incoming photon, and is transferred to an excited electronic state. Through collisions, the molecule is relaxed to the lowest vibrational state within the excited electronic state. After a certain time, the molecule returns to the electronic ground state by emitting a photon. Relaxation to lower vibrational states may follow. The mean lifetime in the excited state is called the fluorescence lifetime, τ.

Photo-bleaching

A fluorophore molecule, unfortunately, does not live for ever. After a certain number of excitation-emission cycles, it decomposes. This process, called photo-bleaching, makes it necessary to avoid unnecessary illumination of the specimen. Bleaching may be promoted by heat and certain chemicals (for example oxygen). In order to reduce bleaching, a specimen can be stored in dark containers in a cold environment. The bleaching processes differ between different fluorophores. Some fluorophores bleach very rapidly, others don't.

Excitation, beam splitters and filters

The choice of excitation light depends on the fluorophore used. It is desirable to use a wavelength close to the maximum of the absorption spectrum. In this manner, a large part of the exciting light is absorbed by the specimen, and the intensity of the illuminating source can be low.

The dichroic beam splitters in the confocal microscope are chosen with regard to the wavelength of the exciting light and the emission spectra of the fluorophores used. In addition to beam splitters, so called emission filters are placed between the pinholes and the detectors, Fig. 3b. Just as in an ordinary epi-fluorescence microscope, these emission filters have to be included to block the last remnants of excitation light that is present due to non-ideal performance of the dichroic beam splitters. In applications with more than one fluorophore, care should be taken so that only light from the right fluorophore may pass the emission filters (this is often difficult, or even impossible, in practice). Below, the excitation wavelengths, beam splitters and detector filters in the Zeiss LSM are listed. With an external laser, other excitation wavelengths can be obtained.

Excitation wavelengths (nm): 488, 543, 633.
Dichroic beam splitters (DBS 1 in Fig. 3b): FT510 ($\lambda < 510$ reflected, $\lambda > 510$ transmitted), FT 560, FT 665
Emission filters: LP520 (transmits $\lambda > 520$), LP570, OG590, RG665

► **Preparatory exercise 3:** A specimen has been stained with two fluorophores called FITC and TRITC (see Fig. 7). Use the scheme in figure 8 to suggest a combination of laser wavelengths, dichroic beam splitters, and emission filters that could be used to image these two dyes simultaneously. Will the fluorophores be efficiently excited? Will the fluorescent light be efficiently detected?

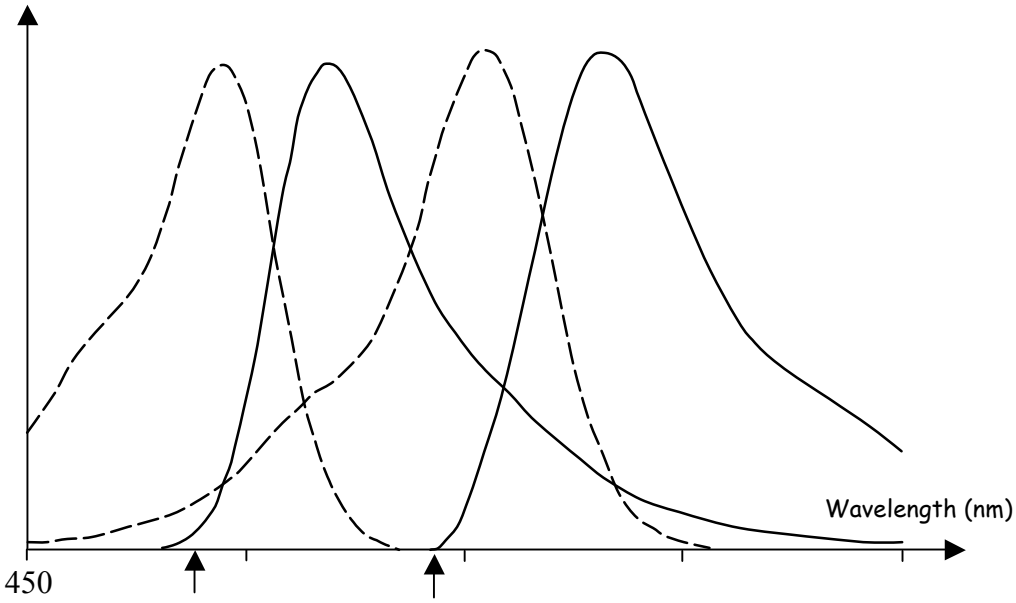


Fig. 7. Excitation and emission spectra for two commonly used fluorophores, FITC (fluorescein-isothiocyanate) and TRITC (tetramethylrhodamine-isothiocyanate). The excitation spectra show how efficiently different wavelengths can excite the fluorophores. Two commonly used excitation wavelengths, 488 and 543 nm, are indicated with arrows.

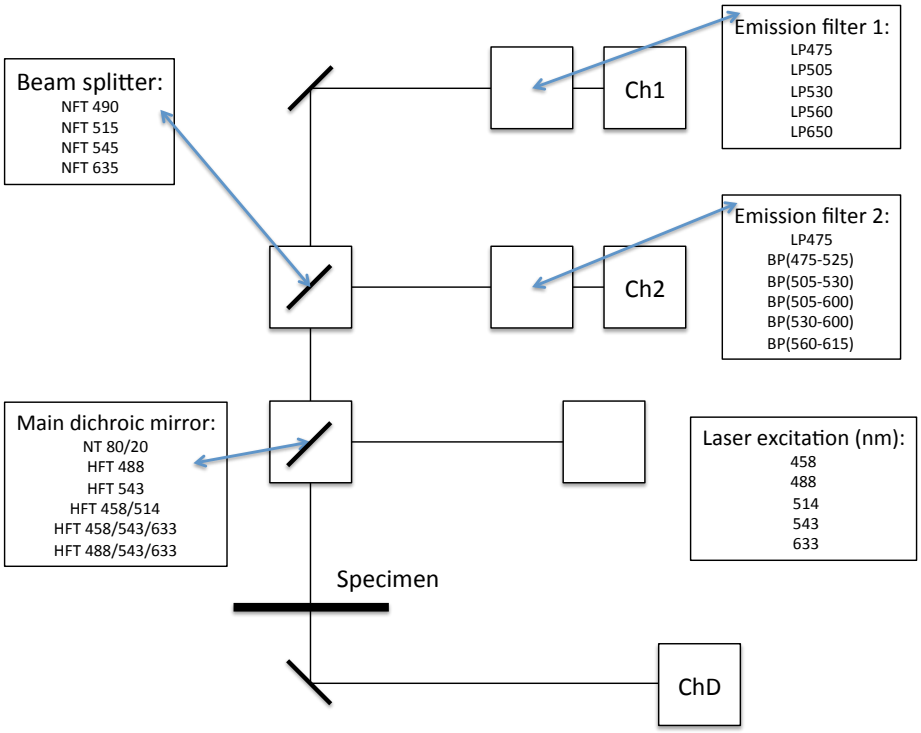


Fig. 8. Layout of the Zeiss 5 Pascal microscope showing options for laser excitation, main dichroic mirror (HFT=Haupt Farb Teiler), beam splitter (NFT=Neben Farb Teiler) and emission filters (BP=band pass, LP=long pass). Suggest a setup where both dyes in figure 7 can be imaged simultaneously.

. Experiments

Before confocal scanning is attempted you should get acquainted with the microscope and its controls. The best way to do this is to use it as a conventional microscope for viewing a specimen. An important difference between this microscope and the ones you have used in previous lab sessions, is that this is an inverted microscope. This means that the microscope has been turned "upside down." Thus, the condenser is located above the specimen and the objective below. Such microscopes are often used in biological and medical research.

Important! ***Make notes of results etc. during the lab work. Make sure you store all images according to the instructions. You will need these later for evaluation and for writing the report (see "Experiment 7").***

Experiment 1: *Get acquainted with the microscope*

First familiarize yourself with the microscope and its controls. This is essential knowledge in order to do confocal microscopy.

- View a specimen (provided by your supervisor) in transmitted halogen light using a 10X objective (see App. A, "Ordinary viewing"). Operate the focus control and the joystick that move the specimen on the stage so you get a "feel" for these controls.

When doing confocal scanning it is necessary to keep track of quite a few parameters, for example what dichroic beam splitters and emission filters that are used. Fig. 3b shows the location of beamsplitters and filters (in Appendix A you can find more detailed information). We will start confocal scanning by looking at a very simple specimen.

- Place a small piece of Post-It paper on top of an empty specimen glass in the microscope. Select the 10X objective, 488 nm illumination, dichroic beam splitter HFT488 for the excitation beam and NFT490 and LP505 on the emission side. Pinhole size is set to approximately 1 Airy unit, laser transmission to 24%, gain approximately 850 and image size to 512 x 512 pixels.
- Start scanning and adjust focus so that you can see a thin line of blue-green light moving quickly back and forth over the paper. Make fine adjustments of focus and contrast so that an image of suitable brightness is seen on the screen. Notice that unless the microscope is properly focused, the image will be completely black. This is very different from an ordinary microscope.

➡ ***Let your supervisor see what you have done, and discuss your results, before you proceed!***

Experiment 2: Influence of cover glass, pinhole size and numerical aperture on optical sectioning

You will now investigate the instrument properties by using a very simple reflecting specimen, namely a flat mirror. In this and the following experiments, a number of images will be stored on disk. You should store all your images on C:\IMG\stud\gr_*x*\, where *x* is the number of your lab group.

- Create folder D:\students\gr*x*\, x = number of your group.
- Place a surface-reflecting mirror on the specimen stage with the reflecting surface downwards facing the objective.
- Select the 20/0.45 objective
- Dichroic beam splitter 488

NO emission filter (use "plate")

- Laser transmission should be set to 0.35%
- Detector pinhole = 0.3 Airy units (see Appendix A for an explanation of what the pinhole size numbers mean).

- Start continuous scanning of the specimen. The mirror is probably out of focus, resulting in a completely black image (possibly mixed with more or less blue pixels). With the mirror defocused, adjust the Brightness control so that only a few blue pixels can be seen (see App. A for explanation and further details).

- Now refocus the microscope and adjust the Contrast control, so that the image brightness is suitable when the mirror is in optimum focus. The image should be as bright as possible, but not saturated. There should be no red pixels indicating detector saturation.

- Make a z-scan (see App. A, "Recording a depth profile"):
- step-length 0.1 μm in z direction
- 512 steps
- Create "database" in your folder and store the image.
- Remove the mirror specimen from the microscope.
- Apply a small drop of immersion oil on the reflecting surface of the mirror (yes, it will create a mess on the beautifully polished mirror, and you will have to clean away the oil afterwards)
- gently press down a cover glass onto the drop of oil (only a thin layer of oil should be present between cover glass and reflecting surface).
- Again place the mirror in the microscope and make a z-scan (focus position and Contrast may have to be changed). Store the image.
- Using the same specimen (mirror + oil film + cover glass) we will now investigate how the pinhole size influences the optical section thickness.
- Make z-scans with the 20/0.50 objective
- step-length of 0.1 μm

- Record stacks with 8-10 different pinhole sizes from 0.3 -10 airy units.
- One z-scan for each pinhole size. Store each z-scan image with a suitable name.
- As the pinhole size changes, the Gain and transmission settings **must** be adjusted to get a suitable brightness in the images (don't use too high gain setting: <900 is ideal), that is: **absolutely no red pixels** and only a few blue pixels.

➡ ***Let your supervisor see what you have done before you proceed!***

The following steps are carried out in lab. session 4:

- Use ImageJ to make intensity profiles in the z direction (see App. B). Determine the FWHM in all cases.

- Plot FWHM as a function of pinhole size for the 20X objective (mirror specimen ***with*** cover glass). Suggest a suitable pinhole for use with the 20X objective. Compare the 20X objective used with and without cover glass. ***These results must be included in the written report!***

- Compare the FWHM numbers and suitable pinhole size with the results from Prep. exercises 1 & 2. Conclusions? ***These results must be included in the written report!***

Experiment 3: Influence of light intensity on the noise in images.

The number of photons detected per pixel will determine the maximum signal-to-noise ratio (SNR) that can be obtained in an image. In this experiment you will investigate whether the instrument performance is limited by photon quantum noise, or if other sources of noise are predominant.

- **First, turn off the lights in the ceiling to avoid stray light!!!**
- Scan (ordinary xy, not z-scan) the same half-silvered mirror that will be used in experiment 5 (clean it if necessary).
- Select the 10X objective.
- Use the same instrument settings as in Experiment 2, i.e.:
- **No** emission (barrier) filter
- Dichrioc beam splitter = 488
- Pinhole size of 15 Airy units. A very large pinhole is used because we don't need optical sectioning, and it's important to get a uniform intensity in the image.
- Move the specimen while scanning, and select a clean-looking part (i.e. as little dust and dirt as possible). Use a rather low gain setting (480-500) and high light intensity (laser trans. = 50%) when doing this initial check, so that the noise level is low.
- Then select low laser light transmission (0.1%) and increase the gain setting to a value between 700-800. If necessary, defocus the specimen a bit so that no saturation occurs in the image.
- The image should now look uniform (except for noise and some occasional dust particle).
- Scan and store an image, **plus a dark image with the laser off**.
- Then increase transmission to 1%, and reduce the gain setting to avoid saturation (no red pixels). Scan and store an image **and dark image**.
- Repeat this process for transmissions 5% and 10%.

➡ ***Let your supervisor see what you have done before you proceed!***

The following step is carried out in lab. session 4:

- Open all images in ImageJ, and transform them into a stack (Image > Stacks > Convert Images to Stack). Displaying the most noise-free image of the stack (attenuation = 30) select a uniform rectangular area (about 1000 pixels in size) near the center of the image for measurement. The selected area must not display any structure in the form of dust particles etc. Measure the average pixel intensity and standard deviation for all images. (The same area will be measured in all images if you move the slider at the bottom of the image window to change images, and give the

command “Analyze > Measure”[♣] without moving the rectangular measurement window). Calculate the SNR in the four images recorded with laser light, compensating the mean values by subtracting the mean values from the dark images. From the transmission factors you know the relative light intensities in all cases. Are the data consistent with photon quantum noise limited performance? If this is the case, how many photons were (on average) detected per pixel in the different images? ***These results must be included in the written report!***

[♣] If the standard deviation is not displayed, click on ”Analyze > Set Measurements” and select Standard deviation.

Experiment 4: Three-dimensional recording and reconstruction of a biological specimen.

You will now collect a stack of confocal images (a set of consecutive optical sections recorded at different depths in the specimen) of a biological specimen. From this image stack you will create 3-D images of the specimen structure. The specimen selected for this task is a piece of lung tissue stained with Lucifer Yellow and embedded in epoxy (courtesy of Dr. Eben Oldmixon, Brown University, USA).

- So far you have been working with reflecting specimens (mirrors), but now you will study a fluorescent specimen. Therefore you need to get rid of all reflected light by using an "emission filter" in front of the detector, see Fig. 3b.

- Select laser wavelength, dichroic beam splitter, and emission filter to suit the specimen (compare Preparatory exercise 3).

- Select the 40/1.3 oil immersion objective
- Set laser transmission to 1%.
- Pinhole size should be approximately 1 airy units.
- Start continuous scanning of 512 x 512 images.
- Adjust focus and Contrast until an image is seen on the computer screen.
- Move the focus position slightly up and down and observe the changes in the recorded image.
- Move the focus setting to a position where the optical section is located close to the upper surface of the specimen (the "upper" surface is located towards the floor, because we are using an inverted microscope).
- Adjust Contrast and/or laser attenuation so that the brightest parts in the image are close to, but <u>not</u>, saturated (i.e. no red pixels).
- Create a directory called, for example, *lu* on D:\students\gr*x*\ (the home directory of your group).
- You are now going to scan and store in this directory a series of 300 images with a vertical spacing of 0.4 μm between consecutive images. To do this:
- Find the window showing "Z-sectioning"
- Z Interval = 0.4 μm
- Number of sections = 300
- Current Section Pos = 1
- Press "start"
- Save inage stack in your folder

<u>Let your supervisor see what you have done before you proceed!</u>

<u>The following step is carried out in lab. session 4:</u>

- Using ImageJ (see Appendix B), calculate "Brightest point" projections of the

specimen volume. Make a number of suitable projections to be shown as an animation, creating the impression of a rotating specimen on the screen. You may try different projection parameters until you are satisfied with the result. You can also try different projection methods.

- Produce two images that differ in rotation by approximately 6 degrees, to be used as a stereo pair. View the images as a red/green anaglyph (see App. B) on the computer screen, and use red/green glasses. You may also want to display them side-by-side on the computer screen, and try to view them as a stereo pair without any technical aids. Unsuccessful? Don't get depressed - many people fail this test! There is also a stereo pair printout available that can be viewed with a stereoscope.

- ***Include your most successful images in your lab report!***

Experiment 5: Scale distortion due to refractive index.

In this experiment you will investigate the influence of specimen refractive index on the depth scale in confocal microscopy. This effect is described in section 2.3 and App. V of ref. 2. For the experiments a cover glass is attached to a half-silvered mirror with a tiny drop of immersion oil, see Fig. 9. To get a suitably thin film of oil, don't let the drop of oil grow so large that it falls down onto the half-silvered mirror. Instead, as soon as a small drop forms at the end of the nozzle, gently bring it in contact with the specimen approximately halfway between the center and the edge. Apply the cover glass and wait for the oil film to spread uniformly. Do not press on the glass, because this will probably result in oil being squeezed out onto the mirror surface outside of the cover glass.

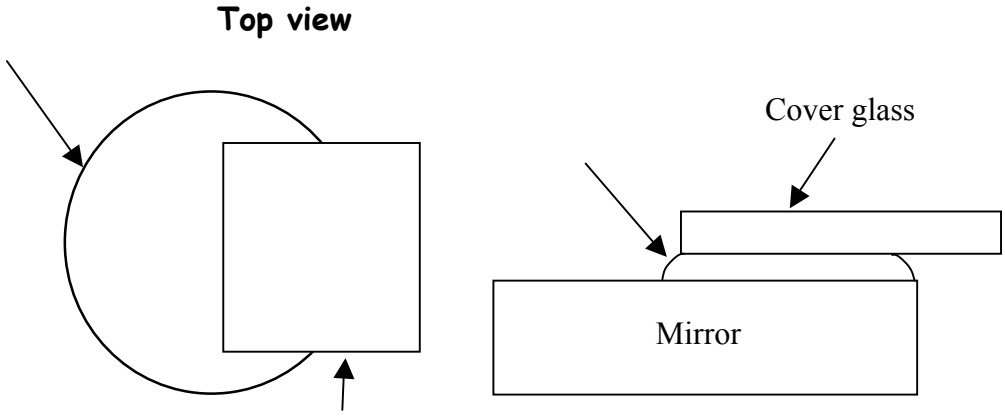


Fig. 9. Specimen used for studying scale distortion due to refractive index mismatch.

- Select the 10/0.3 objective.
- Set pin hole size to 1 airy units
- This is now (again) a reflecting specimen, change emission filter accordingly.
- Start continuous scanning
- Adjust focus and contrast until you see some surface in the image. This can be either the half silvered mirror or the cover glass.
- Move the microscope table so that you have the vertical edge of the coverglass in the center of the image.
- Change focus up and down so you have the focus position roughly in between the mirror and the cover glass.
- If you see a strange striped area in half the image, the oil has probably seeped out from under the cover glass and covered the mirror:
    - Try to move the microscope table along (parallell to) the coverslip edge to find another place where not so much oil is out on the mirror surface.
    - If that is not possible, you have to take off the coverslip, clean the mirror and apply new oil and a new coverglass.

- Make an xz-scan (depth profile), step length = 1.0 μm
- Make sure that both the upper surface of the cover glass and the mirror surface are recorded.
- Store the image.

Let your supervisor see what you have done!

The following step is carried out in lab. session 4:

- Measure the z-positions of the reflecting surfaces with ImageJ. From the measured positions, calculate the refractive index for glass (immersion oil has a refractive index almost identical to that of glass).

The lab report should include the depth profile image with an explanation of what is seen in the image. Also include measured z positions, as well as the equation for calculating the refractive index for glass (and the result of the calculations).

Experiment 6: Clean the instrument and specimens after use.

Cleanliness is a virtue in every microscopic laboratory. Before you leave you should clean the specimens and microscope objectives from immersion oil (as well as dirt, dust etc. that is visible). You learnt how to do this cleaning in lab #2, and now you have an opportunity to practice your skills. There is always the supervisor to ask if you need assistance.

Experiment 7 (after lab. session 4): Writing a lab report. (Done at home)

It is mandatory to write a report (in English) of the laboratory work. The writing of this report is an important part of the lab exercise. The report should contain a short account of the experiments performed, as well as the results and conclusions. An appropriate layout is as follows:

- On the front page there should be the report title as well as the names and email addresses of the author(s). The title should clearly indicate the contents of the report.

- The first page of the report (after the front page) should contain a summary. A summary describes, very briefly, the experiments performed and the results obtained.

- Then follows the main report text, which briefly describes what experiments have been made, how they were made, the results obtained, and what conclusions could be drawn. This text should be divided into different sections with appropriate headings.

- Appendices (if any) may contain calculations that have been made to arrive at the results, derivations of formulas etc. If measured values (primary data) are included in the report they should be placed in an appendix.

Remember that the person who reads the report doesn't know the results beforehand.

The report should be written in such a way that it can be read and understood by a student such as yourself before you made the laboratory work, or even read the lab instructions.

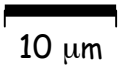
Therefore it is meaningless to give the pinhole size in "digital units" - give the size in μm. Likewise, "contrast" and "brightness" numbers are meningless to a general reader. What you actually do when you change these parameters is that you change detector amplification and signal offset.

You are, of course, welcome to ask the examiner if in doubt.

Below is a check-list of results that should be included in the report.

- FWHM results obtained in experiment 2.

- SNR values obtained in experiment 3.

- An image showing a 3D reconstruction of the biological specimen (experiment 4). Remember that in all published microscopic images there must be some sort of scale bar, indicating, for example, 10 or 100 μm. Example: 

Also include data concerning laser wavelength, filters, fluorophore etc.

- Quantitative results from experiment 5.

Reports should be finished as soon as possible after the final lab exercise (= normally within one month), and can be submitted, preferably in electronic form, to the lab supervisor or examiner.

References

1. Carlsson, K., Imaging Physics, KTH 2009.
2. Carlsson, K., Light Microscopy, KTH 2007.

Appendix A. How to use the image processing software (ImageJ).

Some of the confocal images will be processed or evaluated using the program ImageJ. This program was developed at the National Institutes of Health in USA and can be downloaded via internet free of charge (http://rsb.info.nih.gov/ij/). It is used by many scientists worldwide, especially in connection with microscopy.

How to get started

Start ImageJ by double-clicking on the icon on the desktop. The main window, see Fig. 11, is then shown. By clicking "File > Open" you can open the desired image file. Squares, lines or arbitrarily shaped regions (selected by buttons in the Main window) can be drawn as overlays in the image.

Analyzing an image

Data within an overlay region can be calculated and displayed by clicking "Analyze" and making the appropriate choice. By choosing Measure, a window is opened that displays, for example, area, mean pixel value and standard deviation. If an interesting parameter (e.g. standard deviation) is missing in the list, click on "Analyze > Set Measurements" and select the desired parameter(s). To plot a profile of pixel intensities along an arbitrary line in the image, the desired line is first drawn. By then clicking "Analyze > Plot profile" a new window is opened that shows the profile. For rectangular selections, the "Plot profile" command displays a "column average plot", where the x-axis represents the horizontal position within the selection window and the y-axis the vertically averaged pixel intensity. To average horizontally, hold down the **alt** key. Plotted profiles can be printed out (File > Print), or the values can be stored in a file (click Save in plot window). In addition, plots can be roughly evaluated on-screen by moving the cursor and reading the x- and y-positions.

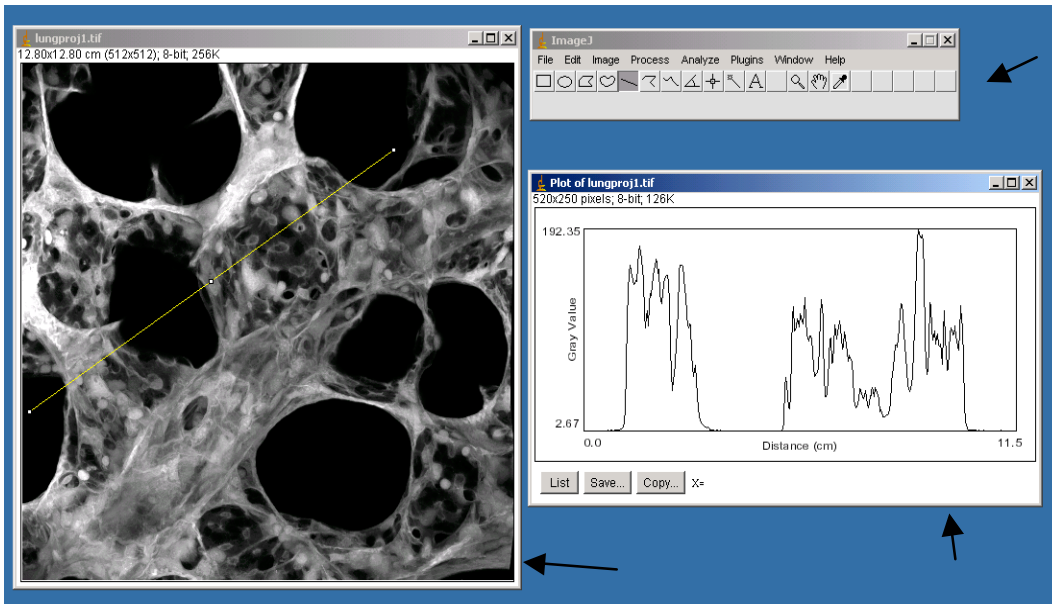


Fig. 11. Examples of windows shown in ImageJ. In this case a profile of pixel intensities along the yellow line in the image is displayed.

Image stacks

ImageJ is also convenient for displaying and processing stacks of images. Let's start by displaying the individual optical section images. Through the sequence "File > Import > Image Sequence" you can open a stack of images. By moving the slider at the bottom of the image widow you can browse through the stack of images one by one. In this way you can get a feel for the image information you have in the stack. If some blue (and possibly red) pixels are seen in the images (indicating zero and saturated pixels) they are removed by selecting Image > Type > 8-bit.

Three-dimensional reconstructions of the image data in a stack can be done in many different ways. The most common technique is to make some sort of projection image through the stack. One example of this is illustrated in Fig. 12.

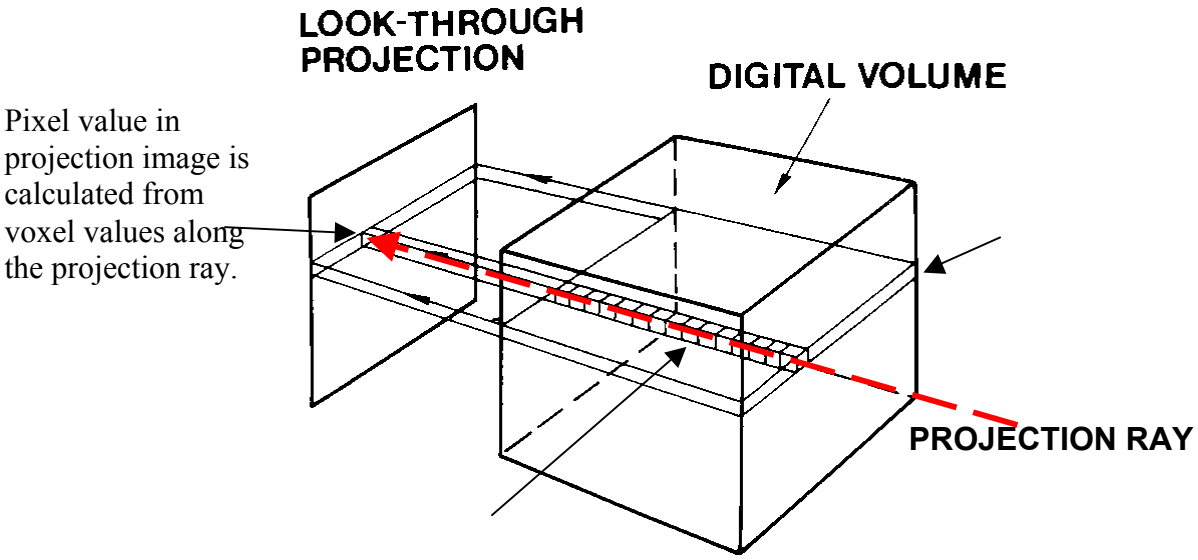


Fig. 12. A stack of consecutive optical sections from a confocal microscope represents a specimen volume. Each individual pixel value is a measure of the light emission from a tiny specimen volume. Pixels in optical section images are often called voxels.

To calculate the pixel values in a projection image, we let a number of imaginary "projection rays" penetrate the volume (one of these is illustrated as a dashed arrow in Fig. 12). The pixel values in the projection image are calculated from the values encountered along each projection ray. For example, the mean or maximum voxel value can be selected. To make projection images in ImageJ, the stack of images must first be opened as described above ("File > Import > Image Sequence"). Then select "Image > Stacks > 3D Project." A window is then displayed where a large number of parameters can be selected. Some of these are rather self-explanatory, whereas others are not. In many cases, however, the default parameters will work well. For a first attempt, you can try "Axis of Rotation: Y-axis", "Projection Method: Brightest Point" and use defaults for all the rest.
After projection images have been calculated, the brightness and contrast of all projections can be adjusted simultaneously by selecting "Image > Adjust > Brightness/contrast". "Image > Stacks > Start animation" can be used to automatically browse through the calculated projection images (or optical sections, depending on which window is selected). This can

create an illusion of a rotating specimen volume on the screen. The speed and mode of browsing (for example back and forth) can be selected in “Animation options.”

Stereo images

To produce a red/green anaglyph of a stereo pair, proceed as follows. Left and right images of the stereo pair are obtained by making two projections through the image stack (typically, +3 and -3 are suitable angles). Then: Image > Color > Convert stack to RGB. The result is a so-called anaglyph, where the left and right images of the stereo pair are displayed in different colors. Using red/green glasses for viewing, a stereo impression is obtained.

An ImageJ manual is available that describes the different parameters. You can also discuss parameter choice with the supervisor.