Biomicroscopy II
Practical Part
Course Book

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Contents

Chapter 1

Introduction

The idea of this course book is to guide you through the familiarisation with modern light microscopes. It is centred around practical experiments aiming to deepen your understanding of typical set-ups and applications especially in the field of life-science. Each chapter is covering one topic and is consisting of individual experiments. It is the idea of the practical part that you do these experiments as autonomously as possible. The questions at the end of each exercise serve as control about the learning outcome.

Chapter 2

Transmission light microscopy

2.1 Introduction

The idea of this section is to familiarize yourself with the set-up of a modern transmission light microscope, the concept of conjugated planes and to experience the most commonly used contrasting techniques in life-science (Phase Contrast, Differential Interference Contrast and Dark-field).
Before going to the microscope recapitulate the different components of a light microscope. This comprises parts like the objective, the eyepiece, the tube lens, the various stops and especially how they are assembled to a functioning microscope.
Additionally you are exposed to the concept of Köhler illumination. This method guarantees even illumination over the field of view as well as optimal resolution and it is indispensable for optimal performance of the various contrasting methods. Therefore you will have to switch the set-up in order to do all the experiments. Ideally you should arrange this with your colleagues who are working next to you at the beginning of the session.

2.2 Set-up of the microscope

2.2.1 Exercises

- Identify whether you are working on an upright or an inverted microscope.
- Find and locate the different components of the microscope.
- Learn and experience how the different components can be exchanged or adjusted.
- Talk to your colleagues which are working on the alternate set-up. Find similarities and differences.

2.2.2 Questions

- Which of the component(s) of the microscope are not accessible?
- What is the difference between the set-up of an upright or an inverted microscope?
- Find the advantages/disadvantages of the different set-ups.

2.3 Köhler illumination

2.3.1 Work-flow

The idea of Köhler illumination it to place and to adjust the field-stop as well as the aperture stop in the light-path of the microscope so that it guarantees even illumination conditions and optimal resolution in the resulting image. In order to realize this the following steps have to be followed:

- Mount the specimen in the sample holder of the microscope.
- Open field-stop and aperture-stop completely.
- Focus the specimen.
- Close the field stop.
- Move the condensor up and down until the field stop is seen sharp through the ocular.
- Open field stop that it is just as big as the field of view
- Remove the eye-piece and close the aperture stop so that 2/3 of the back-focal plane remain open.

2.3.2 Exercises

- Use the diatom sample for this exercise.
- Realize Köhler illumination. Start with the 10 x, NA=0.3 objective and then switch to the 20 x, NA=0.8 objective.
- Take different images with the camera with different settings of the aperture stop (stop should be completely closed and completely open). Compare the images and describe the differences?
- Repeat the experiment with the 63x NA=1.4 objective and compare the images with the previous experiment.
- Repeat the experiment by changing the size of the aperture stop. Compare the results.

2.3.3 Questions

- Why is it useless to do a Köhler alignment without a specimen?
- Why is it recommended to start Köhler illumination with objectives with rather low magnification
- How is size of the aperture stop influencing the image?
- Why should the aperture stop not be used in order to change the contrast/brightness of the image?

2.4 Differential Interference Contrast (DIC)

2.4.1 Work-flow

The idea of this section is to familiarize with Differntial Interference Contrast (DIC) one of the most popular contrasting methods for unstained specimens in life-science. The advantage of DIC is that it works also with thick specimens. To practically realize DIC it is necessary to add at least for different optical elements into the beam-path of the microscope (two prisms, polariser, analyser). The detailed workflow (for an inverted microscope) is as follows:

- Set up Koehler illumination in transmission
- Move specimen out of the light path
- Insert polariser, analyser and lower Wollaston prism
- Remove (one) eyepiece
- Shift Wollaston prism to place dark line in the center
- Turn polarizer until the line is seen mostly dark
- Insert eyepiece back into the eye-tube
- Insert upper Wollaston prism (in condenser)
- Move the specimen back into the light path
- Move lower Wollaston prism to get required contrast

2.4.2 Exercises

- Make the necessary modifications on the microscope in order to be able to do DIC imaging. Attention: the prism you have to use in order to obtain proper DIC images is objective dependant.
- Take images of at least two different samples (e.g. diatoms, tissue).

2.4.3 Questions

- How does a DIC image compare to a transmission image? Find differences and similarities.
- Try to establish "images-features" with which you can recognize a DIC image.

2.5 Phase Contrast

2.5.1 Exercises

Before starting the exercises please make sure that you are using the right microscope. Only the **inverted** microscopes are properly equipped to obtain phase-contrast images

- Take images of at least two different samples (e.g. diatoms, tissue).

2.5.2 Questions

- How does a Phase-contrast image compare to a transmission image? Find differences and similarities.
- Why do you need to increase the transmission light intensity when using phase contrast (compared to regular transmission light microscopy)?
- Try to establish "images-features" with which you can recognize a phase-contrast image.

2.6 Resolution

2.6.1 Exercises

- Start with the 10x N.A 0.3 objective for the following exercise.
- Use the R1L1S1N resolution and distortion target (place it on a cover slip 24 mm x 60 mm).
- Find the part of the target with the line gratings of different frequencies (LP/mm). Place the pattern with the lowest number of LP/mm in the middle of the field of view.
- Realize Köhler illumination. Close the aperture stop completely and remove one eypiece.
- Move the test-pattern in and out of the field of view and see how the pattern is changing.
- Move from the line grating with the lowest number to one with the highest number and see how the pattern you observe is changing.
- Acquire an image of the grating with the lowest and the highest frequency (avoid saturation). Measure/calculate the contrast with the following formulae:

$$contrast = \frac{I_{max} - I_{min}}{I_{max} + I_{min}}$$

2.6.2 Questions

- How can you explain the pattern of the grating you see when you remove the eyepiece?
- How is the pattern changing when moving form gratings with lower to higher frequencies (LP/mm)?
- How is the pattern changing if you bring different filter-cubes into the light path (ideally switch from green to far red)?
- How is the pattern changing if you work with an objective with a higher NA?
- How is this experiment linked to the resolution of the set-up?

Chapter 3

Fluorescence Microscopy

3.1 Introduction

The idea of this chapter is to make you familiar with the underlying principles of fluorescence microscopy with special emphasis on applications if life-science. Understanding the optical set-up as well as the underlying photo-physical phenomena of fluorescence is clearly the basis and necessary in order to master this technology properly. Nevertheless is should be noted that sample preparation is also a very important part of the work flow in order to obtain optimal results. Suboptimal sample preparation can never be cured with instrumentation ("garbage in, garbage out") as well as suboptimal image acquisition cannot be corrected via image processing. Therefore it is utterly important to be aware of the labelling strategy and the sample preparation protocol. During this course it will be unfortunately not possible for you to prepare your samples yourself. Nevertheless you can find a brief description of the sample preparation in this chapter

3.2 Sample

3.2.1 Fluorescent cells

Every immunostaining for adherent cells is consisting of a fixation and permeabilization step followed by the application of the primary and the secondary antibody.
The secondary antibody is typically carrying the fluorescent dye. As one primary antibody molecule can be recognized by several secondary antibody molecules this approach can enhance the labelling density and thus the intensity of the signal. Nevertheless it is possible to omit the secondary antibody if the primary antibody is already labelled with a fluorescent reporter.

- Hela cells (human, immortal cell line derived from cervial cancer cells) cultured at 37 C for 24-48h.

- Staining
  DNA: 4',6-Diamidin-2-phenylindol (DAPI)
  Actin: Phalloidin-Alexa488[1]

[1] Alexa fluorophores are a trademark of lif technologies TM and the exact properties of the fluorophores can be found under: http://www.lifetechnologies.com

- Immuno-staining
  Mitochondria:
  Rabbit antibody anti-TOM20 + Goat anti-Rabbit antibody conjugated to F(ab)'2 Alexa Fluor 555

  Tubulin:
  Mouse antibody anti-Tubulin + Goat anti-Mouse antibody conjugated to F(ab)'2 Alexa Fluor 647

3.3 Cellular substructures

3.3.1 Exercise

Sample: Fluorescent Cells

- Use the 10x objective on order to find the correct focal plane. Then switch to the 20x objective (refocus with the fine focus knob if necessary).
- Image the samples with the different fluorescence filter-cubes of the microscope. Which one/s is/are giving a reasonable image?
- Acquire an image with the camera of each cellular substructure (avoid image saturation).
- Acquire a z-stack of at least two biological substructure.

3.3.2 Questions

- Which biological structures can be seen with which filtercube?.
- Why do you need to have different camera exposure times for the different biological substructures?
- What is slowing down the acquisition of the z-stack? How could one speed it up?

3.4 Objective comparison

3.4.1 Exercise

Sample: Fluorescent Cells

- Use the 63x NA=1.4 objective.
- Acquire 3-4 images of the nuclei with the camera (avoid saturation).
- Open the images with ImageJ and measure the pixel intensity of the nuclei (store the results).
- Repeat the above described experiment with the 40x NA=1.3 objective.
- Compare the measured pixel intensities of the two experiments.

3.4.2 Questions

- Which parameter of the objective is influencing the resolution of the image?
- Which objective is giving a brighter image? Why?
- Which factors are influencing the pixel intensity?

3.4.3 Exercise

Sample: Fluorescent Cells

- Use the 63x or the 40x objective.
- Acquire images of one biological substructure with the camera by varying the exposure time of the camera(avoid saturation).
- Acquire images of one biological substructure with the camera by varying the binning of the camera(avoid saturation).
- Open ImageJ and measure the mean intensity of the camera field of view for the different exposure times.
- Measure the mean intensity of the camera field of view for different camera binnings.
- Plot the mean intensity versus the exposure time by using ImgagJ/Fiji (Analyse->Tools Curve Fitting)

3.4.4 Questions

- Why is it essential to avoid saturation for the above described experiments?
- What is the physical explanation for camera saturation?
- What do you think could be the advantage of binning done on the camera chip compared to binning done via image processing (post acquisition)?

3.5 Fluorescence Illumination

3.5.1 Exercises

Sample: Fluorescent slide (Chroma)

- Select the 10x objective. Find a suitable filter-cube for your slide by using the eyepiece.
- Take an image of the slide with the camera. Avoid saturation in the image.
- Repeat the experiment with the 20x objective and compare the results with the previous experiment.
- Analyse the image further by using the “Line Profile” tool of ImageJ.

3.5.2 Questions

- What do the images tell you? Are they reflecting the excitation or the emission behaviour of the microscope? Why? How could the images be used to correct for uneven illumination?
- How would the profile change in case the image would be saturated?

Chapter 4

Confocal microscopy

4.1 Introduction

The idea of this chapter is to make you familiar with the concept of confocal microscopy. It is important to know the underlying working principle of this technique in order to understand imaging parameters like pixel-dwell time, scanning speed, pixel size, detector gain and resolution.

4.2 Cellular substructures

Sample: Fluorescent Cells
Objective: 20x NA 0.8

4.2.1 Exercises

- Try to find reasonable setting for the confocal microscope in order to be able to acquire an image of the nucleus (dye: DAPI) and actin (dye Alexa488):. Avoid saturation and image both channels simultaneously.
- Repeat the above describes experiment (=same settings) by only using either 405 nm or 488 nm for excitation.
- Repeat the first experiment by using sequential scanning settings. Compare it with the first experiment. What are the differences?
- Try to find reasonable setting on the confocal microscope in order to be able to acquire an image of the DAPI, the Alexa488nm and the “red” labelled structure (no saturation).
- Find an area with nicely separated microtubules. Acquire the same field of view with at least three different pixel sizes (under sampled, over samples, Nyquist sampling). Measure the full width at half maximum (FWHM) of a microtubule.
- find an area with separated nuclei. Acquire the same field of view by varying a) the detector gain b) the dwell time c) laser intensity; at least four different setting for each parameter. Plot the pixel intensity of one nucleus vs. the dwell-time and the detector gain and laser intensity.

- Acquire the same field of view with three different structures and four pinhole sizes
- Acquire a 3D stack with at least two different cellular substructures.
- Acquire a transmission image.

4.2.2 Questions

- What happens if you increase the gain of the Alexa488 channel under excitation with 405 nm? Try to find an explanation of your observation (tip: fluorescence emission spectra).
- Explain the FWHM results.
- How do you explain that the pixel intensity is independent of the pixel dwell time?

4.3 Illumination Fluorescence/Confocal microscopy

Sample: Fluorescent slide (Chroma)

- Select the 10x objective. Find a suitable filter-cube for your slide by using the eyepiece.
- Find suitable settings to image the slide using the confocal scan-head (avoid saturation, remember the Hi-Lo lookup-table).
- Acquire an image of the slide. What does the image tell you? Analyse the image further by using the “Line Profile” tool.
- Repeat the experiment with different “zoom” settings (also unzoom!). What are the differences? Try to find an explanation for your observation (tip: how does the scanning mechanism work optically?)

Chapter 5

Annexe

5.1 Material

5.1.1 Microscopes

ZEISS LSM 700 INVERT

- Modality:Confocal AND Widefield
- Two photomultiplyers for fluorescence detection
- One photomultiplyers for transmission detection (non-descanned)
- Stand: INVERT Zeiss AxioObserver Z1
- XY-stage: IN1-manual, IN2-motorised
- Software: ZEN 2009
- Camera: Axiocam MRm (B/W)
  - resolution: 1388 pixel x 1040 pixel
  - chip size: 8.9 mm x 6.7 mm
  - detector size: 6.45 µm x 6.45 µm
  - frame rate: 11 frames/sec
  - dynamical range: 12bit
- C-mount: 1.0x
- Objectives

	Mag./NA	Imm. media	Contrast	Working distance
EC Plan-Neofluar	10x/0.30	air	DIC	5.20mm
Plan-Apochromat	20x/0.8	air	DIC	0.55mm
Plan-Apochromat	40x/1.30	oil	DIC	0.21mm
Plan-Apochromat	63x/1.40	oil	DIC	0.19mm
EC Plan-Neofluar	10x/0.30	air	PH 1	5.2mm
Plan-Apochromat	20x/0.80	air	PH 2	0.55mm

- Light sources
  - Tungsten-Halogen lamp (transmission)
  - LED Lumencore SOLA (fluorescence)
  - Diode lasers 405 nm, 488 nm, 555 nm, 639 nm

- Fluorescence filters

Short name	Excitation	Beams-splitter	Emission
Blue	SP 365	FT 395	BP 420-470
Green	BP 450-490	FT 510	BP 515-565
Red	BP 546-552	FT 560	BP 575-640
Far-red	BP 615-655	FT 660	BP 665-715

Leica SP8 Upright

- Modality:Confocal AND Widefield
- Three photomultiplyers for fluorescence detection (2x HyD + 1 PMT)
- One photomultiplyers for transmission detection (non-descanned)
- Stand: Upright Leica DM6 CS
- XY-stage: motorised
- Software: LAS-X
- Camera: DFC7000 GT (B/W)
  - resolution: 1920 pixel x 1440 pixel
  - chip size: 8.8 mm x 6.6 mm
  - detector size: 4.54 µm x 4.55 µm
  - frame rate: upto 50 frames/sec
  - dynamical range: 8-12bit
- C-mount: 1.0x
- Objectives

	Mag./NA	Imm. media	Contrast	Working distance
HC PL Fluotar	5x/0.15	air	DIC	13.70 mm
HC PL Fluotar	10x/0.3	air	DIC	11.0 mm
HC Fluotar	16x/0.60	imm	Corr	2.5 mm
HC APO	20x/0.50	water	DIC	3.5mm
HC PL APO	20x/0.75	air	DIC	0.62 mm
HC PL APO	40x/1.25	gluyc	DIC	0.14 mm
HC PL APO	63x/1.4	oil	DIC	0.14 mm

- Light sources
  - Tungsten-Halogen lamp (transmission)
  - LED Lumencore Sola SMII (fluorescence)
  - Diode lasers 405 nm, 488 nm, 552 nm, 638 nm

- Fluorescence filters

Short name	Excitation	Beams-splitter	Emission
DAPI	BP 350/50	FT 400	BP 460/50
GFP	BP 450-490	FT 495	BP 500-550
RHOD	BP 546/10	FT 560	BP 585/40
Y5	BP 620/60	FT 660	BP 700/75

Leica SP8 inverted

- Modality:Confocal AND Widefield
- Three photomultiplyers for fluorescence detection (2x HyD + 1 PMT)
- One photomultiplyers for transmission detection (non-descanned)
- Stand: Upright Leica DM6 CS
- XY-stage: motorised
- Software: LAS-X
- Camera: DFC7000 GT (B/W)
  - resolution: 1920 pixel x 1440 pixel
  - chip size: 8.8 mm x 6.6 mm
  - detector size: 4.54 µm x 4.55 µm
  - frame rate: upto 50 frames/sec
  - dynamical range: 8-12bit
- C-mount: 1.0x
- Objectives

	Mag./NA	Imm. media	Contrast	Working distance
HC PL Fluotar	5x/0.15	air	DIC	13.70 mm
HC PL Fluotar	10x/0.3	air	DIC	11.0 mm
HC PL APO	20x/0.75	air	DIC	0.62 mm
HC PL APO	40x/1.25	gluyc	DIC	0.14 mm
HC PL APO	63x/1.4	oil	DIC	0.14 mm

- Light sources
  - Tungsten-Halogen lamp (transmission)
  - LED Lumencore Sola SMII (fluorescence)
  - Diode lasers 405 nm, 488 nm, 552 nm, 638 nm
- Fluorescence filters

Short name	Excitation	Beams-splitter	Emission
DAPI	BP 350/50	FT 400	BP 460/50
GFP	BP 450-490	FT 495	BP 500-550
RHOD	BP 546/10	FT 560	BP 585/40
Y5	BP 620/60	FT 660	BP 700/75