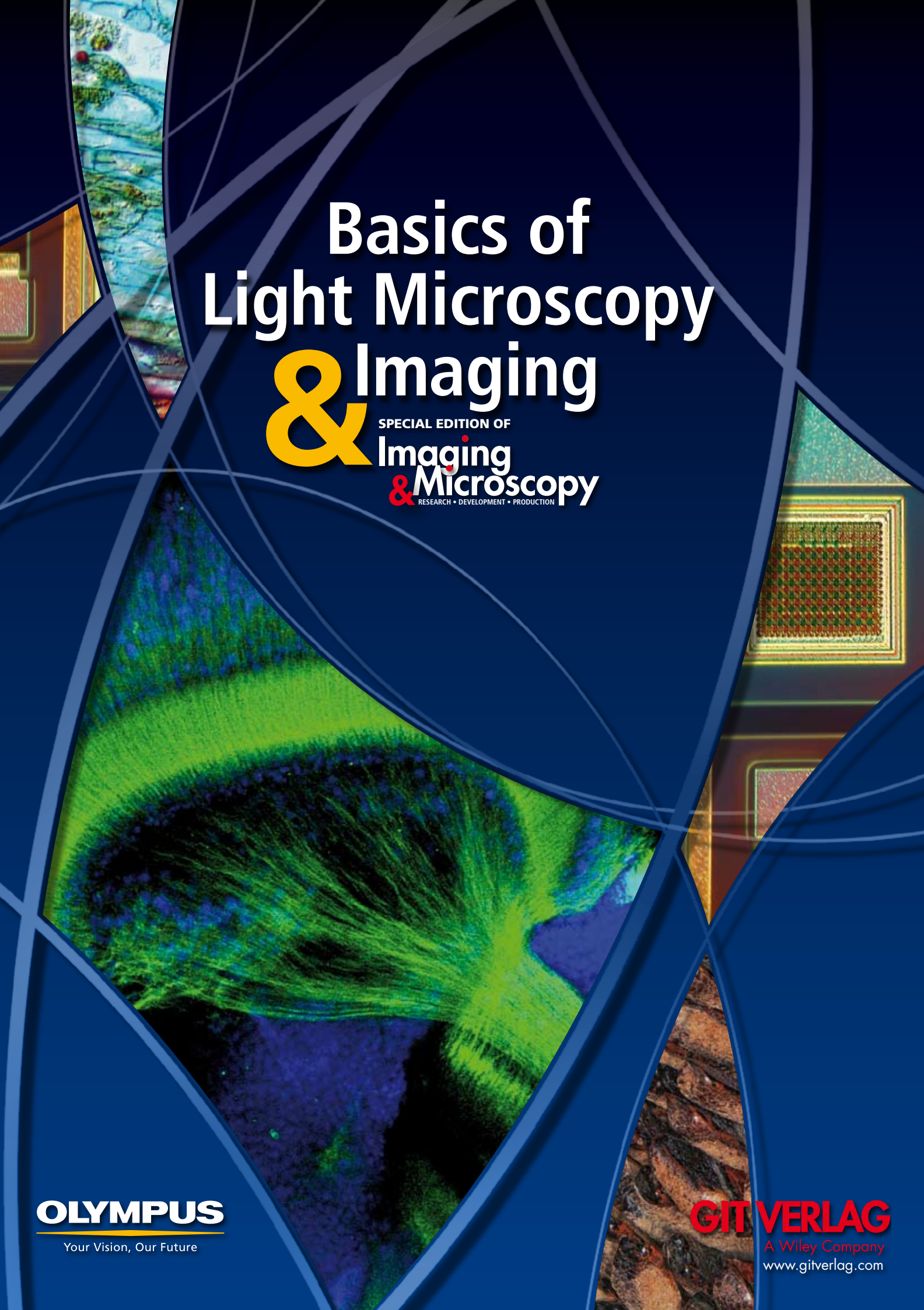
Basics of Light Microscopy & Imaging

SPECIAL EDITION OF

Imaging & Microscopy

RESEARCH • DEVELOPMENT • PRODUCTION



GIT VERLAG

A Wiley Company

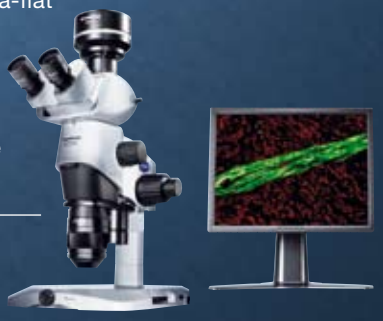
www.gitverlag.com

OLYMPUS

Your Vision, Our Future

GIVE YOUR EYES TIME TO ADJUST TO THE HIGH LIGHT EFFICIENCY. OLYMPUS SZX2.

If you're used to other stereo microscopes, you'd better be prepared before you start working with the new Olympus SZX2 range. The peerless resolution and exceptional light efficiency enable you to see fluorescence with brightness and brilliance like never before, while providing optimum protection for your specimens at the same time. All this makes Olympus SZX2 stereo microscopes the perfect tools for precise fluorescence analysis on fixed and living samples. Importantly, the innovations present in the SZX2 series go far beyond fluorescence. The optimised 3-D effect offers superior conditions for specimen preparation and precise manipulation. The unique ultra-flat LED light base provides bright and even illumination of the entire field of view at a constant colour temperature. Moreover, the Comfort*View* eyepieces and new trinocular observation tubes make sure your work is as relaxed as it is successful. The future looks bright with Olympus.



For more information, contact:
Olympus Life and Material Science Europa GmbH
Phone: +49 40 2 37 73 54 26
E-mail: microscopy@olympus-europa.com
www.olympus-europa.com

O L Y M P U S M I C R O S C O P Y

Introduction

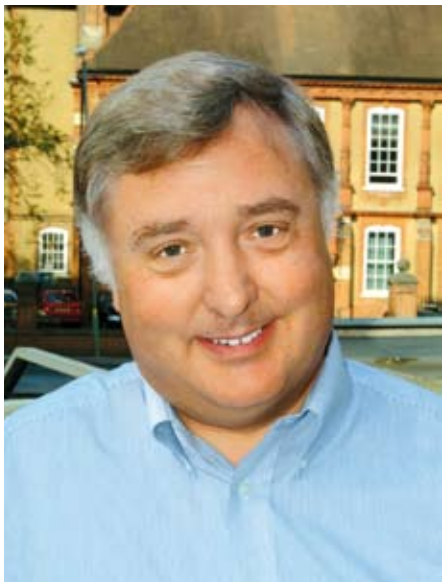
In this wide-ranging review, Olympus microscopy experts draw on their extensive knowledge and many years of experience to provide readers with a highly useful and rare overview of the combined fields of microscopy and imaging. By discussing both these fields together, this review aims to help clarify and summarise the key points of note in this story. Encompassing the creation of highly detailed specimen views through to final image capture and processing, the story will also be backed up by useful insights, examples, as well as handy hints and tips along the way.

To make it easy to dip in and dip out of this comprehensive overview, the review is clearly structured into several chapters. These take the reader from explanations of the physics of light, colour and resolution, through details of contrast and fluorescence techniques, to finish up with discussion on 3D imaging. Highlighting and summarising key points made within this review are a series of information boxes. These also aim to provide useful insight on techniques, as well as expert advice on microscope set up.

Content

Dear Reader

"A Huge Number of Techniques in a Relatively Short Space"



Tony Wilson Ph.D
Professor of Engineering Science
University of Oxford
United Kingdom

Although microscopes are becoming more and more easy to use, it still remains important to have an appreciation of the fundamental principles that determine the resolution and contrast seen in microscope images. This series of articles, by authors from Olympus, aims to provide an overview of the physical principles involved in microscope image formation and the various commonly used contrast mechanisms together with much practically oriented advice. Inevitably it is impossible to cover any of the many aspects in great depth. However, their approach, which is to discuss applications and provide practical advice for many aspects of modern day microscopy, will prove attractive to many.

The articles begin by setting the scene with a discussion of the factors that determine the resolving power of the conventional microscope. This permits the introduction of important concepts such as numerical aperture, Airy disc, point spread function, the difference between depth of focus and depth of field and the concept of parfocality. Common contrast mechanisms such as darkfield and phase contrast are then introduced, followed by differential interference contrast and polarisation contrast imaging. Throughout the discussion, the importance of digital image processing is emphasised and simple examples such as histogram equalisation and the use of various filters are discussed.

The contrast mechanisms above take advantage of the fact that both the amplitude and phase of the light is altered as it passes through the specimen. Spatial variations in the amplitude (attenuation) and/or the phase are used to provide the image contrast. Another extremely important source of image contrast is fluorescence, whether it arise naturally or as a result of specific fluorescent labels having been deliberately introduced into the specimen. The elements of fluorescence microscopy and techniques of spectral unimixing are discussed and brief mention is made of more advanced techniques where spatial variations in, for example, fluorescence lifetime are used to provide image contrast. Finally, techniques to provide three-dimensional views of an object such as those afforded by stereo microscopes are discussed together with a very brief mention of the confocal microscope.
The authors have attempted the very difficult task of trying to cover a huge number of techniques in a relatively short space. I hope you enjoy reading these articles.

"Unique presentation of technical aspects in connection with image processing"

Dr. Martin Friedrich
Head Imaging & Microscopy
GIT Verlag, A Wiley Company
Germany

As a regular reader of "Imaging & Microscopy," the title of this issue, "Basics of Light Microscopy & Imaging," must certainly seem familiar to you. From March 2003 to November 2005, we had a series of eleven articles with the same name and content focus.

In collaboration with the authors, Dr Manfred Kässens, Dr Rainer Wegerhoff, and Dr Olaf Weidlich of Olympus, a lasting "basic principles" series was created that describes the different terms and techniques of modern light microscopy and the associated image processing and analysis. The presentation of technical aspects and applications in connection with image processing and analysis was unique at that time and became the special motivation and objective of the authors.

For us, the positive feedback from the readers on the individual contributions time and again confirmed the high quality of the series. This was then also the inducement to integrate all eleven articles into a comprehensive compendium in a special issue. For this purpose, the texts and figures have been once more amended or supplemented and the layout redesigned. The work now before you is a guide that addresses both the novice and the experienced user of light microscopic imaging. It serves as a reference work, as well as introductory reading material.

A total of 20,000 copies of "Basics of Light Microscopy & Imaging" were printed, more than double the normal print run of "Imaging & Microscopy." With this, we would like to underline that we are just as interested in communicating fundamental information as in the publication of new methods, technologies and applications.

Enjoy reading this special issue.

"You Can Get Keywords for Expanding Your Knowledge"

Alberto Diaspro Ph.D
Professor of Applied Physics
University of Genoa
Italy

There is a great merit for publishing "Basics of LIGHT MICROSCOPY and IMAGING: From Colour to Resolution, from Fluorescence to 3D imaging". It is a great merit since it is very important to define, to clarify, and to introduce pivotal elements for the comprehension and successive utilisation of optical concepts useful for microscopical techniques and imaging. The niche still occupied by optical microscopy, within a scenario where resolution "obsession" plays an important role, is mainly due to the unique ability of light-matter interactions to allow temporal three-dimensional studies of specimens. This is of key relevance since the understanding of the complicate and delicate relationship existing between structure and function can be better realised in a 4D (x-y-z-t) situation. In the last years, several improvements have pushed optical microscopy, from confocal schemes [1-6] to multiphoton architectures, from 7-9 folds enhancements in resolution to single molecule tracking and imaging allowing to coin the term optical nanoscopy [7]. Advances in biological labelling as the ones promoted by the utilisation of visible fluorescent proteins [8-12] and of overwhelming strategies like the "F" techniques (FRAP – fluorescence recovery after photobleaching, FRET – fluorescence resonance energy transfer, FCS – fluorescence correlation spectroscopy, FLIM – fluorescence lifetime imaging microscopy) [4, 13-15] collocate optical microscopy in a predominant position over other microscopical techniques. So it becomes mandatory for primers, and more in general for all those researchers looking for answers about their biological problems that can be satisfied by using the optical microscope, to have a good starting point for finding the optimal microscopical technique and for understanding what can be done and what cannot be done. This long note on Basics can be a good point for starting. It brings the reader through different concepts and techniques. The reader can get the keywords for expanding her/his knowledge. There are some important concepts like the concept of resolution and the related sampling problems, spectral unmixing and photon counting that are introduced for further readings. This article reports some interesting examples and a good link between the different mechanisms of contrast, from DIC to phase contrast until fluorescence methods. Treatment is not rigorous, but it keeps the audience interested and is sufficiently clear. I read it with interest even if I would prefer to have more bibliographic modern references to amplify the achievable knowledge.

References

[1] Amos B. (2000): Lessons from the history of light microscopy. Nat Cell Biol. 2(8), E151-2.
[2] Wilson T., Sheppard C. (1984): Theory and practice of scanning optical microscopy. Academic Press, London.
[3] Diaspro A. (2001): Confocal and two-photon microscopy : foundations, applications, and advances. Wiley-Liss, New York.
[4] Pawley JB. (2006): Handbook of Biological Confocal Microscopy, 3rd edition, Plenum-Springer, New York.
[5] Hawkes PW., Spence JCH. (2006): Science of Microscopy, Springer, New York.
[6] Masters BR. (2006): Confocal Microscopy And Multiphoton Excitation Microscopy: The Genesis of Live Cell Imaging, SPIE Press Monograph Vol. PM161, USA.
[7] Hell SW. (2003): Toward fluorescence nanoscopy. Nat Biotechnol. 21, 1347.
[8] Jares-Erijman EA., Jovin TM. (2003): Nat Biotechnol. 21(11), 1387.
[9] Tsien RY. (2006): Breeding molecules to spy on cells. Harvey Lect. 2003-2004, 99, 77.
[10] Tsien RY. (1998): The green fluorescent protein. Annu Rev Biochem. 67, 509.
[11] Pozzan T. (1997): Protein-protein interactions. Calcium turns turquoise into gold. Nature. 388, 834
[12] Diaspro A. (2006): Shine on ... proteins. Microsc Res Tech. 69(3),149
[13] Periasamy A. (2000): Methods in Cellular Imaging, Oxford University Press, New York.
[14] Becker W. (2005): Advanced Time-Correlated Single Photon Counting Techniques, Springer, Berlin.
[15] Periasamy A., Day RN. (2006): Molecular Imaging : FRET Microscopy and Spectroscopy, An American Physiological Society Book, USA.

Imprint

Published by
GIT VERLAG GmbH & Co. KG

Sector Management
Anna Seidinger
a.seidinger@gitverlag.com

Journal Management
Dr. Martin Friedrich
m.friedrich@gitverlag.com

Authors
Dr. Rainer Wegerhoff
rainer.wegerhoff@olympus-europa.com
Dr. Olaf Weidlich
olaf.weidlich@olympus-sis.com
Dr. Manfred Kässens
manfred.kaessens@olympus-sis.com

Editorial Assistance
Lana Feldmann
l.feldmann@gitverlag.com

Production Manager
Dietmar Edhofer
d.edhofer@gitverlag.com

Layout
Ruth Herrmann
r.herrmann@gitverlag.com

Litho and Coverdesign
Elke Palzer
palzer@gitverlag.com

GIT VERLAG GmbH & Co. KG
Rösslerstrasse 90
64293 Darmstadt
Germany
Tel.: +49 6151 8090 0
Fax: 49 6151 8090 133
www.gitverlag.com

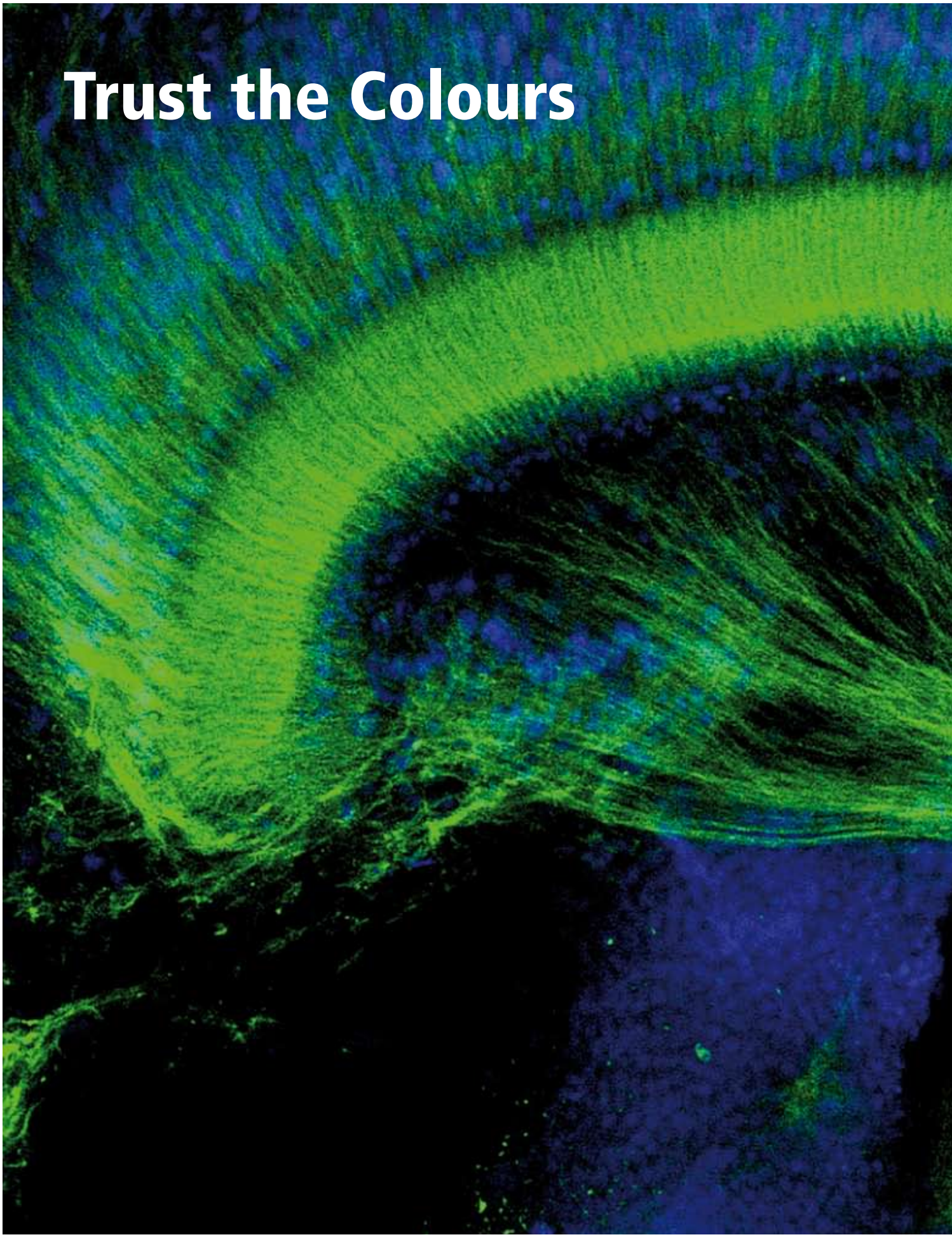
In Cooperation with
Olympus Life and Material Science Europa GmbH
Wendenstrasse 14–18
D-20097 Hamburg, Germany
Tel.: +49 40 23772-0
Fax: +49 40 23 77 36 47
www.olympus-europa.com

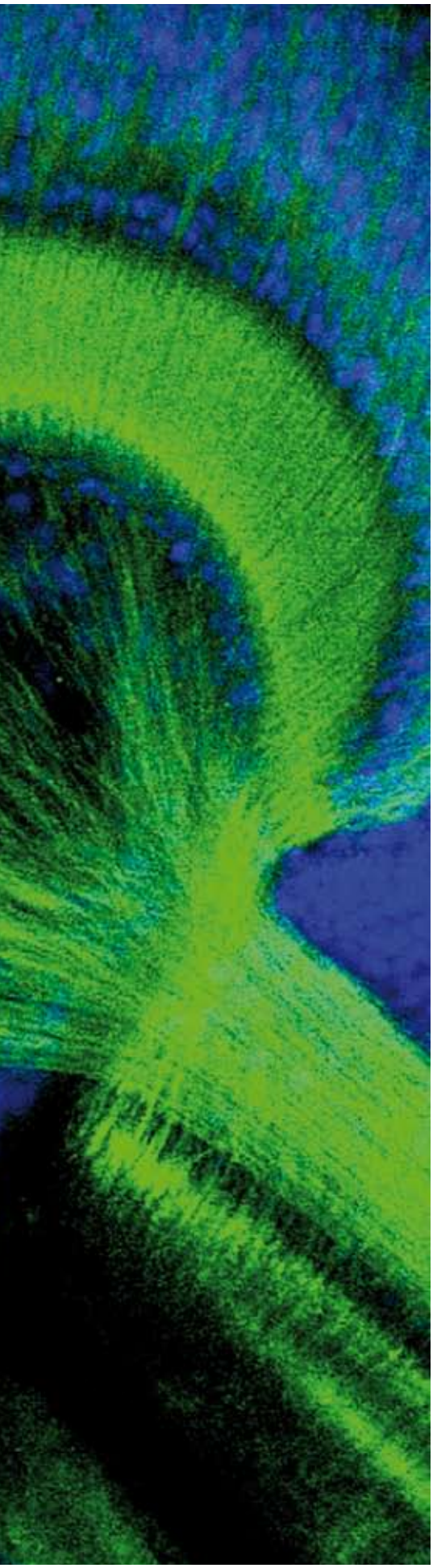
Printed by
Frotscher Druck
Riedstrasse 8, 64295 Darmstadt, Germany

Circulation
20,000 copies

GIT VERLAG
A Wiley Company

Trust the Colours





Light

How do we describe light that reaches our eyes? We would probably use two key words: brightness and colour. Let us compare these expressions to the way light is characterised in natural sciences: It is looked upon as an electromagnetic wave having specific amplitude and distinct wavelength. Both, the workaday and the scientist's perspective essentially mean the same. The wave's amplitude gives the brightness of the light, whereas the wavelength determines its colour. Figure 1 shows how colour and wavelength are related.

Colour

Colour always arises from light. There is no other source. The human eye can perceive light in the colours of a wavelength range between 400 and 700 nm. But instead of having just one distinct colour, light is usually composed of many fractions with different colours. This is what a spectrum of a light implies. For example look at the spectrum of our life source, the central sun, which emits light of all different colours (fig. 2).

White light

All colours of a light source superimpose. So light sources have the colour dependent on the predominant wavelength palette. A candle for example may look yellowish, because the light mainly comprises wavelengths in the 560–600 nm range. Light from sources emitting in the whole spectral range with somewhat comparable amounts appears as white light.

Green grass

What makes us see objects, which are not light sources themselves? There are different processes, which happen when light meets physical matter. They are called reflection, refraction, diffraction and absorption. They all happen together, but usually one process dominates. See for example grass or other dense physical matters: What we see when observing grass is mainly reflected light. But why does grass appear to be green? The reason is that grass reflects only portions of the white daylight. At the same time it absorbs the red and blue portions of the daylight. Thus the green light remains.

Colour models

Much that we know and observe related to colour can be mathematically described by different colour models, i.e. in different colour spaces. These are what the tokens HSI, RGB, and CYMK stand for. Each of these models gives a different perspective and is applied in fields where it can be used more easily than the other models. But nevertheless each model describes the same physical reality.

HSI

Let us stick to the grass and assume it is fresh spring grass. How would we describe it in everyday language? We would characterise it as green – and not as blue or orange. This is the basic hue of a colour. We would probably characterise it additionally as "full" or "deep" – and not as pale. This is the saturation of a colour. Then one would describe it as bright – and not as dark. This is the brightness or intensity of a colour. Hue, Saturation, and Intensity form the HSI colour model. The Munsell Colour Tree (fig. 3) gives a three-dimensional geometrical representation of this model. Here, the hue value is represented by an angle, the saturation by a radius from the central axis, and intensity or brightness by a vertical position on the cylinder.

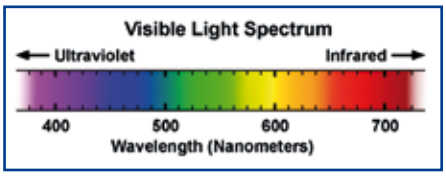


Fig. 1: Visible light spectrum.

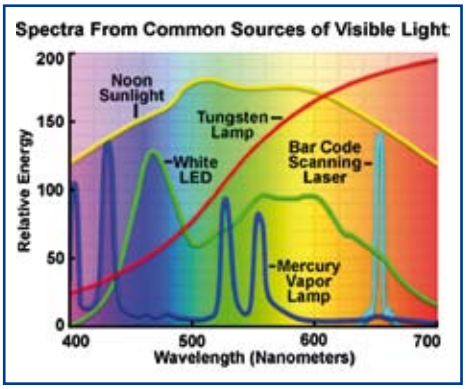


Fig. 2: Spectrum of the sun, and spectra of common sources of visible light.

RGB

The HSI model is suitable to describe and discern a colour. But there is another colour model, which better mirrors how our human perception mechanism works. The human eye uses three types of cone

cell photo receptors which are sensitive to light, respectively in the red V(R), green V(G), and blue V(B) spectral range. These colours are known as primary colours. The clue is that all of the existing colours can be produced by adding various combinations of these primary colours. For example, the human eye perceives an equal amount of all the three colours as white light. The addition of an equal amount of red and blue light yields magenta light, blue and green yields cyan, and green and red yields yellow (fig. 4). All the other colours are generated by stimulation of all three types of cone cells to a varying degree. The practical application of the RGB model is many-fold: For example, besides human perception, digital cameras, monitors, and image file formats also function in a way which can be described by the addition of the three primary colours.

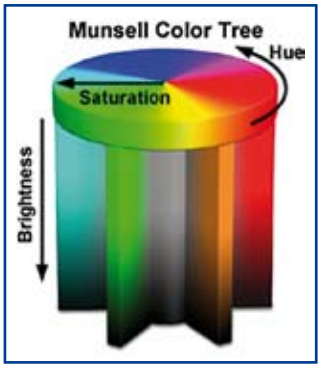


Fig. 3: Munsell Colour Tree.

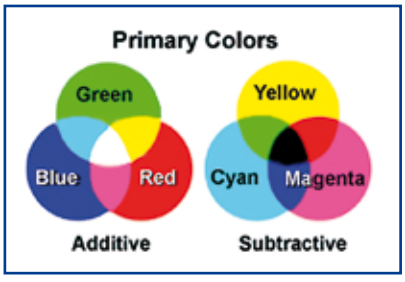


Fig. 4: Primary colours.

CYM

The overlap of the three primary additive colours red, green, and blue creates the colours cyan (C), yellow (Y), and magenta (M). These are called complementary or primary subtractive colours, because they are formed by subtraction of red, green, and blue from white. In this way, yellow light is observed, when blue light is removed from white light. Here, all the other colours can be produced by subtracting various amounts of cyan, yellow, and magenta from white. Subtraction of all three in equal amount generates black, i.e. the absence of light. White cannot be generated by the complementary colours (fig. 4). The CYM model and its more workable extension, the CYMK model, find their applications in the technology of optical components such as filters as well as for printers, for example.

Colour shift

Let us now examine a colour phenomenon which astonishes us in daily life: the colour shift. This also gives us the chance to take the first step into light microscopy and look closer into its typical light sources halogen bulb, xenon arc lamp and mercury arc lamp.

It is a common experience to buy a pair of black trousers that is obviously dark blue when you look at them back home. This inconvenient phenomenon of colour shift is not restricted to the blue trousers. The so-called “white light” that is generated by a halogen bulb at a microscope, differs a lot from light from a xenon burner. At first glance it is the intensity that is obviously different, but even if you reduce the light intensity of a xenon burner, the halogen light will give you a more yellowish impression when projected onto a white surface. Furthermore, dimming the halogen bulb light can make the colour even more red-like. This can be easily observed at the microscope if you focus on a white specimen area and increase the light power slowly. The image observed will change from a yellow-red to a more bluish and very bright image. This means that with increasing power, the intensity or availability of different wavelengths (colours) has been changed. An additional aspect to consider here are the subsequent light perception and interpretation. They are

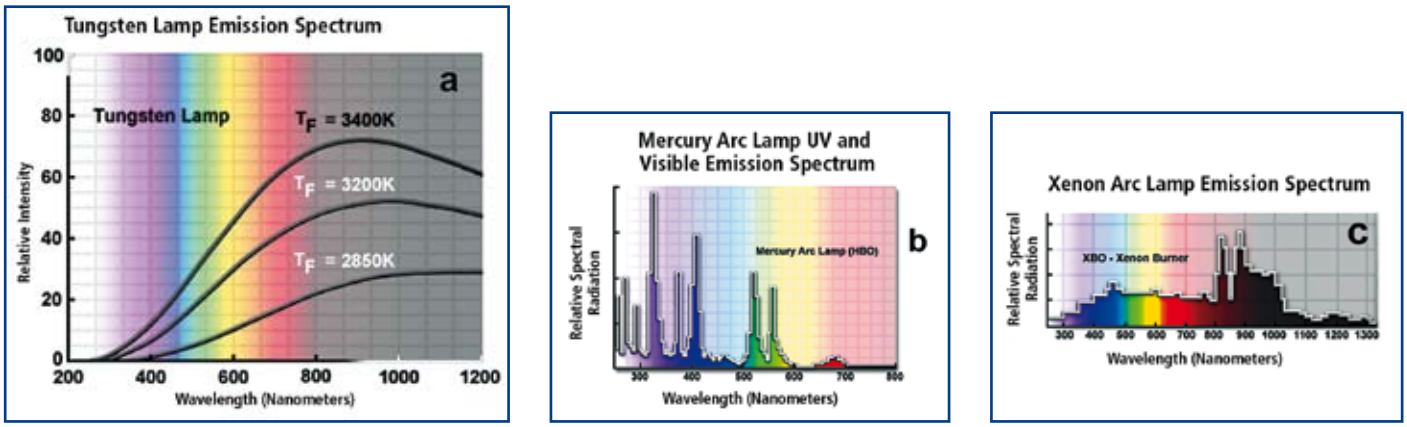


Fig. 5a-c is showing the emission spectra of three typically used light sources at a microscope: (a) the tungsten halogen bulb, T_F = colour temperature at different power settings, (b) the mercury burner, (c) the xenon burner.

done by eye and brain and result in the eventually recognised colour.

But let us come back to the light generation. The overall spectrum of a light source at a defined power setting is described with the term colour temperature. The term colour temperature is a help to describe the spectrum of light sources as if a black piece of ideal metal is heated up. If a temperature of about 3500 K is reached, this metal will have a yellowish colour. This colour will change into a bluish white when it reaches 6000K.

For a 12V / 100W tungsten halogen lamp at +9 Volt the colour temperature is approximately 3200K (fig. 5a) whereas for a 75 W xenon burner it is 6000K. So the colour temperature gives us a good hint about the overall colour shift. Yet it will not give an idea of the individual intensities at defined wavelengths. This knowledge is of high importance if we have to use a light source for example for fluorescence microscopy. In this case the light source has to produce a sufficient intensity of light in a range of wavelengths that match the excitation range of the fluorochrome under observation.

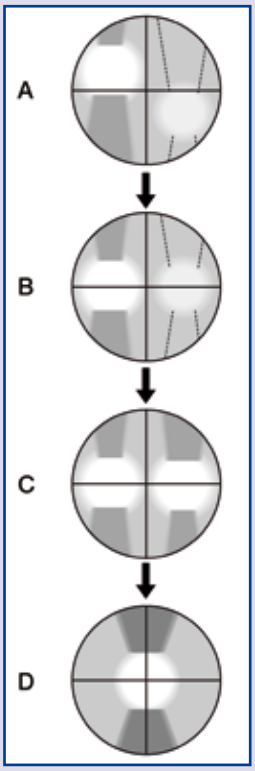
Microscope Light Sources

Tungsten – halogen bulbs

Nearly all light microscopes are equipped with a halogen lamp (10W–100W) either for general use, or as an addition to another light source. A wide range of optical contrast methods can be driven with this type of light source, covering all wavelengths within the visible range but with an increase in intensity from blue to red. Additionally, the spectral curve alters with the used power (fig. 5a). To achieve similar looking colours in the prevalent brightfield microscopy the power setting should be kept at one level, e.g. 9V (T_F= 3200 K; colour temperature at +9V). This light intensity level is often marked at the microscope frame by a photo pictogram.

But here a problem arises: If the light intensity is to be kept fixed in the light microscope the light might be too bright for observation. In daily life if sunlight is too bright sunglasses help – but they

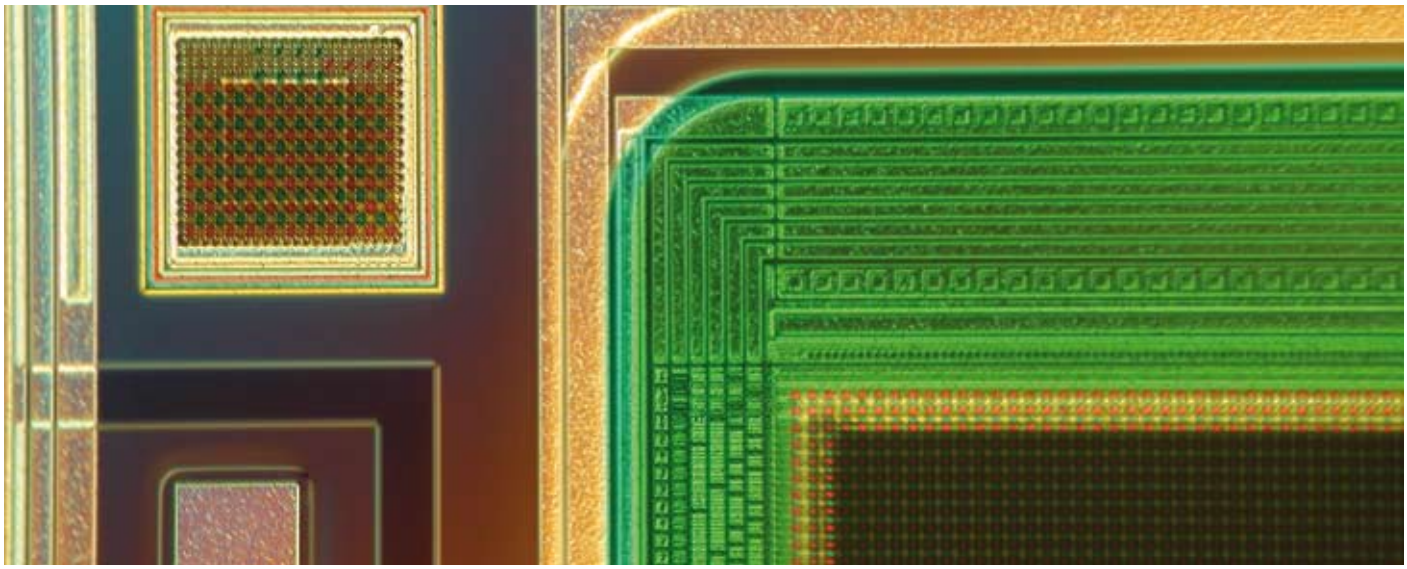
Box 1: Centring of a mercury burner



Depending on the type (inverted or upright) and manufacturer of the microscope there will be some individual differences but the strategy remains the same.

Please also see the instruction manual of the microscope.

1. Start the power supply for the burner, use a UV-protection shield and ensure that a mirror-cube is in the light path.
2. Locate a white paper card on the stage of the microscope and open the shutter. If the light is too bright insert optional available neutral density filters (e.g. ND25 – only 25 % of the light intensity will pass through).
3. Bring the focus to the lowest position.
4. Get a free objective position or on upright microscopes a 20x objective in the light path.
5. If available, open the aperture stop and close the field stop.
6. Optimise brightness with the burner centring knobs (mostly located at the side of the lamp house)
7. From the lamp house, an image of the arc of the burner itself and the mirrored image are projected on the card. To see them in focus, use a collector focussing screw (Figure A).
8. If only one spot can be located or the second is not the same size, the mirror (Figure B) has to be re-centred as well. At most lamp houses there are screws at the back for screwdrivers for this option. Rotate them until the images have the same size as shown in (Figure C).
9. Locate both images parallel to each other and overlay them by using the burner centring screws (Figure D).
10. Defocus the images with the collector focusing screw and open the field stop.
11. Remove the white paper card and bring a homogenous fluorescent specimen into focus (e.g. try some curry on the cover slip).
12. Fine adjustment of the homogenous illumination can only be performed under observation: if necessary readjust the collector focusing screw so that the total field of view is equally illuminated.
13. If digital acquisition is of prime interest, the fine adjustment can also be performed at the monitor, to optimise the illumination for the size of the CCD.



might not only reduce the intensity but also let us see the world in other colours. In light microscopy there are light filters that only reduce the intensity. These filters are called neutral density filters (ND) or neutral grey filters. They are characterised by the light that they will transmit. Therefore, a ND50 will allow half the light intensity to pass through whereas a ND25 will reduce the intensity to 25 % without changing the colours. If the spectrum is changed, we will have colour filters. There is a huge variety of colour filters available but here we will only discuss the so-called light balancing daylight filter (LBD).

This filter is used together with the halogen light source to compensate for the over distribution of the long (red) wavelengths. This enables us to see the colours of a stained pathology section for example on a neutral white background in brightfield microscopy (fig. 11).

Mercury Arc lamp

The mercury burner is characterised by peaks of intensity at 313, 334, 365, 406, 435, 546 and 578nm and lower intensities at other wavelengths (see fig. 5b). This feature enables the mercury burner to be the most used light source for fluorescence applications. Whenever the peak emissions of the burner match the excitation needs of the fluorochromes a good (depending on the specimen) signal can be achieved. However, these benefits are reduced by a relative short lifetime of the burner of about 300 hours and a small change of the emission spectrum due to deposits of cathode material to the inner glass surface of the burner with ongoing lifetime.

Xenon Arc lamp (XBO)

Xenon burners are the first choice light sources when a very bright light is needed for reflected microscopy, such as differential interference contrast on dark objects, or quantitative analysis of fluorescence signals as for example in ion ratio measurement. They show an even intensity across the visible spectrum, brighter than the halogen bulb but they do not reach the intensity peaks of the mercury burners. The xenon burner emission spectrum allows the direct analysis of intensities at different fluorescence excitation or emission wavelengths. The lifetime is of about 500–3000 hours depending on use (frequent on/off switching reduces the lifetime), and the type of burner (75 or 150W). For optimisation of illumination especially with the mercury and xenon burners the centring and alignment is very important.

Table 1: Comparison of different light sources and their colour temperature performance

Light source	Colour temperature
Vacuum lamp (220 W / 220 V)	2790 K
Nitraphot (tungsten filament) lamp B (500 W / 220 V)	3000 K
Photo and cinema lamps as well as colour control lamp (Fischer)	3200 K
Photo and cinema (e.g., nitraphot (tungsten filament) lamp S)	3400 K
Yellow flash bulb	3400 K
Clear flash bulb	3800 K
Moonlight	4120 K
Beck arc lamp	5000 K
White arc lamp as well as blue flash bulb	5500 K
Electron flash unit	5500-6500 K
Sun only (morning and afternoon)	5200-5400 K
Sun only (noontime)	5600 K
Sun and a cloudless sky	6000 K
Overcast sky	6700 K
Fog, very hazy	7500-8500 K
Blue northern sky at a 45° vertical angle	11000 K
International standard for average sunlight	5500 K

Coming to the point

To ensure that the light source is able to emit the required spectrum is one part of the colour story; to ensure that the objective lenses used can handle this effectively is another. When “white” light is passing through the lens systems of an objective refraction occurs. Due to the physics of light, blue (shorter) wavelengths are refracted to a greater extent than green or red (longer) wavelengths. This helps to create the blue sky but is not the best for good colour reproduction at a microscope. If objectives did not compensate this aberration then as outlined in fig. 6 there would be focus points

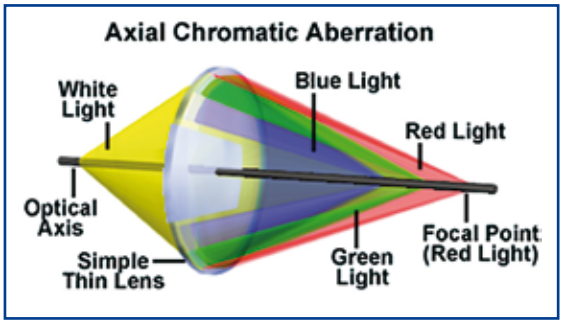


Fig. 6: Schematic of axial chromatic aberration.

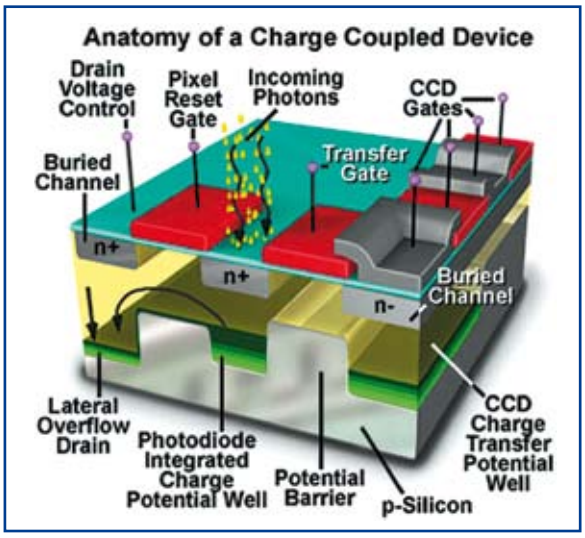


Fig. 7: The principle behind how CCD cameras function.

for all colours along the optical axes. This would create colour fringes surrounding the image.

To reduce this effect achromatic lens combinations have been used since the 18th century. These types of lenses combine the blue and red wavelengths to one focus point. Further colour correction at the visible range can be achieved by adding different sorts of glass systems together to reach the so-called Fluorite objectives (the name originally comes from the fluorspar, introduced into the glass formulation). They are also known as Neofluar, Fluotar, or Semi-apochromat. To correct the complete range from near infrared (IR) to ultraviolet (UV) the finest class of objectives, apochromate, has been designed. Beside colour correction the transmission power of an objective can also be of prime interest. This is especially the case if high power near UV is needed at e.g. 340 nm (excitation of the calcium sensitive dye Fura-2) or IR is needed for 900 nm IR-DIC.

The look of colour

Let us now go one step further and see how the light with its colours – after having passed the microscope – is detected in modern camera systems. Here, digital cameras have become standard for acquisition of colour or monochrome images in the microscopy field. But how do these cameras work and how can it be guaranteed that the colours in the images they provide are accurate? This is of critical importance because it is a primary prerequisite for all activities such as image documentation, archiving or analysis.

Detecting colour

Light detection functions in the same way for both colour and monochrome cameras. Fig. 7 illustrates how CCD digital cameras (Charged Coupled Device) function. In colour cameras, mosaic filters are mounted over the CCD elements (fig. 8). These filters ensure that only green, red or blue light is permitted to come into contact with the light-sensitive part of the single sensors or pixels (picture element). The proportion of colours is generally two green pixels to one red and one blue pixel. The colour image is actually generated by the computer initially via complex algorithms. This involves assessing and processing the respective signals of the green, red and blue pixels accordingly. For example, a single pixel with a bit depth of 8 becomes a 24-bit colour pixel (fig. 9).

Depending on the imaging method, the reflected or transmitted light of the object is focused onto a light-sensitive CCD sensor. So what is a CCD (Charge Coupled Device) element? This is semiconductor architecture designed to read out electric charges from defined storage areas (figs. 7, 10). Due to the special readout method, the light-sensitive area of a pixel is restricted to just 20 % of the actual pixel surface. This is why special

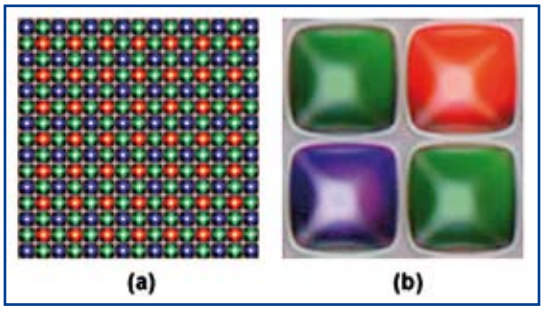


Fig. 8: Bayer colour filter mosaic array and underlying photodiodes.

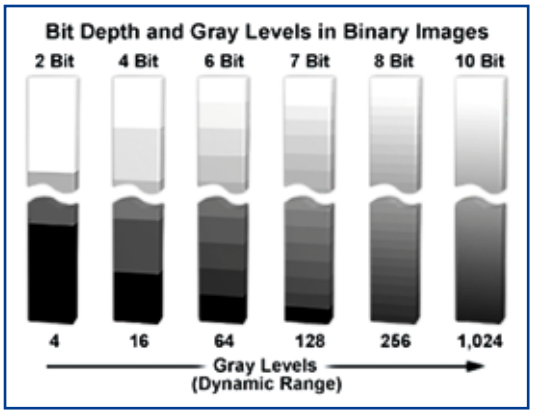


Fig. 9: Bit depth and grey levels in digital images.

lenses above the sensor focus all incoming light onto the light-sensitive area of the pixel surface. The light generates electron-hole pairs via the photoelectric effect. These electrical charges are collected, combined into charge packets and subsequently transported through the entire CCD sensor. The charge is converted into a voltage first because processing voltage is significantly easier than processing current. This analogue output signal is then amplified firstly on the chip itself and then again outside the chip. An analogue/digital converter converts the voltage (the signal) into a binary format.

There are various bit depths, with the standard being 8 bit. This means that 256 combinations (intensity values) are available for each pixel (figs. 9, 10). Currently, many new cameras appear on the market with bit depths of 12 bit (4096 intensity levels). There are even cameras offering bit depths up to 16 bit (65656 intensity levels). The number of pixels a camera has depends on the CCD chip used in it and the technology to create the image. At the moment cameras are on offer with a resolution of up to 12.5 million pixels.

Colour temperature and white balance

Let us now continue on the colour temperature subject of the microscope light sources and review its impact on the colours in digital imaging. As we know light is essential for both microscopes and cameras. There are many types of light, as was explained above for microscope light sources. The sun may yield different colours depending on the time of day and in the microscope there are variations depending on the source used as well as on the acquisition environment. The reason for this is what is referred to as colour temperature. As we have seen this term describes a specific physical effect – i.e., the spectral compositions of differing light sources are not identical and furthermore are determined by temperature (see Table 1). This means that colour temperature is of tremendous significance with regard to digital image acquisition and display as it influences both colour perception and colour display to such a critical degree. A person's eyes automatically correct against this effect subconsciously by adapting to changing lighting conditions. This means that he/she will see those things that are known to be white as white.

Digital or video cameras are unfortunately not intelligent enough to register changing light conditions on their own and to correct against the resulting colour shifts. This is why these kinds of cameras often yield images whose colours are tinged. Correcting colour tinge(s) in true-colour images is known as white balance (see fig. 11). Many cameras tend to adapt image colours at acquisition, this kind of colour displacement can be corrected retroactively. To do so, requires an image with an area where the user knows there should be no colour. In fact, this particular image area should be black, white or grey.

Automated white balance adjustments

Most imaging software programmes offer embedded algorithms to correct for colour tinges. To do this, the user has to define a section interactively within the image area where it is certain that the pixels should be white, black or grey (but at present are tinged). First of all, three correction factors will be calculated based on the pixels within the section – one for each of the three colour components Red (R), Green (G), Blue (B). These correction factors are defined such that the pixels within the section will be grey on average – the section will have no colour at all. The whole image is then corrected automatically using these correction factors (fig. 11c, d).

The following is a more detailed description of what takes place. The average intensity I for each pixel (n) within the section will be calculated: I_n= (R+G+B)/3. The colour factor (F) for each colour component, of each pixel within the section will then be determined based on this calculation, e.g., for the red colour factor: F_n (R) = (I_n/R).

Take a look at this example. A pixel has the following colour components: (R,G,B) = (100,245,255) – thus an average intensity of I_n =200 and a red colour factor of F_n (R) = (200/100) = 2.0. The three colour factors will be averaged for all pixels within the circle, meaning that a correction factor (<F(R)>, <F(G)> and <F(B)>) will be determined for each colour component. And now, the colour components of all the image's pixels will be multiplied by the corresponding correction factor(s).

Why do colours not match easily?

It would be ideal to be able to pick any digital camera, monitor or printer at random and have the resulting colours on-screen and in the printed output be acceptably close to the original. But unfortunately, getting colours to match is hard to achieve.

For one thing, colour is subjective. This means that colour is an intrinsic feature of an object and colours are purely subjective – as interpreted by the visual system and the brain. Another critical aspect is that lighting affects col-

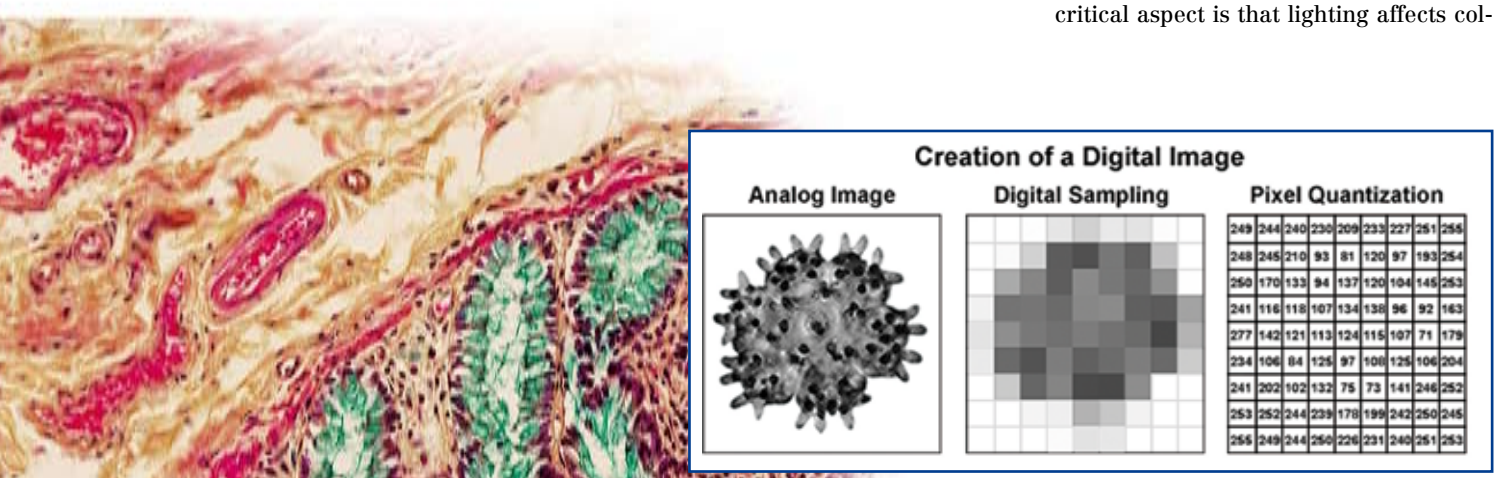


Fig. 10: Creation of a digital image.

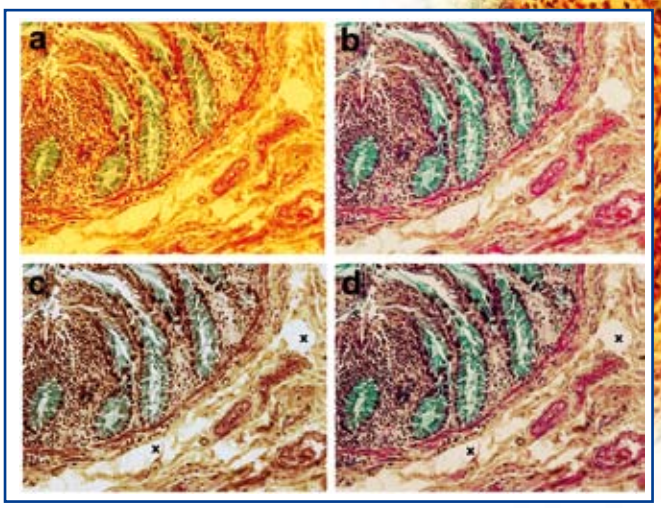


Fig. 11: White balance adjustment using microscope and software options on a histological specimen.
a: Image with wrong microscope settings (low power 3V, no LBD filter), note that areas that are not stained (background) show a yellowish colour.
b: image using optimised microscope settings (9V + LBD filter + Neutral density filer ND6), note that the background becomes white.
c: Image after digital white balancing of image (a), note that with the help of a software white balancing on the yellowish image (a) the colours can be recalculated to some extend to a well balanced image (compare with b or d).
d: Image after digital white balancing of image (b), note that an already well balanced image (b) can still be enhanced in quality. Areas that are used to define (neutral background) regions of interest (ROI), are marked with (x).

our. So a printout will vary depending on the lighting. It will look different under incandescent light, fluorescent light and in daylight. Furthermore, colours affect other colours. This means your perception of a colour will change depending on the colours surrounding that colour. In addition, monitors can display colours that printers cannot print. Printers can print colours that monitors cannot display. Cameras can record colours that neither monitors nor printers can produce. A colour model is at the very least simply a way of representing colours mathematically. When different devices use different colour models they have to translate colours from one model to another. This often results in error. Given all the limitations inherent in trying to match colours, it is important to understand the difference between correctable colour errors and non-correctable errors. Correctable errors are ones where you can do something about them via software – such as with a colour management system or white balance. Non-correctable errors are the ones that you can't do anything about because the information you need to correct them simply does not exist. Better lenses, better optical coatings and improved CCD arrays can all minimise the non-correctable errors. Correctable errors, however, require colour management through software.

If every program used the same approach to colour management and every printer, monitor, scanner and camera were designed to work with that colour management scheme, colour management would obviously be a snap. If the hardware devices all used the same standard colour model, for example, or came with profiles that would translate colour information to and from a standard as needed, you'd be able to move colour information around – from program to program or scanned image to printer – without ever introducing errors.

Conclusion

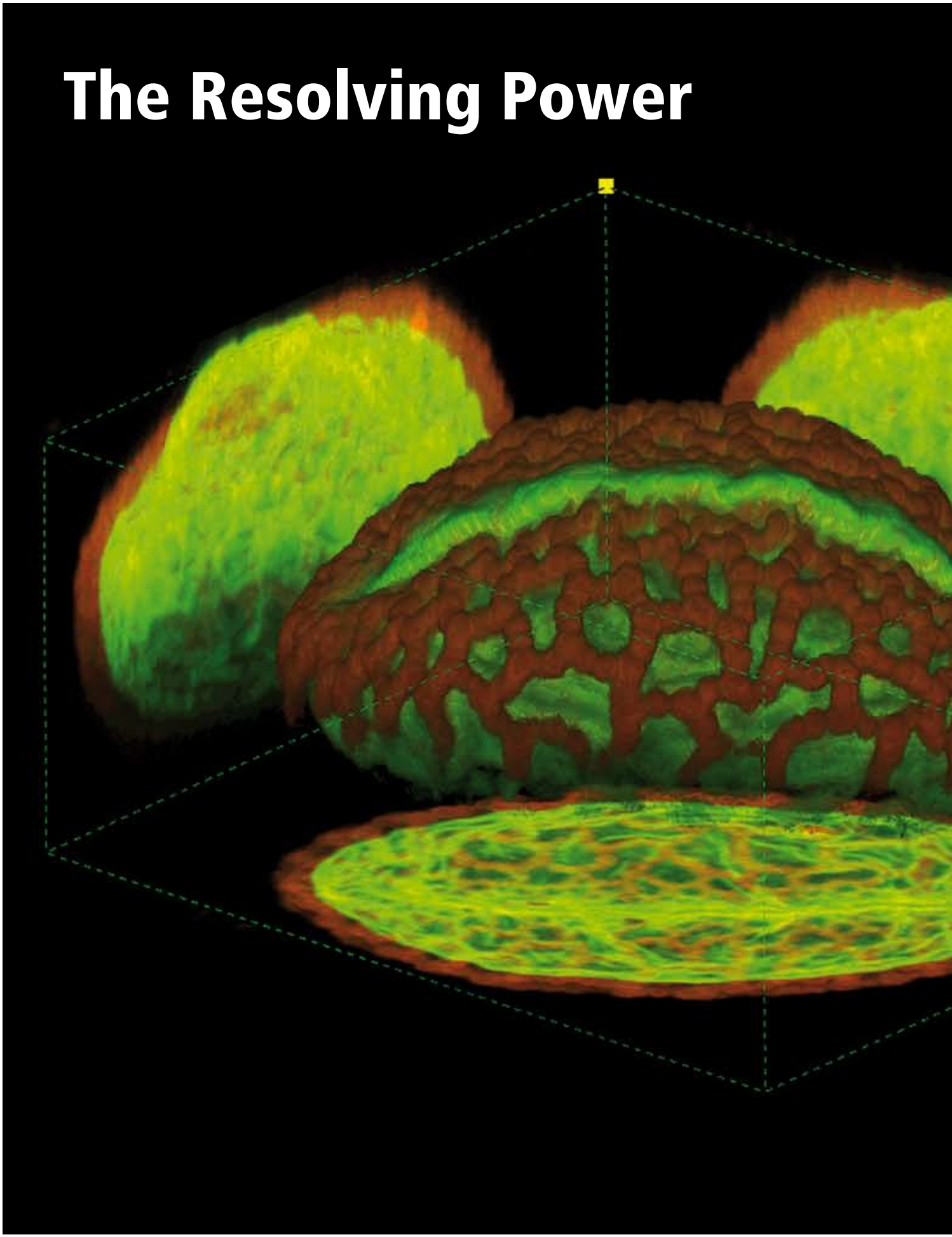
The light that reaches our eye consists of many different colours, and different light sources produce a different mix of these. Different objects in the world absorb and reflect different wavelengths – that is what gives them their colour.

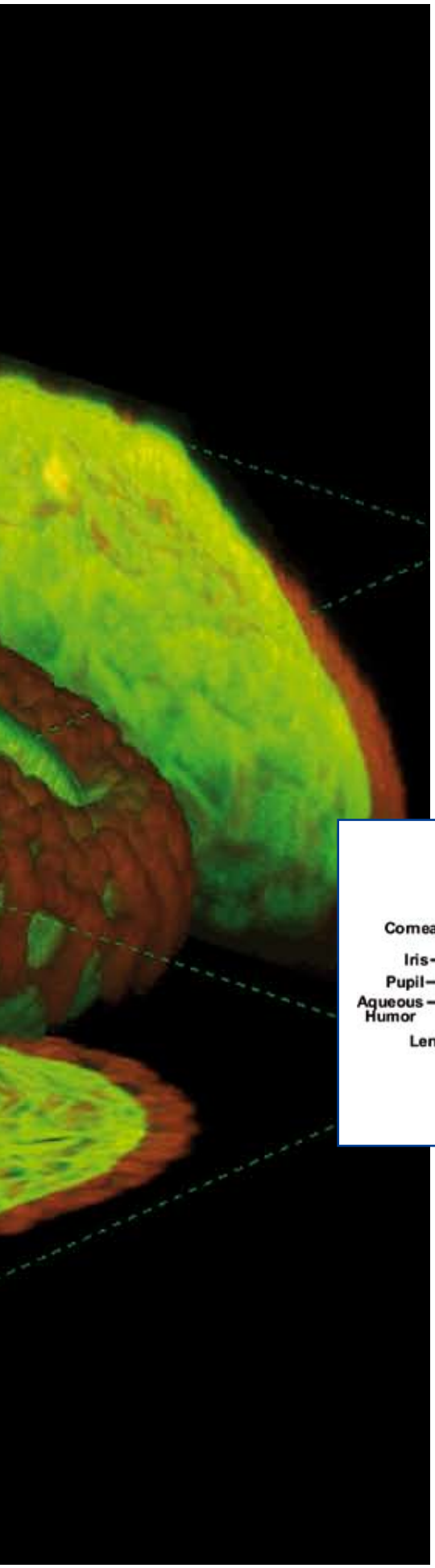
There are many colour models to map this multi-dimensional world, each describing the same physical reality but having different applications. Our vision systems give us only a partial view of it.

For effective imaging, the microscopist must be aware of the complexity of the world of light. There is a variety of light sources, objectives and filters, to enable the assembly of a system with the appropriate properties for a particular application. The photographer in turn works with an imaging system which takes partial information and processes it to achieve colour balance. Printers and monitors in turn repeat the process – and remove one further from the original data.

Several hundred years in the development of optics have managed to hide some of the complexity from the casual user. But microscopy is a good deal more complicated than it might seem. An awareness of what is really going on – in our eyes, in our brains, in the optics, on the CCD and on the screen – enables us to appreciate just what a wonderful achievement that final image is, and how much care is required to ensure the best results.

The Resolving Power





Human vision

For most of us, seeing something is normal and we are accustomed to evaluate things by looking at them. But the seeing act uses many different and complex structures that enable us not only to catch images but to process and interpret them at the same time. Every image that is projected on the retina of our eye is transformed into neuronal impulses creating and influencing behaviour: this is what we call seeing. Therefore, what we see is not necessarily what another person sees.

Compared to these processes within our brain, the first steps of vision seem to be much simpler. To create a projection onto the layer of the retina, where millions of light sensitive sensory cells are located, the light passes through an optical system that consists of cornea, aqueous humour, iris, pupil, focus lens and the vitreous humour, see fig. 12. All these elements together create what the raster framework of sensory cells can translate within their capability into neuronal activity.

Eagles and mice

Those two different set ups – the optical system and the sensor system – restrict the options of human vision at the very first step. Details can be found at www.mic-d.com/curriculum/lightandcolor/humanvision.html. Having eagle eyed optical systems alone will not enable us to see mice from far away. To resolve a small object (the mouse) against a large background needs special physical properties of the optics and also requires a certain sensitivity and number of sensors used to translate the information.

If this text is getting too small, you have to come closer to ..... This means your eyes can get light from the small letters in a wider angle than before. You are now focusing on a smaller area, spread over the total amount of sensory cells. More individual sensors can catch differences and translate them into a recognised image.

Unfortunately, we can move closer only up to a certain distance, after which our flexible optical system is no longer able to project the image clearly onto the retina. If the words are typed too small, like these ["you can not resolve it without optical help"] "You can not resolve it without optical help". our resolution is reached.

The wavelength that transports the information of the image also influences what we can resolve. Shorter blue wavelengths carry finer details than longer ones from the same object. Using a microscope it would be easy to read the text and to document it via the sensors of a camera. But these tools are also restricted by the resolution of their optical system and the number and sensitivity of sensors – the pixels.

What is resolution?

Resolution or resolving power of a microscope can be defined as the smallest distance apart at which two points on a specimen can still be seen separately. In the transmitted light microscope, the xy-resolution R is determined essentially by three parameters: the wavelength λ of the illuminating light, and the numerical aperture (NA) of the objective (NA^{obj}) as well as the condenser (NA^{cond}).

$$R = 1.22 * \lambda / (NA^{obj} + NA^{cond}) \quad (1)$$

When the aperture of the condenser is adjusted to that of the objective, i.e. the aperture of the condenser is essentially the same as the objective aperture, the equation (1) simplifies to:

$$R = 0.61 * \lambda / NA^{obj} \quad (2)$$

This equation is used for both transmitted light microscopy and for reflected

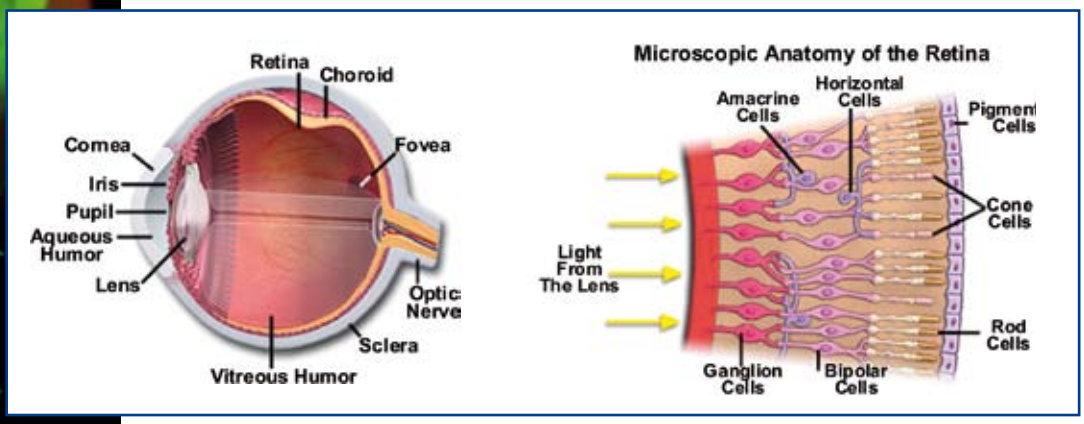


Fig. 12: Anatomy of the human eye and cross section of the retina.

light microscopy. Note here that resolution is NOT directly dependent on the magnification. Furthermore the end magnification should not be higher than 1000x the NA of the objective, because then the image will be only enlarged but no further resolution will be visible. This is called empty magnification.

What is the numerical aperture of an objective?

The numerical aperture of a microscope objective is a measure of its ability to gather light and thus resolve fine specimen detail at a fixed objective distance. The numerical aperture is calculated with the following formula:

$$NA = n*(\sin \mu) \qquad (3)$$

n is the refractive index of the medium between the front lens of the objective and the specimen cover glass. It is n = 1.00 for air and n = 1.51 for oil. μ is one half the angular aperture, as can be seen in fig. 13. The bigger μ, the higher is the numerical aperture. Working in air, the theoretical maximum value of the numerical aperture is NA = 1 (μ = 90°). The practical limit is NA = 0.95.

Numerical aperture in practice

For all microscope objectives the resolving power is mentioned with the number for the numerical aperture shown directly following the index for magnification (fig. 14), e.g. a UPlanFLN 60x/0.9 objective produces a 60x magnification with a numerical aperture of 0.9. The Numerical Aperture strongly differs depending on the type of objective or condenser, i.e. the optical aberration correction. Table 2 lists some typical numerical apertures for different objective magnifications and corrections.

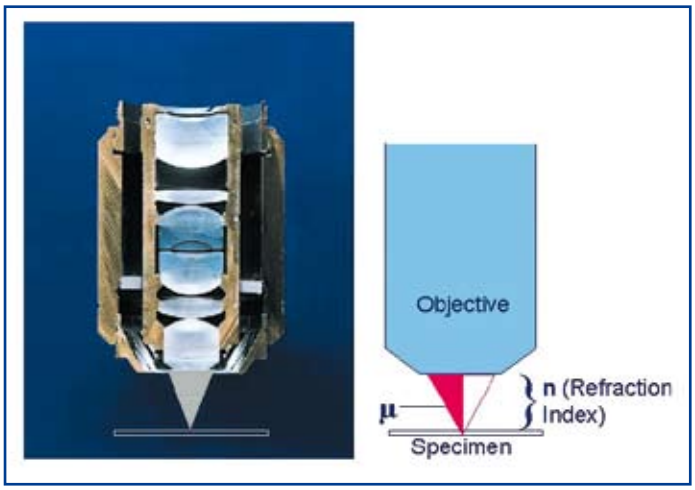


Fig. 13: Angular aperture of an objective.

Table 2: Numerical Apertures (NA) for different types of objectives and magnifications

Magnification	Plan Achromat	Plan Fluorite	Plan Apochromat
4 x	0.1	0.13	0.16
10x	0.25	0.3	0.4
20x	0.4	0.5	0.75
40x	0.65	0.75	0.9
60x	0.8 (dry)	0.9 (dry)	1.42 (oil immersion)
100x	1.25 (oil)	1.3 (oil)	1.4 (oil)

To achieve higher NA than 0.95 for objectives they have to be used with immersion media between front lens and specimen. Oil or water is mostly used for that purpose. Because objectives have to be specially designed for this, the type of immersion media is always mentioned on the objective like on the UPlanFLN 60x/1.25 Oil Iris objective. This objective needs oil as an immersion media and can not produce good image quality without it.

For special techniques like the Total Internal Reflection Fluorescence Microscopy (TIRFM), objectives are produced with very high NA leading by the Olympus APO100x OHR with a NA of 1.65. The resolution that can be achieved, particularly for transmitted light microscopy, is depending on the correct light alignment of the microscope. Köhler illumination is recommended to produce equally distributed transmitted light and ensure the microscope reaches its full resolving potential (see box 2).

What resolution can be reached with a light microscope?

To make the subject more applicable, some resolution numbers shall be given here. Using a middle wavelength of 550 nm, the Plan Achromat 4x provides a resolution of about 3.3 μm, whereas the Plan Apochromat reaches about 2.1 μm. The Plan Achromat 40x provides a resolution of 0.51 μm and the Plan Apochromat of 0.37 μm. The real-world limit for

Box 2: How to set Köhler illumination

To align a microscope that can change the distance of the condenser to the area of the specimen for Köhler illumination you should:

1. Focus with a 10x or higher magnifying objective on a specimen, so that you can see at least something of interest in focus. (If your condenser has a front lens please swing it in when using more then 10x magnifying objectives.)
2. Close the field stop at the light exit.
3. Move the condenser up or down to visualise the closed field stop. Now only a round central part of your field of view is illuminated.
4. If the illumination is not in the centre the condenser has to be moved in the XY direction to centre it.
5. Finely adjust the height of the condenser so that the edges of the field stop are in focus and the diffraction colour at this edge of the field stop is blue green.
6. Open the field stop to such an amount that the total field of view is illuminated (re-centring in xy direction may be necessary).
7. For best viewing contrast at brightfield close the aperture stop to an amount of 80 % of the objective numerical aperture.

In practice, you can slowly close the aperture stop of your condenser while looking at a specimen. At the moment when the first change in contrast occurs, the NA of the condenser is getting smaller then the NA of the objective and this setting can be used. For documentation the condenser NA should be set according to that of the objective. To visualise the aperture stop directly you can remove one eyepiece and see the aperture stop working as a normal diaphragm.

the resolution which can be reached with a Plan Apochromat 100x is often not higher than R = 0.24 µm.

To give a comparison to other microscopes that do not work with visible light, the resolution reached nowadays in a scanning electron microscope is about R = 2.3 nm. In a transmission electron microscope, structures down to a size of 0.2 nm can be resolved. Scanning probe microscopes even open the gates to the atomic, sub Angstrøm dimensions and allow the detection of single atoms.

Table 3: Optical resolution and number of needed pixels. The number of pixels a 1/2 inch chip should have to meet the Nyquist criterion (2 pixels per feature) and the optimum resolution (3 pixels per feature). The number for LP/mm is given for the projection of the image on the CCD.

Objective	Magnification	NA	Resolution (µm)	Lp/mm (on CCD)	CCD resolution 1/2˝ Nyquist limit 2 pixel/lp	CCD resolution 1/2˝ Necessary resolution 3 pixel/lp
PlanApoN	2	0,08	4,19	119	1526 x 1145	2289 x 1717
UPlanSApo	4	0,16	2,10	119	1526 x 1145	2289 x 1717
UPlanSApo	10	0,4	0,84	119	1526 x 1145	2289 x 1717
UPlanSApo	20	0,75	0,45	111	1420 x 1065	2131 x 1598
UPlanSApo	40	0,9	0,37	67	858 x 644	1288 x 966
UPlanSApo	100	1,4	0,24	42	534 x 401	801 x 601

What are airy disks?

Every specimen detail that is illuminated within a microscope creates a so-called diffraction pattern or Airy disk pattern. This is a distribution of a bright central spot (the Airy disk or primary maximum), and the so called secondary maxima separated by dark regions (minima or rings of the Airy disk pattern; see fig. 15, 16) created by interference (More details: www.mic-d.com/ curriculum/lightandcolor/diffraction.html). When two details within the specimen are closely together we can only see them separated if the two central spots are not too close to each other and the Airy disks themselves are not overlapping. This is what the Rayleigh criterion describes. Good distinction is still possible when one Airy disk just falls in the first minimum of the other (fig. 15).

The smaller the Airy disks, the higher the resolution in an image. Objectives which have a higher numerical aperture produce smaller Airy disks (fig. 16) from the same specimen detail than low NA objectives. But the better the resolution in the xy direction, the less is the specimen layer that is in sharp focus at the same time (Depth of field), because the resolution also gets better in the z direction. Like higher magnification, higher resolution always creates less depth of field. Also in most cases the increase of NA means that the objective gets closer to the specimen (less working distance) compared to an objective of lower NA but with same magnification. Therefore, choosing the best objective for your application may not only depend on the resolving power.

Resolution in digital images – is it important?

The next step is to go from the optical image to the digital image. What happens here? The "real" world conversion from an optical to a digital image works via the light sensitive elements of the CCD chips in digital cameras, for example. Or, a video camera sensor may provide voltage signals that are read out and digitised in special frame grabber cards. But what is of more interest here is the principle which lies behind the actual realisations. The optical image is continuous-tone, i.e. it has continuously varying areas of shades and colour tones. The continuous image has to be digitised and quantified; otherwise it cannot be dealt with in a computer. To do so, the original image is first divided into small separate blocks, usually square shaped, which are called pixels. Next, each pixel is assigned a discrete brightness value. The first step is called digital sampling, the second pixel quantisation. Both convert the continuous-tone optical image into a two-dimensional pixel array: a digital image.

Digital resolution – what is it for?

The pixel quantisation of the image intensities depends on the bit depth or dynamic range of the converting system. The bit depth defines the number of grey levels or the range of colour values a pixel can have, and thus determines a kind of a brightness or colour resolution. Yet it is the digital sampling which defines the spatial resolution in a digital image. Both spatial and brightness resolutions give the image the capability to reproduce fine details that were present in the original image. The spatial resolution depends on the number of pixels in the digital image. At first glance, the following rule makes sense: the higher the number of pixels within the same physical dimensions, the higher becomes the spatial resolution. See the effect of different numbers of pixels on the actual specimen structure in fig. 17. The first image (175 x 175) provides the image information as reasonably expected, whereas specimen details will be lost with fewer pixels (44 x 44). With even fewer, the specimen features are masked and not visible any more. This effect is called pixel blocking.

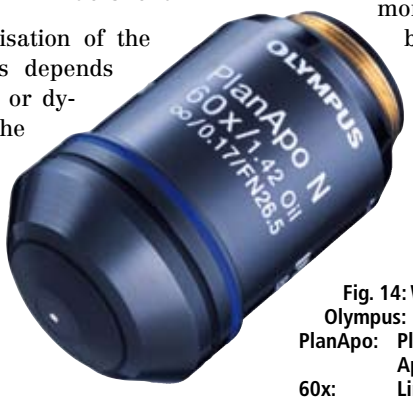


Fig. 14: What is what on an objective?

Olympus:	Manufacturer
PlanApo:	Plan: Flat field correction; Apo: Apochromatic;
60x:	Linear magnification
1,42 Oil:	Numerical Aperture (needs oil immersion)
∞:	Infinity corrected optic (can not be mixed with finite optics that belongs to the 160 mm optics)
0.17:	Cover slip correction. Needs a cover slip of 0.17mm thickness.
Note:	Take care of this parameter. There can also be a "0" for no coverslip, a "1" for 1mm thickness or a range of mm. Wrong usage of objectives will create "foggy" images (spherical aberration).
FN 26.5:	Field number 26.5 (When using an ocular and tube that can provide a FN of 26.5 you may divide this number with the magnification to achieve the diameter in your field of view in mm. 26.5/60 = 0,44 mm).

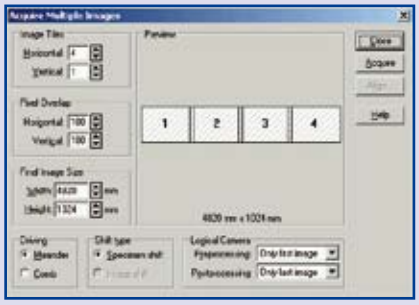
Box 3:

Multiple Image Alignment (mia)

Multiple image alignment is a software approach to combine several images into one panorama view having high resolution at the same time.

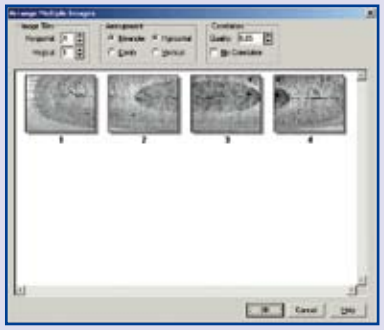
Here, the software takes control of the microscope, camera, motor stage, etc. All parameters are transferred to the imaging system through a remote interface. Using this data, the entire microscope and camera setups can be controlled and calibrated by the software. After defining the required image size and resolution, the user can execute the following steps automatically with a single mouse click:

1. Calculation of the required number of image sections and their relative positions



2. Acquisition of the image sections including stage movement, image acquisition and computing the optimum overlap

3. Seamless "stitching" of the image sections with sub-pixel accuracy by intelligent pattern recognition within the overlap areas.



4. The overlap areas are adjusted automatically for differences in intensity

5. Visualisation of the full view image.

Is there an optimum digital resolution?

Thus, the number of pixels per optical image area must not be too small. But what exactly is the limit? There shouldn't be any information loss during the conversion from optical to digital. To guarantee this, the digital spatial resolution should be equal or higher than the optical resolution, i.e. the resolving power of the microscope. This requirement is formulated in the Nyquist theorem: The sampling interval (i.e. the number of pixels) must be equal to twice the highest spatial frequency present in the optical image. To say it in different words: To capture the smallest degree of detail, two pixels are collected for each feature. For high resolution images, the Nyquist criterion is extended to 3 pixels per feature.

To understand what the Nyquist criterion states, look at the representations in fig. 18 and 19. The most critical feature to reproduce is the ideal periodic pattern of a pair of black and white lines (lower figures). With a sampling interval of two pixels (fig. 18), the digital image (upper figure) might or might not be able to resolve the line pair pattern, depending on the geometric alignment of specimen and camera. Yet a sampling interval with three pixels (fig. 19) resolves the line pair pattern under any given geometric alignment. The digital image (upper figure) is always able to display the line pair structure.

With real specimens, 2 pixels per feature should be sufficient to resolve most details. So now, we can answer some of the questions above. Yes, there is an optimum spatial digital resolution of two or three pixels per specimen feature. The resolution should definitely not be smaller than this, otherwise information will be lost.

Calculating an example

A practical example will illustrate which digital resolution is desirable under which circumstances. The Nyquist criterion is expressed in the following equation:

$$R * M = 2 * \text{pixel size} \qquad (4)$$

R is the optical resolution of the objective; M is the resulting magnification at the camera sensor. It is calculated by the objective magnification multiplied by the magnification of the camera adapter.

Assuming we work with a 10x Plan Apochromat having a numerical aperture (NA) = 0.4. The central wavelength of the illuminating light is λ = 550 nm. So the optical resolution of the objective is R = 0.61* λ/NA = 0.839 µm. Assuming further that the camera adapter magnification is 1x, so the resulting magnification of objective and camera adaptor is M = 10x. Now, the resolution of the objective has to be multiplied by a factor of 10 to calculate the resolution at the camera:

R * M = 0.839 µm * 10 = 8.39 µm. Thus, in this setup, we have a minimum distance of 8.39 µm at which the line pairs can still be resolved. These are 1 / 8.39 = 119 line pairs per millimetre.

The pixel size is the size of the CCD chip divided by the number of pixels.

A 1/2 inch chip has a size of 6.4 mm * 4.8 mm. So the number of pixels a 1/2 inch chip needs to meet the Nyquist cri-

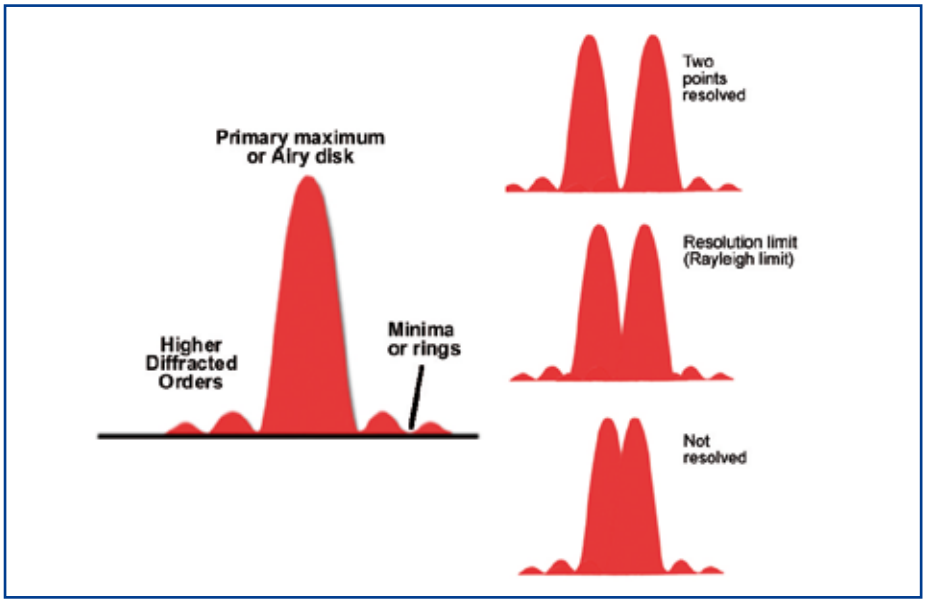


Fig. 15: Intensity profiles of the Airy disk patterns of one specimen detail and of two details at different distances.

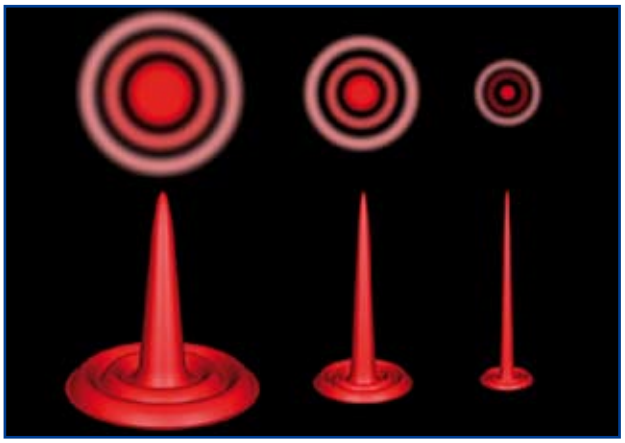


Fig. 16: Airy disk patterns of different size as an example of the resolving power for low NA (left) and high NA (right) objectives.

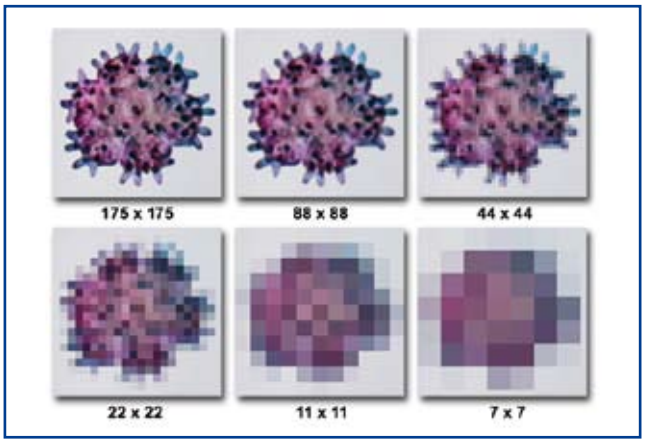


Fig. 17: Four representations of the same image, with different numbers of pixels used. The numbers of pixels is written below each image.

terion with 2 pixels per feature, is 1/(R * M) * chip size * 2 = 119 line pairs / mm * 6.4 mm *2 = 1526 pixels in horizontal direction. If you want 3 pixels per line pair, the result is 2289 pixels. This calculation can be followed through for different kind of objectives. Please check out, which numbers of pixels we need for a 1/2 inch chip in table 3.

What might be astonishing here is the fact, that the higher the magnification, the fewer pixels the chip of a CCD camera needs! Working with a 100x Plan Apochromat objective combined with an 1/2 inch chip, we need just 800 x 600 pixels to resolve digitally even the finest optically distinguished structure. The higher number of pixels of about 2300 x 1700 is necessary only at lower magnifications up to 10.

Which camera to buy?

The resolution of a CCD camera is clearly one important criterion for its selection. The resolution should be optimally adjusted to your main imaging objective. Optimum resolution depends on the objective and the microscope magnification you usually work with. It should have a minimum number of pixels to not lose any optically achieved resolution as described above. But the number of pixels also should not be much higher, because the number of pixels is directly correlated with the image acquisition time. The gain in resolution is paid for by a slow acquisition process. The fastest frame rates of digital cameras working at high resolution can go up to the 100 milliseconds or even reach the second range, which can become a practical disadvantage in daily work. Furthermore, unnecessary pixels obviously need the same amount of storage capacity as necessary pixels. For example, a 24 bit true colour image consisting of 2289 x 1717 pixels has a file size of almost 12 MB, if there is no compression method applied. The slow frame rate and the file size are just two aspects which demonstrate, that the handling of high resolution images becomes increasingly elaborate.

High resolution over a wide field of view

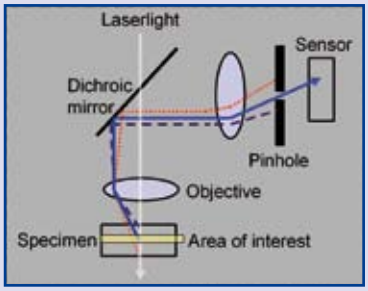
The xy-resolution desired is one aspect which makes an image 'high quality'. Another partially contradicting aspect is that we usually want to see the largest possible fraction of the sample – in best case the whole object under investigation. Here, the microscope's field of view becomes important. The field of view is given by a number – the so called field number – e.g. if the microscope is equipped for the field number 22 and a 10x magnifying objective is in use, the diagonal of the field of view (via eyepieces and tube that supports the given FN) is 22/10 = 2.2 mm. Using lower magnifying objectives will enable a larger field of view and using large field tubes and oculars can enlarge the field number to 26.5 (e.g. field number 26.5 and 4x objective allows a diagonal of 26.5/4 = 6.624 mm), but unfortunately low magnifying objec-

Box 4: Using hardware to increase the resolution in fluorescence microscopy

Several hardware components are available, to enable the acquisition of images that are not disturbed by out of focus blur. For example, grid projection, confocal pinhole detection and TIRFM.

Since out of focus parts of a specimen produce blur in an image, there is the simple option of eliminating this stray light from the image. With most confocal microscopes, a small pinhole is located in front of the sensor and only those light beams that are originally from the focus area can pass through, others are simply absorbed. The resulting point image only contains information from the focus area. To create an image of more then just one point of the focus area, a scanning process is needed.

This scanning process can be performed with the help of spinning disks for an ordinary fluorescence microscope or by a scanning process with a laser beam in a confocal laser scanning microscope (cLSM) set-up. Each system produces different levels of improvement to the resolution. However, all of them need a professional digital sensor system to display the images.



TIRFM (Total Internal Reflection Fluorescent Microscopy) uses a completely different concept. With this method, a very thin layer of the specimen (around 200 nm) is used to create the image. Therefore, TIRFM is ideal to analyse e.g. single molecule interactions or membrane processes. To achieve this target, a light beam is directed within a critical angle towards the cover slip.

Because of the higher refractive index of the cover slip compared to the specimen, total internal reflection occurs. This means that almost no direct light enters the specimen – but due to the physics of light a so called evanescent wave travels in the specimen direction. This wave is only strong enough to excite fluorochromes within the first few hundred nanometres close to the cover slip. The fluorescent image is restricted to this small depth and cannot be driven into deeper areas of the specimen, and also does not contain out of focus blur from deeper areas. (More information about TIRF can be found at www.olympusmicro.com/primer/techniques/fluorescence/tirf/tirfhome.html).

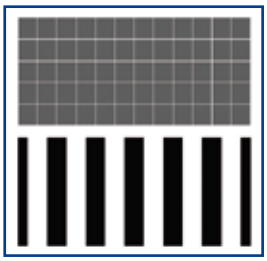


Fig. 18: Line pair pattern achieved with 2 pixels per line pair. Please refer to the text for the description.

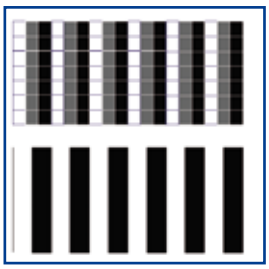


Fig. 19: Line pair pattern resolved with 3 pixels per line pair. Please refer to the text for the description.

Box 5: Changing objectives but keeping the digital live image in focus – Parfocal alignment.

High class objectives are designed to be parfocal. That means even when you change from a 4x magnifying objective to a 40x objective – the structure under observation remains in focus. This is especially a need for imaging with automated microscopy. To use this feature at the microscope the following short guideline will help:

We assume that the microscope in use is in proper Köhler illumination setting, and the digital camera is connected via a camera adapter (c-mount) that allows focus alignment.

(Some adapters are designed with a focusing screw; some can be fixed with screws in different distance positions.)

1. Use a high magnifying objective (40x or more) to get well recognisable detail of a specimen into focus (camera live image), with the help of the course and fine focus of the microscope frame.
2. Change to a low magnifying objective (e.g. 4x), and **do not** change the course or fine focus at the microscope, but, align the focus of the camera adapter until the camera live image shows a clear in focus image.
3. Changing back to high magnification – the image is still in focus – you do not believe? Have a try.

tives can not reach the same maximum NA as high magnifying objectives. Even when we use the best resolving lens for a 4x objective, the N.A of this Apochromat (0.16) is still lower then the lowest resolving 20x Achromat with a N.A. of 0.35. Having a lower NA produces a lower resolution. In a number of applications, a field of view of several millimetres and a resolution on the micrometer or nanometre scale is required simultaneously (fig. 20). How can we overcome this problem, especially when we recognise that the CCD sensor of the camera reduces the field of view (monitor image) once more? Some image processing can offer an alternative. In the first step, these systems automatically acquire individual images at the predefined high resolution. In the next step the software performs intelligent pattern recognition together with a plausibility check on the overlapping parts of the individual image sections to align them all in one image with excellent accuracy (better than a pixel). The computed result shows one combined image, maintaining the original resolution. So you get an image which has both high resolution and a large field of view (fig. 21). Please check box 3 for the description of the image processing procedure. Additionally, box 7 describes its sophisticated and extended follower called Digital Virtual Microscopy.

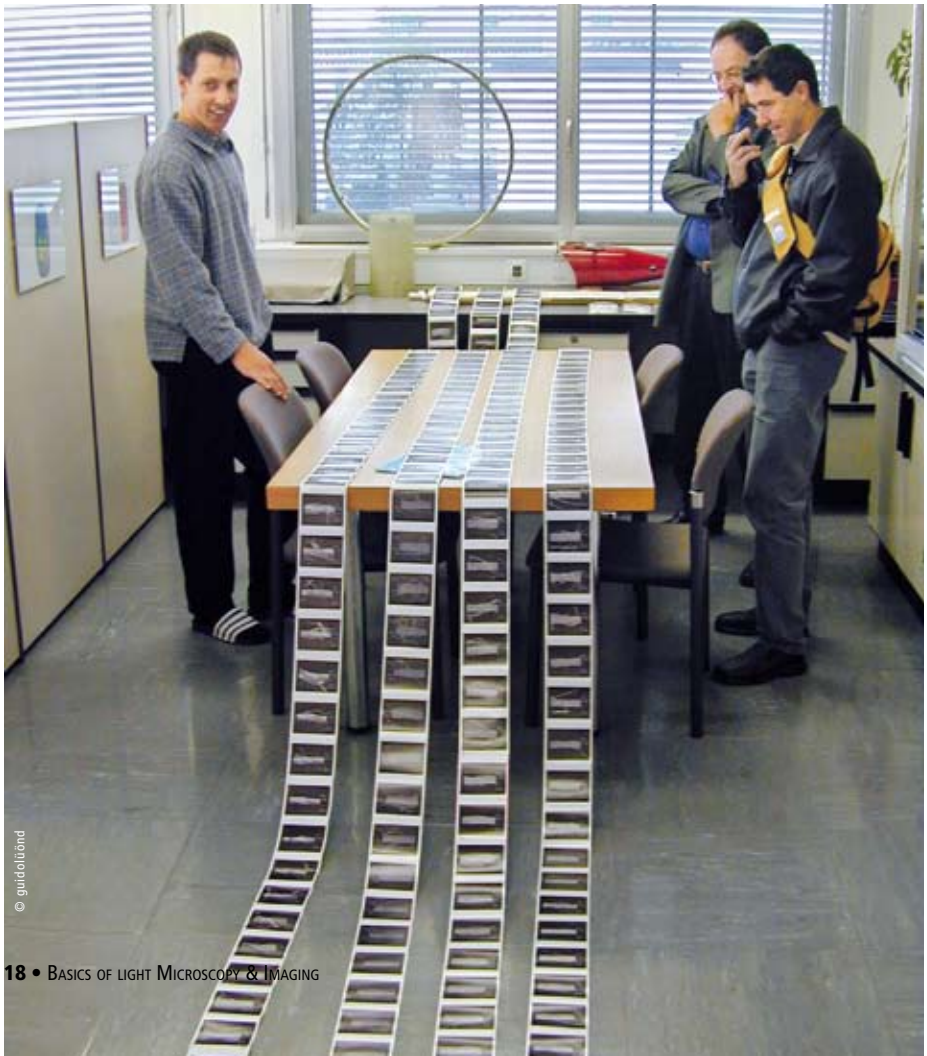


Fig 20: Mr. Guido Lüönd, Rieter AG, Department Werkstoff-Technik DTTAM, Winterthur, Switzerland, works on fibres using a microscope. For their investigations they often have to glue the single images into an overview image. Previously this was a considerable challenge. Now a piece of software takes over his job.

Physical limits and methods to overcome them

As described before, the resolution of a microscope objective is defined as the smallest distance between two points on a specimen that can still be distinguished as two separate entities. But several limitations influence the resolution. In the following we cover influence of image blurring and resolution depth capacity.

Convolution and deconvolution

Stray light from out of focus areas above or below the focal plane (e.g. in fluorescence microscopy) causes glare, distortion and blurriness within the acquisition (fig. 22.a). These image artefacts are known as convolution and they limit one's ability to assess images quickly, as well as make more extensive evaluations. There are several sometimes sophisticated hardware approaches in use which allow reducing or even avoiding out of focus blur at the first place when acquiring the images. Please see the description in box 4. A different approach is the so-called deconvolution which is a recognised mathematical method for eliminating these image artefacts after image ac-

quisition. It is called deconvolution. If the point spread function (PSF) is known, it is possible to deconvolute the image. This means the convolution is mathematically reversed, resulting in a reconstruction of the original object. The resulting image is much sharper, with less noise and at higher resolution (fig. 22.b).

What exactly is the point spread function?

The point spread function is the image of a point source of light from the specimen projected by the microscope objective onto the intermediate image plane, i.e. the point spread function is represented by the Airy disk pattern (fig. 15, 16). Mathematically, the point spread function is the Fourier transform of the optical transfer function (OTF), which is in general a measurement of the microscope's ability to transfer contrast from the specimen to the intermediate image plane at a specific resolution. PSF (or OTF) of an individual objective or a lens system depends on numerical aperture, objective design, illumination wavelength, and the contrast mode (e.g. brightfield, phase, DIC).

The three-dimensional point spread function

The microscope imaging system spreads the image of a point source of light from the specimen not only in two dimensions, but the point appears widened into a three-dimensional contour. Thus, more generally speaking, the PSF of a system is the three dimensional diffraction pattern generated by an ideal point source of light. The three-dimensional shapes of the PSF create the so-called out-of-focus blur, which reduces the resolution and contrast in images, e.g. in fluorescence microscopy. This blurring or haze comes from sections within the specimen which are outside of the focal plane of the actual image. So, an image from any focal plane of the specimen contains blurred light from points located in that plane mixed together with blurred light from points originating in other focal planes (fig. 23).

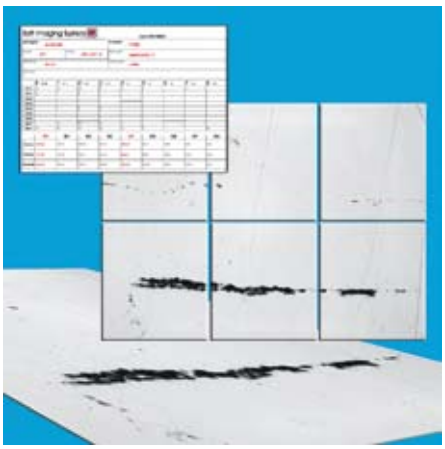


Fig. 21: The analysis of non-metallic inclusions according to DIN, ASTM and JIS requires the processing of areas up to 1000 mm² while the sulphide and oxide inclusions to be analysed are themselves are less than micrometres wide. No camera is available offering such a large CCD sensor. The image processing solution stitches single overlapped images to give one high resolution image together.

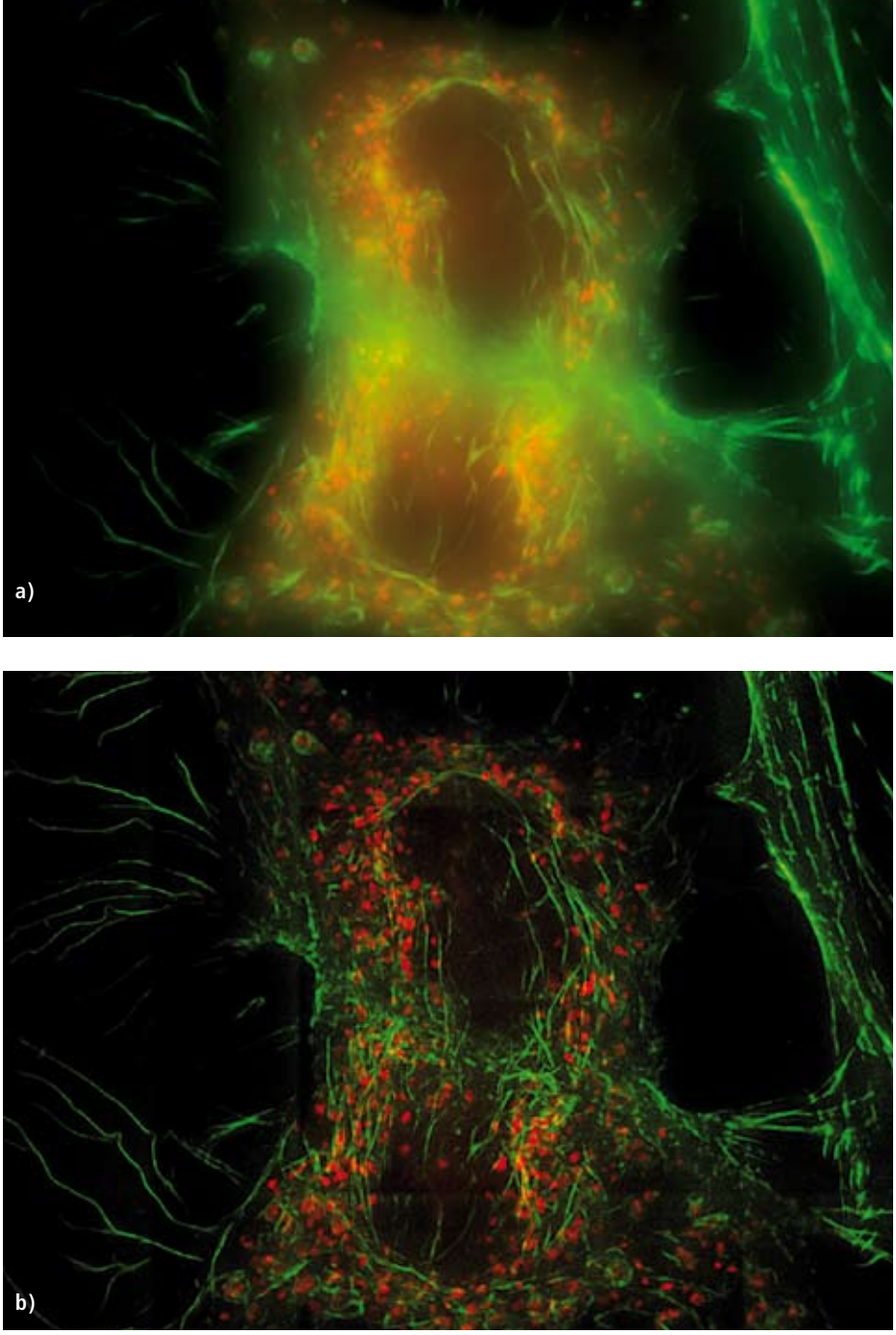


Fig. 22: Via deconvolution artefacts can be computed out of fluorescence images. a) These artefacts are caused by the stray light from non-focused areas above and below the focus level. These phenomena, referred to as convolution, result in glare, distortion and blurriness. b) Deconvolution is a recognised mathematical procedure for eliminating such artefacts. The resulting image displayed is sharper with less noise and thus at higher resolution. This is also advantageous for more extensive analyses.

What is deconvolution used for?

When the PSF of a system is known, it can be used to remove the blurring present in the images. This is what the so-called deconvolution does: It is an image processing technique for removing out-of-focus blur from images. The deconvolution algorithm works on a stack of images, which are optical sections through the specimen and are recorded along the z-axis of the microscope. The algorithm calculates the three-dimensional PSF of the system and reverses the blurring present in the image. In this respect, deconvolution attempts to reconstruct the specimen from the blurred image (fig. 23).

Depth of focus versus depth of field

Two terms – depth of focus and depth of field – are often used to describe the same optical performance, the amount of specimen depth structures that can be seen in focus at the same time. However,

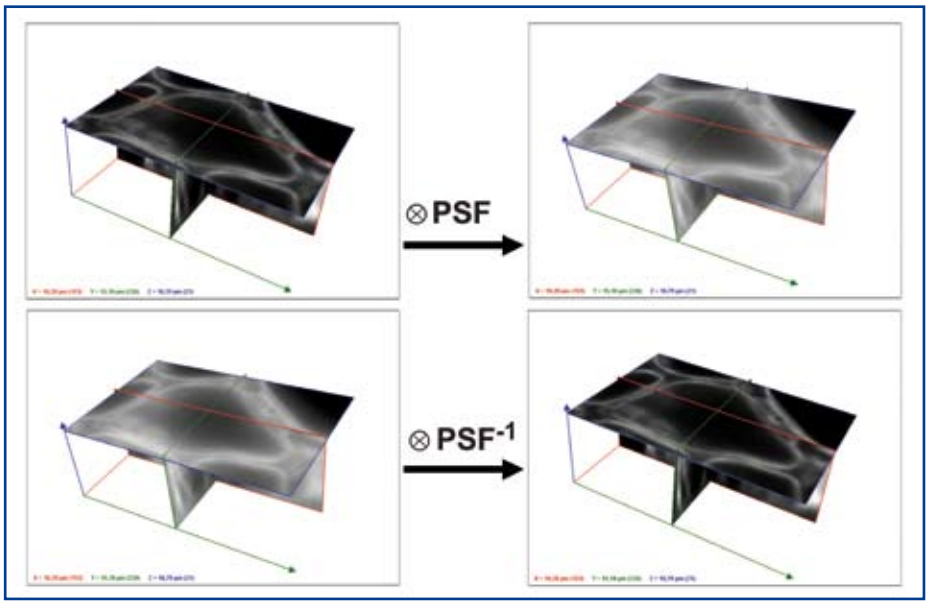


Fig. 23: Stray light originating from areas above and below the focal plane results in glare, distortion and blurriness (convolution) especially in fluorescence microscopy and histology. Deconvolution is a recognised mathematical method for correcting these artefacts. The degree to which an image is distorted is described by what is known as the point spread function (PSF). Once this is known, it becomes possible to "deconvolute" the image. This means that the convolution of the image is mathematically reversed and the original contours of the specimen are reconstructed. The greater the precision with which the degree of distortion – i.e., PSF – is known, the better the result. What is this result? A sharper and noise-free version of the image at higher resolution with enhanced image quality.

only the term "Depth of Field" should be used for this feature. As we will point out later the term "Depth of Focus" is needed for a different optical feature.

An optical system, such as the eye, which focuses light, will generally produce a clear image at a particular distance from the optical components. In a camera, the ideal situation is where a clear image is formed on the chip, and in the eye it is where a clear image is formed on the retina. For the eye, this happens when the length of the eye matches its optical power, and if a distant object is in focus when the eye is relaxed. If there is a difference between the power and length in such a situation, then the image that is formed on the retina will be very slightly out of focus. However, such a discrepancy may be small enough that it is not noticed, and thus there is a small amount of "slop" in the system such that a range of focus is considered to be acceptable. This range is termed the "depth of focus" of the eye. Looking at it the other way round, the eye might be precisely in focus for a particular distance – for example an object one metre away. However, because of the slop in the system, other objects 90 cm and 110 cm away may also be seen clearly. In front of, and behind, the precise focal distance there is a range where vision is clear, and this is termed the "depth of field".

Now look at the physical understanding of depth of focus in microscopy and the depth of field, respectively. Depth of field Δfi in a microscope is the area in front of and behind the specimen that will be in acceptable focus. It can be defined by the distance from the nearest object plane in focus to that of the farthest plane also simultaneously in focus. This value describes the range of distance along the optical axis in which the specimen can move without the image appearing to lose sharpness. Mathematically Δfi is directly proportional to

$$\Delta fi \sim \lambda/2*NA^2 \quad (5)$$

Δfi = depth of field
λ = wavelength of light (emission)
NA = numerical aperture

Δfi obviously depends on the resolution of the microscope. Large lenses with short focal length and high magnifications will have a very short depth of field. Small lenses with long focal length and low magnifications will be much better.
Depth of focus Δfo in a microscope is the distance above and below the image plane over which the image appears in focus. It's the extent of the region around the image plane in which the image will appear to be sharp. Δfo is directly proportional to

$$\Delta fo \sim M^2/NA \quad (6)$$

Δfo = depth of focus
M = magnification
NA = numerical aperture

Δfo refers to the image space and depends strongly on the magnification M, but also on changes in numerical aperture NA.

As a take home message – high resolution will create relative low depth of field, high magnifications will create a higher depth of focus – this is also why the procedure for parfocal alignment will work.

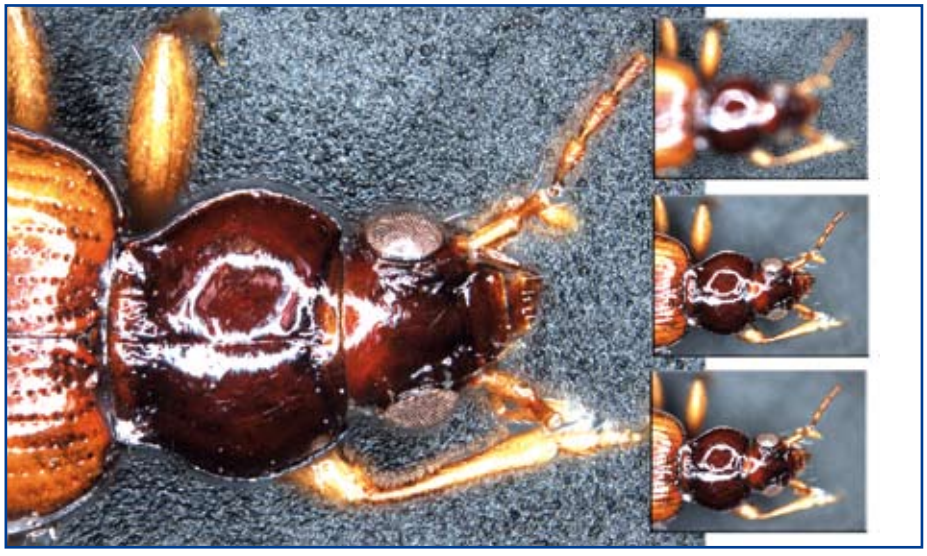


Fig. 24: The software extracts the focused areas from the component images of an image series and reassembles them into one infinitely sharp image. The example shown here is the resulting image computed automatically using 25 images of a *Bembidion tetracolum* sample.

Parfocality

There is a well-known difficulty in conventional light microscopy which refers to the limited depth of focus: If you focus

Box 6: Extended Focal Imaging (efi)

A lack of depth of field in microscope images is an old and familiar problem. The microscope's own depth of field is only capable of focusing a limited height range at the same time. The remaining parts of the image are then blurry. Electronic image processing points the way out of this dead-end street.

For a motorised microscope equipped with a motorised stage the whole process can be automated. The software takes control of the microscope, camera, motor stage, etc. All parameters are transferred to the imaging system through a remote interface. Using these data, the entire microscope and camera set-up can be controlled and calibrated by the software. After having defined the total number of images, and the maximum and minimum height of the stage, the user can execute the following steps automatically with a single mouse click.

1. In the first step the user defines the number of images in the focus series. Using a microscope with a motor stage the user has to define the maximum and the minimum lift of the microscope stage as well as the total number of individual images he intends to acquire.
2. The defined image series at varying focus levels is acquired.
3. Next the composite image of pixel-by-pixel precision will be generated from these images. This is done by extracting the respective focused image areas of each separate image and assembling these into a focused composite image. Most of these solutions take into consideration the typical (due to construction) shift of the optical axis that occurs when focusing stereoscopic microscopes.
4. The "Extended focal image" with practically limitless depth of field is computed.
5. Now the user is able to generate a height map which permits users to reconstruct three-dimensional views e.g. to measure height differences.

Box 7: Virtual microscopy

Light microscopy is one of the classic imaging techniques used in medical education and for routine procedures of pathology, histology, physiology and embryology. Pathology makes use of microscopes for diagnostic investigation of tissue samples to determine abnormal changes. Digitisation has resulted in significant progress in the field of microscopy. However, digital technology up until now has presented some decisive limitations. One problem is that the field of view of any camera is limited for any given magnification. It is usually not possible to have a complete overview of the tissue specimen with just one image at a resolution that allows further analysis. Digital virtual microscopy moves beyond this barrier.

Virtual microscopy is the digital equivalent to conventional microscopy. Instead of viewing a specimen through the eyepiece of the microscope and evaluating it, a virtual image of the entire slide (a 'virtual slide') with perfect image quality is displayed on the monitor. The individual system components (microscope, motor stage, PC, software) are all optimally inter-coordinated and offer speed, precision and reliability of use. The Olympus solution .slide scans the entire slide at the resolution required. Integrated focus routines make sure the image is always in sharp focus. The single images acquired are automatically stitched together into a large montage (the 'virtual slide'). The entire 'virtual slide' can be viewed onscreen. Detail image segments can be selected and zoomed in or out, the equivalent to working with an actual glass slide under the microscope with the same efficiency. With an internet connection, this procedure can be done from anywhere in the world. In addition, users have all the advantages of digital image processing at their fingertips, including structured web archiving of images, analysis results and documentation.

an image with one objective, e.g. 4x, it might be out of focus when you change to a another magnification, e.g. 40x. The term parfocality describes the situation when the structures remain in focus. Parfocality is a characteristic of objectives. Please read box 5 how to assure parfocality with high class objectives.

Fig. 25: This image shows how far money can go: This Singaporean coin detail was captured using a ColorView digital CCD camera and processed using the realignment, extended focus and 3-D imaging software modules. Capture and automatically align multiple component images into a high-resolution composite using the MIA module. Extract the sharpest details within the component images and reassemble into one single image having infinite depth of focus with the module EFI. Finally, create incredibly realistic views using height and texture information attained through the perspective functions of the 3D module.

Automated sharp images

Let us come back to restrictions arising from the limited depth of field. The better the lateral resolution, the smaller your depth of field will be. The problem is in fact physical, and it cannot be circumvented by any adjustments to the optical system. Today's standard light microscopes allow objects to be viewed with a maximum magnification of about 1000x. The depth of field is then reduced to about 1 µm. Only in this area the specimen is perfectly imaged. This physical limitation of inadequate depth of field is a familiar problem in microscope acquisitions. In the metal-processing industry, when analysing or evaluating two-dimensional metallographical objects such as, e.g., a section sample, a section press is generally used in order to obtain an exact orthogonal alignment in relation to the optical axis of the microscope. The sample to be analysed can then be acquired totally focused in a single acquisition.

However, objects which have a distinctly three-dimensional structure or for investigations of, e.g., 3-D wear, at greater magnifications no satisfactory overview image is obtainable via stereo or reflected-light microscope. The microscope can only be focused onto limited areas of the object. Due to the limited depth of field, it is actually impossible to obtain a sharp acquisition of the entire image field. These physical restrictions can only be transcended via digital image analysis. The normally wholly-binding laws of physics are "side-stepped". What happens is that an image series is acquired at varying focus levels. Then special software algorithms are applied to all the images of the image series and distinguish between sharply-focused and unfocused image segments in each image. The sharp image segments of the images are then pieced back together to form a single totally-focused image of the entire sample (fig. 24). Furthermore, measuring height differences and generating three-dimensional images becomes feasible (fig. 25). For further detail please see box 6.

Contrast and Microscopy

All cats are grey in the dark

Why are cats grey in the dark? Here, the term contrast comes into play. Contrast refers to the difference of intensities or colours within an image. Details within an image need intensity or colour differences to be recognised from the adjacent surroundings and overall background. Let us first restrict our view to a greyscale level image, like image fig. 26. While watching the image try to estimate the number of grey scales that you can distinguish – and keep that number in mind.

Let us now compare this number with another example – you enter an office shop in order to buy a selection of card samples in different shades of grey. All of the cards fall down by accident and are scattered over the floor. Now you have to replace them in the correct grey level order – how much can you differentiate now?

Surprisingly, we are only able to differentiate approximately 50–60 grey levels – that means that already the 8 bit image on your monitor with 256 grey scales offers greater possible differentiation than we can discriminate with our own eyes. The card samples in various shades of grey need to a have an approximate difference in contrast level of about 2% in order for us to recognise them as different. However, if we look at the number of image areas (pixels) that represent a discrete intensity (grey level or pixel intensity) within an intensity distribution of the image we can understand and handle contrast and brightness variations more easily. Intensity or grey level distributions are referred to as histograms, see box 8 for further explanation. These histograms allow us to optimise camera and microscope settings so that all the intensity values that are available within the specimen are acquired (fig. 27). If we do not study the available intensity values at the initial stage before image acquisition, they are archived and can not be visualised in additional processing steps.

The familiar view – brightfield contrast

In brightfield transmitted microscopy the contrast of the specimen is mainly produced by the different absorption levels of light, either due to staining or by pigments that are specimen inherent (amplitude objects). With a histological specimen for example, the staining procedure itself can vary the contrast levels that are available for imaging (fig. 27). Nevertheless, the choice of appropriate optical equipment and correct illumination settings is vital for the best contrast.

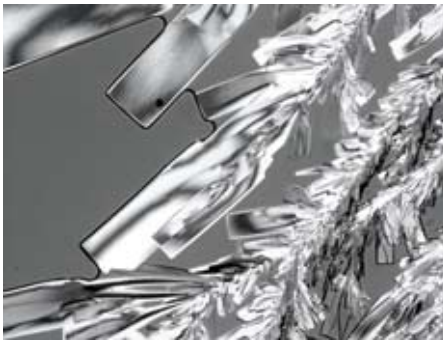


Fig. 26: Vitamin C crystals observed in polarised light.

During our explanation about Köhler alignment (see box 2) we described that at the end of this procedure the aperture stop should be closed to approximately 80% of the numerical aperture (NA) of the objective. This setting is to achieve the best contrast setting for our eyes. Further reduction of the aperture stop will introduce an artificial appearance and low resolution to the image.

For documentation purposes however, the aperture stop can be set to the same level as the NA of the objective – because the camera sensors in use are capable to handle much more contrast levels than our eyes. For specimens lacking natural differences in internal absorption of light, like living cells (phase objects; fig. 28) or

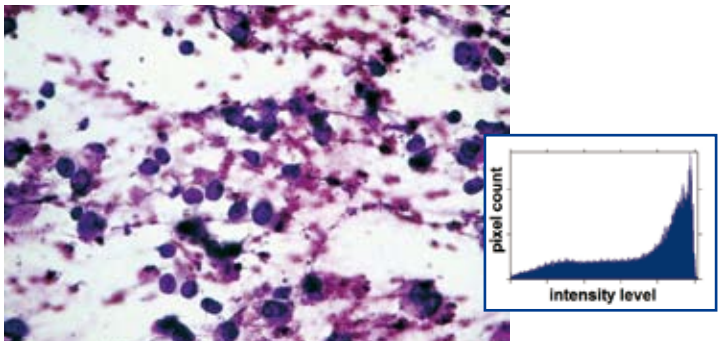
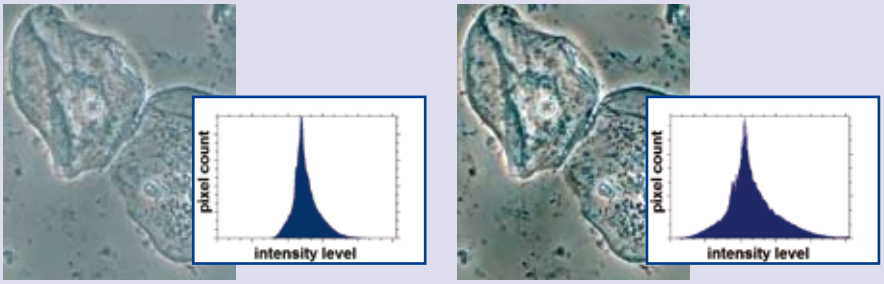


Fig. 27: Histological staining. Cells obtained after a transbronchial needle application. Image and histogram show that the overall intensity and contrast have been optimised.

Box 8: Histogram optimisation during acquisition

An intensity histogram depicts the intensity distribution of the pixels in an image. The intensity values are plotted along the x axis and range from 0–255 in an 8-bit greyscale image or 24-bit (3x8bit) colour image. The number of pixels per intensity value is displayed along the y axis.

The intensity histogram provides the means to monitor general characteristics of a digital image like its overall intensity, its contrast, the dynamic range used, possible saturation, the sample's phases etc. In this respect the histogram gives a more objective criterion to estimate the quality of an image than just viewing it (which is somewhat subjective). When displayed in the live mode during image acquisition, the histogram allows optimising and fine tuning of the acquisition modes and parameters on the microscope as well as the camera. These include microscope alignment, contrast method, microscope settings, or current camera exposure time. So the histogram helps to acquire a better image containing more image information.



Epithel cells viewed with phase contrast: left side with low contrast setting, right sight with contrast optimisation of microscope and camera setting.

Left figure is obviously lit correctly but has poor contrast. The corresponding histogram mirrors this: the peak is roughly located in the middle of the intensity range (x-axis), so the camera's exposure time is correctly set. But all pixels are squashed in the middle range between about 75 and 200. Thus, only about half of the dynamic range of the camera system is used. Aligning the microscope better (e.g., light intensity) and adapting the camera exposure time increases the image contrast (right figure). It also spreads the intensity distribution over the whole dynamic range without reducing information by saturation (see the corresponding histogram). Here, more detailed image structures become visible.

General rule

The overall contrast is usually best when the intensity histogram covers the whole dynamic range of the system; but one should usually avoid creating overflow or saturation at the right side of the histogram, i.e. white pixels.

It is also possible to use the histogram to improve the image contrast afterwards. However, you can not increase the image content; you can only improve the image display.

reflected microscopy specimens without significant three dimensional relief structures, the acquired image has flat contrast. To better visualise the existing image features (fig. 28, original image), subsequent digital contrast optimisation procedures may be applied (fig. 28, improved image). But to visualise more details in those specimens optical contrast methods must be used.

Like stars in the sky – Darkfield Contrast

Dust in the air is easily visible when a light beam is travelling through the air in a darkened room. The visibility is only achieved because the dust particles diffract and/or reflect the light and this light is now travelling in all directions.

Therefore we can see light originating from the particle in front of a dark background even when the particle itself is too small to be resolved or does not show an appropriate contrast under daylight conditions. This phenomenon is also used in darkfield (or dark ground) microscopy. Light is directed to the specimen in a way that no direct light enters the objective. If there is no light scattering particle the image is dark, if there is something that diffracts or reflects the light, those scattered beams can enter the objective and are visible as bright white structures on a black background (fig. 29).
(See also: http://www.olympusmicro.com/primer/techniques/darkfieldindex.html)

Transmitted darkfield

For transmitted microscopy including stereo microscopy, this contrast method is especially used to visualise scattering objects like small fresh water micro-organisms or diatoms and fibres (fig. 30). Almost all upright microscopes can be easily equipped for darkfield illumination.

The easiest way to achieve simple darkfield is a central light stop insert for the condenser. For better illumination or even high resolving darkfield, special darkfield condensers are required (fig. 29).

To ensure that no direct light is entering the objective, the numerical aperture (NA) of the condenser has to be about 15% higher than the NA of the objective. This is in contradiction to all other contrast methods where the objective has a higher or equal NA than the condenser. Remember the NA is a number that describes the angle of the light cone a condenser or objective is using. An objective with high NA like the apochromate is characterised by a high angle of its light cone and therefore it may be possible that some of the direct illumination will also enter the objective. This would destroy the darkfield contrast immediately. For that reason objectives with high NA are

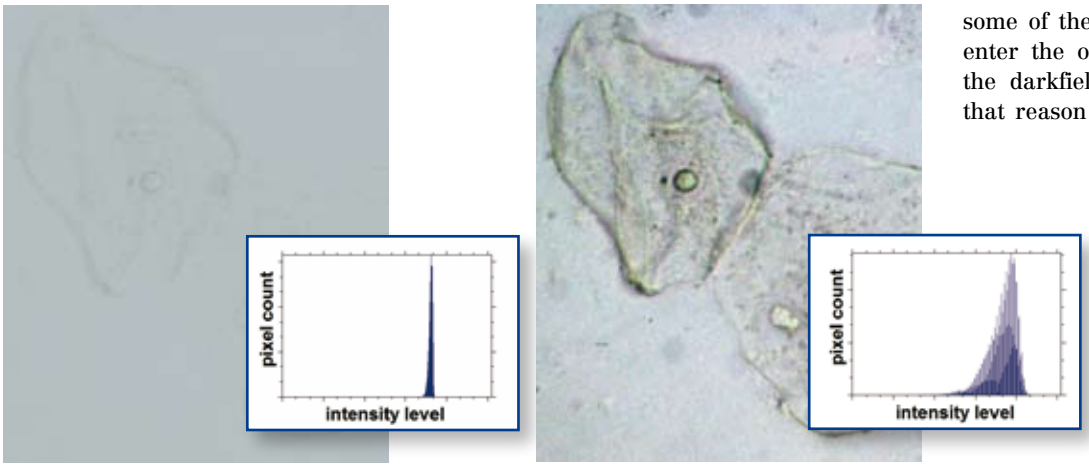


Fig. 28: Brightfield image of living mouth epithelial cells on a slide before and after digital contrast optimisation of the archived image and corresponding histograms. See the section "Making it look better", for further explanation.

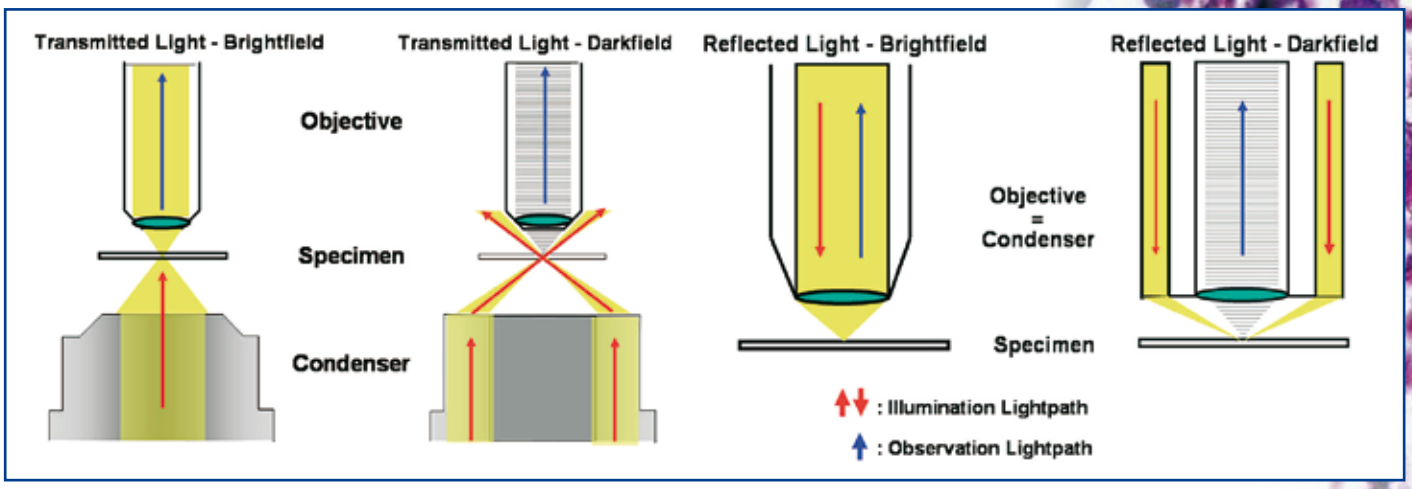


Fig. 29: Light path for darkfield compared to brightfield set up in transmitted and reflected illumination.

available, designed with an internal iris diaphragm to reduce the NA to the appropriate amount for darkfield observation.

Reflected darkfield

Within the reflected microscopy applications the darkfield illumination is a very common contrast technique. It allows the visualisation of smallest scratches and changes in height because of the circular oblique illumination (fig. 31). To achieve a reflected darkfield, the amount of special adaptations at the microscope is more sophisticated. The central light stop is located within a cube of the reflected light attachment and the special brightfield/darkfield objective guides the illumination light in an outer ring to the specimen. Only the scattered light from the specimen runs in the normal central part of the objective as image forming light rays (fig. 29). Those objectives can also be used for normal brightfield observation or other techniques like differential interference contrast (DIC) and/or polarisation.

Creating destructive interference – Phase Contrast

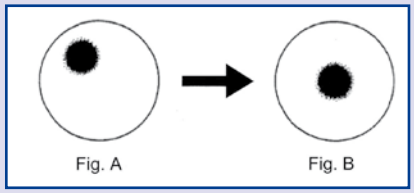
Light that is travelling through part of a specimen and is not absorbed by amplitude objects will not produce a clearly visible image. The intensity remains the same, but the phase is changed compared to the light just travelling in the surrounding areas. This phase shift of about a quarter wavelength for a cultured cell is not visible to our eyes. Therefore, additional optical elements are needed to convert this difference into an intensity shift. These optical elements create a contrast where un-deviated and deviated light are ½ wavelength out of phase, which results in destructive interference. This means that details of the cell appear dark against a lighter background in positive phase contrast (see figures in box 8). (See also: www.olympusmicro.com/primer/techniques/phasecontrast/phaseindex.html)

For phase contrast microscopy two elements are needed. One is a ring slit insert for the condenser, the other is special objectives that contain a phase plate. Objectives for phase contrast are characterised by green lettering and an indication of the size of the phase ring like Ph1, Ph2, Ph3 or PhC, PhL, PhP. Corresponding to these objective phase rings the appropriate inserts for the condenser have to be used.

Both elements are located at the so called back focal planes. They are visible

Box 9: Alignment of a transmitted darkfield condenser

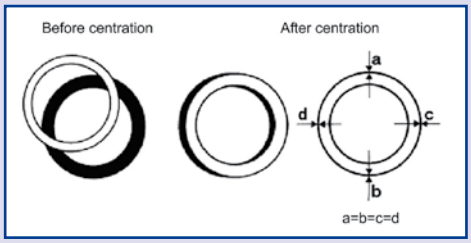
1. Engage the 10x objective and bring the specimen into focus.
2. While looking through the eyepieces and using the condenser height adjustment knob, carefully adjust the height of the condenser until a dark circular spot becomes visible (Figure A).
3. Turn the condenser centring screws to move the dark spot to the centre of field of view (Figure B). This completes the centration.



4. Engage the desired objective. Using the condenser height adjustment knob, adjust until the darkfield spot is eliminated and a good darkfield image is obtained.

Box 10: Alignment of phase contrast

1. A microscope condenser with Köhler illumination has to be in Köhler positioning.
2. Remove one of the eyepieces and look into the empty eyepiece sleeve. When using a centring telescope at the eyepiece sleeve (highly recommended and in some inverted microscopes already build in at the tube e.g. Olympus U-BI90CT – binocular tube), bring the bright ring (condenser ring slit) and dark ring (objective phase plate) into focus by turning the eye lens focus.
3. Use the centring screws for the condenser inserts to centre the phase contrast ring, so that the bright ring overlaps the dark ring within the field of view (see figure).
4. Repeat these steps for each phase and contrast ring set.
5. Remove the centring telescope and replace it with the eyepiece.



6. Widen the field iris diaphragm opening until the diaphragm image circumscribes the field of view.

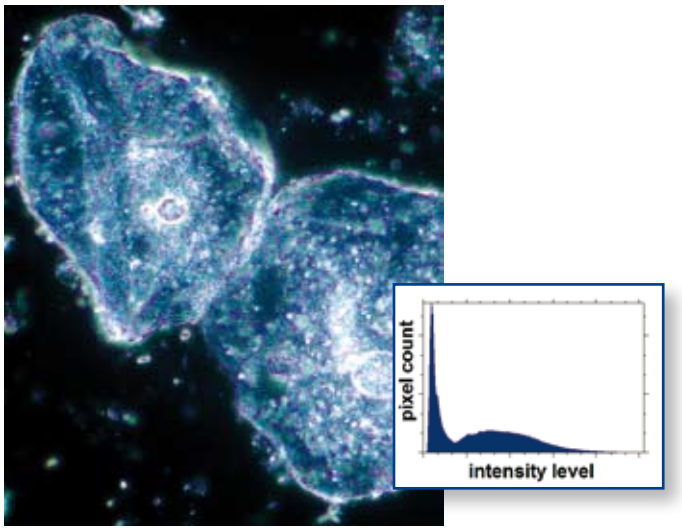


Fig. 30: Epithelial cells with transmitted darkfield contrast.

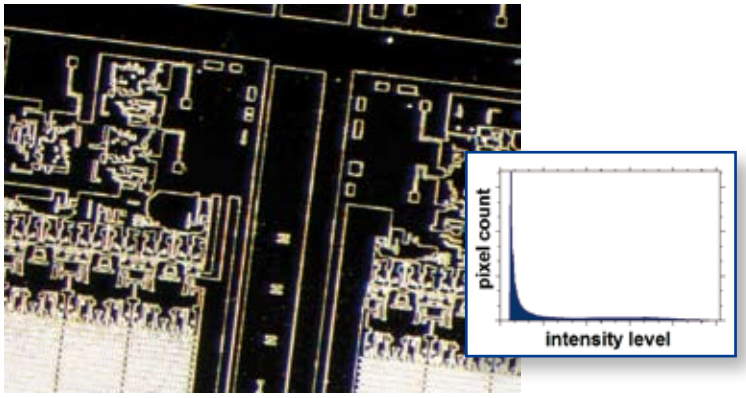


Fig. 31: Wafer at reflected darkfield contrast.

when an eyepiece is removed and can then be aligned under optical control (for better viewing a centring telescope should be used).

Due to the optical principles of phase contrast it allows good contrast in transmitted light when the living specimens are unstained and thin. A specimen should not be more than 10 µm thick. For these specimens the dark contrast is valid, whereas thicker details and overlaying structures produce a bright halo ring. This halo-effect can be so strong and superimposed that a clear analysis of the underlying morphology becomes critical. Nevertheless, the artificial looking image of thicker structures can be used by expert eyes to determine how many cells within an adherent cell culture are undergoing mitosis or have entered cell death pathways. Fig. 32 shows bright halo rings, visible round the relatively thick astrocyte cell bodies, whereas the fine details show dark phase contrast.

When observing cells in a chamber e.g. those of a 24 well plate, the cells which are best for imaging may lie at the border of the well. It is possible to see the cells, but because the light path is changed by the border of the well and the adhesion effect of the medium, the phase contrast is totally misaligned at this position. We can realign the phase rings for this specific specimen position as described below, but will have to realign them again when the stage is moved to other positions. Furthermore, the use of the special Olympus PHC phase contrast inserts instead of the PH1 helps to ensure better contrast in multi well plates where meniscus problems are apparent.

Making it look better – for vision only

It is essential to select the optical contrast method appropriate to the sample investigated and depending on the features which are to be made visible. You can only build upon something which is already there.

Having done the best possible job here you can try to better visualise these features. It often makes sense to adjust the image contrast digitally. Here you can either elevate the overall image contrast or you can emphasise special structures. Whatever you want to do, you should again utilise the histogram to define exactly the steps for image improvement.

Fig. 28 shows a drastic example (left side). The optical contrast is so poor that the cells' relief is hardly visible. The corresponding histogram shows that the

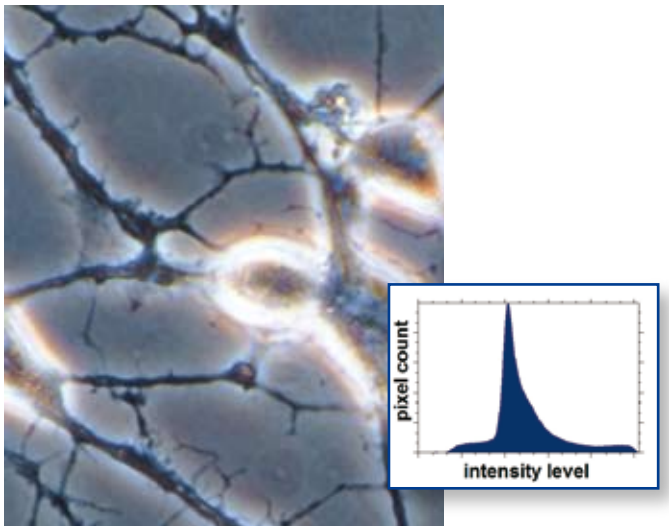


Fig. 32: Astrocytes with phase contrast, note: cell bodies are surrounded by halo rings.

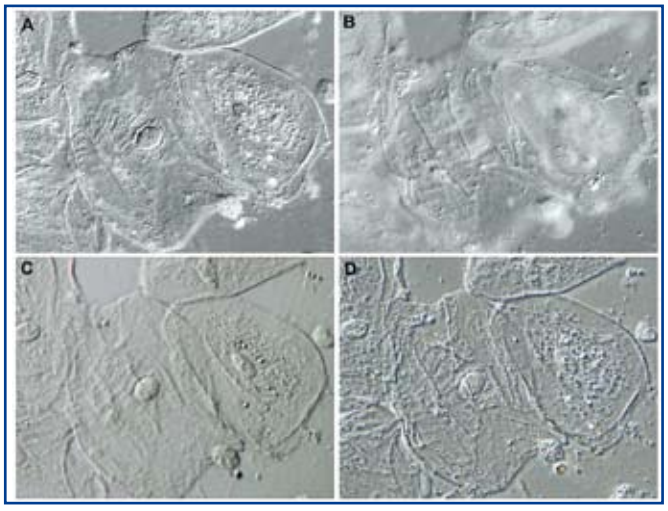


Fig. 33: Epithelial cells scratched with a spoon from the tongue and transferred to a cover slip. A,B: DIC illumination at different focus plane; C: Oblique contrast; D: Olympus Relief Contrast imaged with lower numerical aperture.

pixels are grouped in the centre of the histogram indicating that only a small fraction of the camera's dynamic range has been used (intensity values 165–186 out of 256). The image has only 21 different intensity values. The present features can be made visible by stretching the histogram over the whole dynamic range from 0–255. This operation does not change the information content of the image but lets us at least see what is there (right side).

This stretching comes to its limits as soon as there is a single black and a single white pixel in the image. In this case a moderate cut of up to 3% on the left side (dark pixels) and on the right side (bright pixels) doesn't cause much information loss but increases the contrast of the image's main features in the central intensity range. To selectively accentuate features of a distinct intensity range (so-called phase), an individual transfer function has to be defined which increases the phase's contrast to the background or other phases. The intensity and contrast image operations are usually performed after image acquisition. Often, the camera control of the image analysis software makes it possible to have the contrast stretched image calculated and displayed in the live mode. Alternatively, the contrast can be set manually on the live histogram while the direct results can be monitored in the live image. Whatever way the digital histogram is used, it is a powerful tool to maintain image fidelity as well as to create a clear picture of physical nature.

Light alone is not enough – Differential Interference Contrast (DIC)

If you have ever been skiing on a foggy day, you will know that diffuse light strongly reduces the ability to see differences in the height of the snow – and that can cause major problems. An equally distributed light source does not produce clear shadows and this causes reduced visibility of three dimensional structures. Our human vision is triggered to see three dimensions and is well trained to interpret structures if they are illuminated more or less from one point. The resulting dark and bright areas at the surface of a structure allow us to easily recognise and identify them. Using our experience, we get information of height and distance. Therefore, a contrast method that displays differences in a structure as a pattern of bright and dark areas is something that looks very familiar to us and seems to be easy to interpret. Structures within a specimen can be identified and even though they are only two dimensionally displayed they look three dimensional.

Real three dimensional images can only be observed at a stereo microscope where two light paths of two combined microscopes are used, sending the image to our eyes at a slightly different angle. But this will be a topic to be described later.

Simple from One Side

The simplest way to achieve a contrast method that results in a relief-like image is by using oblique illumination: however, the theory of oblique illumination is more complex. Details can be found at: www.olympusmicro.com/primer/ techniques/oblique/obliquehome.html. Special condensers are available to handle this technique on upright microscopes with a lot of comfort. Oblique illumination is also often used on stereo microscopes to enhance the contrast of a 3D surface simply by illuminating the specimen from one side. For reflected light, this can be done with flexible cold light fibres, or with LED ring light systems that offer reproducible illumination of segments that can even be rotated.

Box 11: How to set up the transmitted DIC microscope (e.g. Olympus type)

- Use proper Köhler positioning of the microscope with a specimen in focus (best with 10x objective).
- Insert polariser (condenser side) and analyser (objective side) into the light path.
- Remove specimen out of the light path.
- Turn the polariser until the image gets to its darkest view (Cross-Nicol position).
- Insert the DIC prism at the condenser corresponding to the objective and slider type in use, immediately the image is bright again.
- Insert the DIC slider at the objective side. By rotating the fine adjustment knob at this slider the amount of contrast of the DIC can be varied.
- The best contrast is achieved when the background shows a grey colour and the specimen is clearly pseudo three dimensional.

Another Way to Achieve this Setting Is the Following:

- Use proper Köhler positioning of the microscope with a specimen in focus (best with 10x objective).
- Insert polariser (condenser side) and analyser (objective side) and the prism slider into the light path.
- Remove specimen out of the light path.
- Remove one eyepiece and if available look through a centring telescope, rotate the slider adjustment knob until a black interference line is visible (box 11- Figure).
- Rotate the polariser until the black line becomes darkest.
- Insert the DIC prism at the condenser and insert the eyepiece for normal observation.
- Fine adjustment of DIC-slider can be done for selecting the amount of interference contrast.

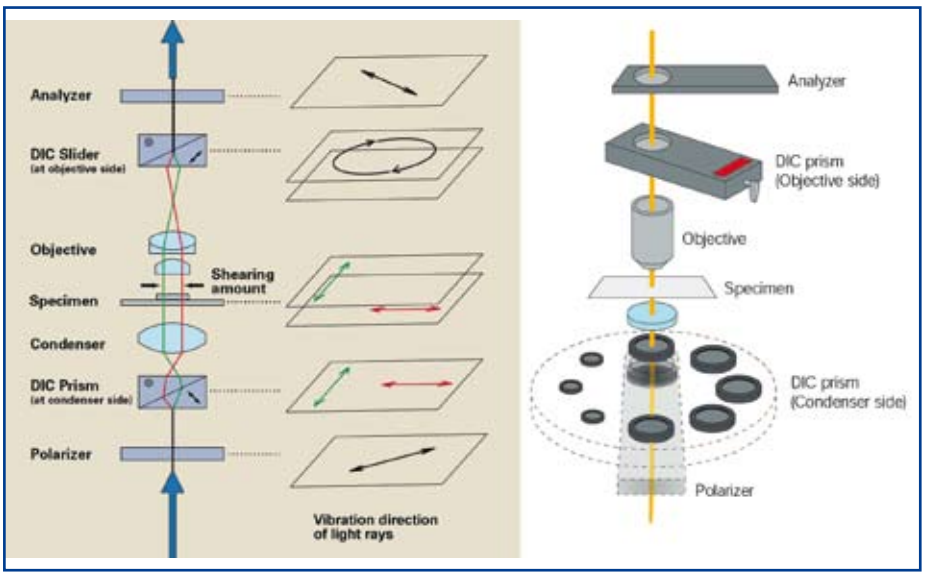


Fig. 34: Simple principle of Nomarski DIC microscopy.

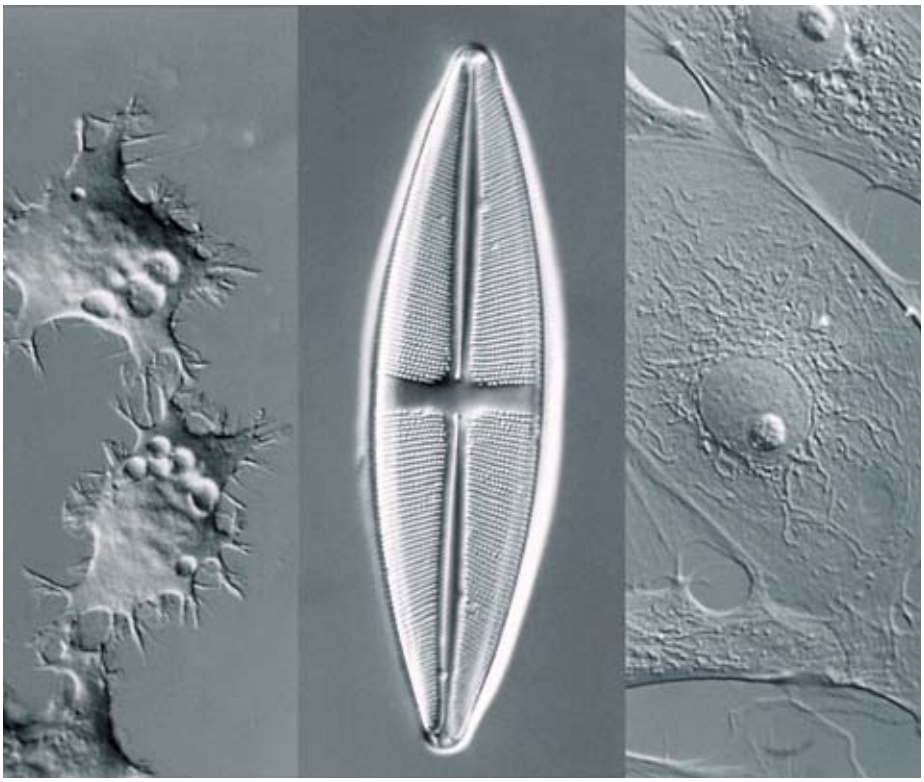


Fig. 35: Specimens imaged with different shearing values. Left side shows thin NG108 cells imaged with the high contrast prism set, the middle image shows a thick diatom specimen imaged with the high resolution prism set and the right image shows PtK2 cells imaged with the general prism set.

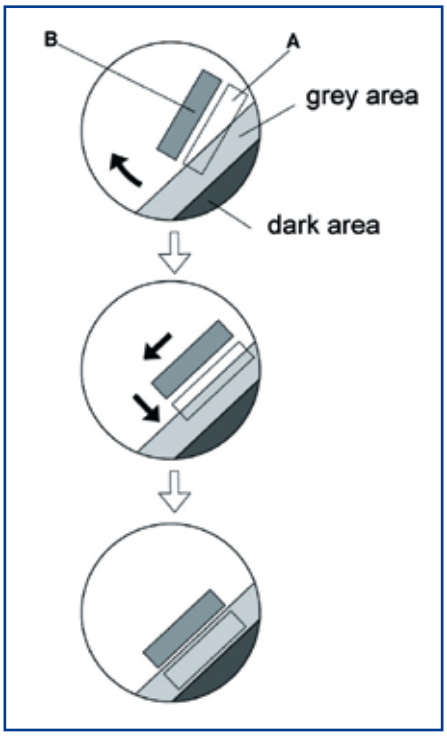


Fig. 36: Alignment of Olympus Relief Contrast or Hoffmann Modulation condenser insert, grey and dark areas are located within the objective and areas A and B are located as an insert within the condenser and can be aligned accordingly.

In transmitted microscopy the oblique condenser (e.g. the Olympus oblique condenser WI-OBCD) has an adjustable slit that can be rotated. After Köhler alignment of the microscope this slit is inserted in the light path and results in illumination from one side so that specimens that vary in thickness and density are contrasted. Rotating the slit enables us to highlight structures from every side. The contrast itself is produced by the complete thickness of the specimen and the resolution of the image is limited due to the oblique illumination (fig. 33c).

To overcome the limitations of oblique contrast, Nomarski Differential Interference Contrast (DIC) is commonly used for high resolving images. The benefit of this method is that the relief like image is only contrasted at the focus area (depth of field). The user can optically section a thicker specimen by changing the focus level.

As shown in fig. 33 the contrasted focus layer can be restricted to the layer of the somata (fig. 33a) or the superficial part of these cells (fig. 33b). In addition, using infrared light (mostly used around 700 or 900 nm) instead of white light, this technique allows a very deep look of more than 100 µm into thick sections, which is often used in neurobiological research. Nomarski DIC creates an amplified contrast of phase differences which occurs when light passes through material with different refractive indices. Detailed information about the theory and use of the DIC method can be found at www.olympusmicro.com/primer/techniques/dic/dichome.html. Here we will concentrate on the basic introduction and the practical use.

To achieve transmitted Nomarski DIC images, four optical elements are needed: a polariser, two prisms and an analyser. For the reflected DIC setup only one DIC prism (the slider) is required.

Let us have a closer look at transmitted DIC. The wave vibration direction of light is unified by a polariser located between the light source and condenser

Fig. 37: Transmitted polarising microscopy; variation of melted chemicals viewed with crossed polariser. Images courtesy of Norbert Junker, Olympus Europa GmbH, Hamburg, Germany.

(fig. 34). In the condenser, a special insert – a Wollaston prism (matching the magnification of the objective) – divides every light ray into two, called the ordinary and the extraordinary, which vibrate at a 90 degree angle to each other. Both light rays then travel a small distance apart, the so called shearing distance. At the specimen, the ray that is passing through e.g. a cell part is delayed compared to the one passing through the surrounding medium. This result in a phase shift of both rays, which are recombined with the help of a second prism located at the objective revolver. Only those combined rays with a phase shift, interfere in a way that they contain vibration planes that pass through the analyser. To create an easily observable pseudo 3D image, a prism can be moved in and out to enhance the phase shift between ordinary and extraordinary ray. At a mid position the background should be dark and the specimens should be visible as if illuminated from the equivalent North-West and South-West simultaneously. When the slider is screwed more to one direction the typical three dimensional view of the specimen will come up, either having the sun in North-West or East-South location. This will only work if no depolarising material (e.g. plastic) is used within the light path. Therefore, a real high resolving DIC method can not be used with plastic Petri dishes or multi well plates. In those cases other methods like Hoffmann Modulation Contrast or the analogous Olympus Relief Contrast are commonly used.

The DIC method does not require special objectives, but the prism has to match the objective used. Suitable prisms are available for most fluorite and apochromat objectives. DIC contrast can be offered with shearing values for every optimisation, a high contrast set-up that is best for very thin specimens (higher shearing value, fig. 35 left), a standard prism slider for general use (fig. 35 right) and a high resolution slider for thick specimens (lower shearing value, fig. 35 mid) like C. elegans or zebra fish embryos.

Different names but the same principle: Hoffman Modulation Contrast – Olympus Relief Contrast

Whenever high resolution is needed and plastic dishes are used, the Hoffman Modulation or Olympus relief contrast method are common techniques for inverted transmission light microscopes e.g. for *in vitro* techniques (fig. 33d).

In principle it combines the oblique illumination of a specimen where the re-

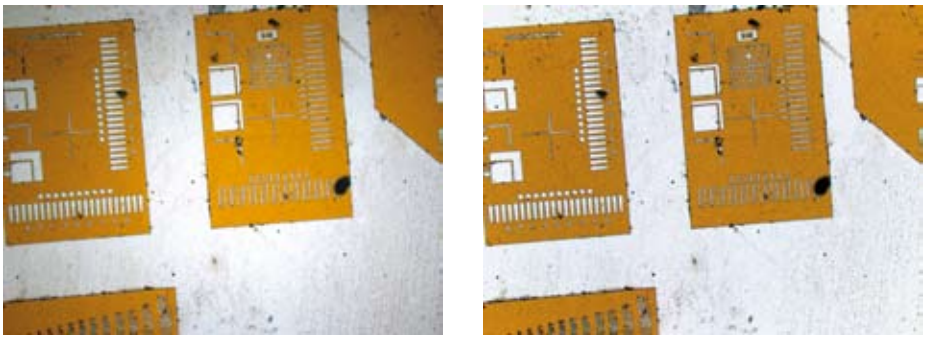
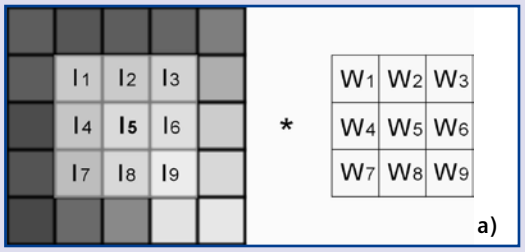


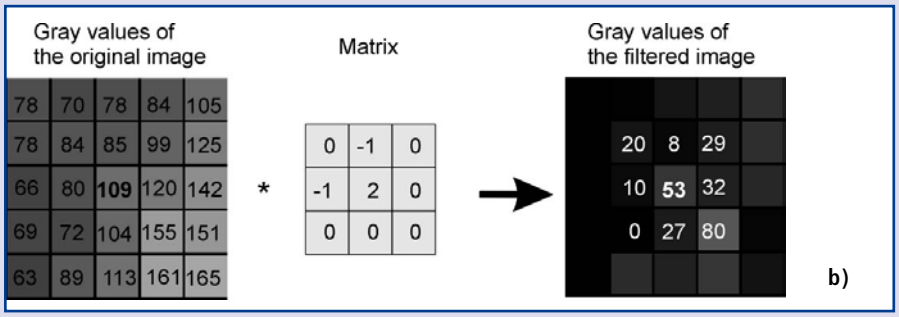
Fig. 38: Brightfield image of a wafer. Left side: The ring shaped background pattern is clearly visible. Right side: The same image with real-time shading correction.

Box 12: How to describe image processing operations mathematically

From the mathematical point of view, an image processing function is a transfer function which is applied to each pixel of the original image individually and generates a new pixel value (intensity/colour) in the resulting imaging. The transfer functions can be roughly divided into point operations, local operations and global operations.

- Point Operations: The resulting pixel is only dependent on the intensity or colour of the original pixel, but it is independent from the pixel places (x/y) and the pixel intensities/colours of the neighbouring pixels. Point operations can be monitored, defined and executed via the histogram. All intensity and contrast operations are point operations. To be exact, point operations are not filters in the more specific sense of the word.
- Local Operations: These look not only at the pixel itself but also at its neighbouring pixels to calculate the new pixel value (intensity / colour). For example, convolution filters are local operations. Many well known noise reduction, sharpening and edge enhancing filters are convolution filters. Here, the filter's transfer function can be described as a matrix consisting of whole positive or negative numbers, known as weight factors. Any original pixel is situated at the centre of the matrix. The matrix is applied to the original pixel and its neighbours to calculate the resulting pixel intensity. See box 12 – Figure 1a as illustration. The 3x3 matrix has nine weight factors W1 – W9. Each weight factor is multiplied with the respective intensities I1 – I9 of the original image to calculate the intensity of the central pixel I5 after filtering. See a concrete numerical example in box 12 – Figure 1 b. In addition to this calculation, the resulting intensity value can be divided by a normalisation factor and added to an offset value in order to ensure that the filter operation does not alter the average image brightness. The matrix weight factors determine the function and the strength of the filter. For example, a 3x3 matrix with all weight factors being one makes a simple average filter used for noise reduction. Sharpen filters consist of matrices having a positive central weight factor surrounded by negative weight factors. The unsymmetrical matrix in fig. 43b creates a pseudo topographical contrast in the resulting image.





- Global operations: All pixels of the original image with regard to their intensity / colour and position (x/y) are used to calculate the resulting pixel. Lowpass and bandpass noise reduction filters are examples. In general, all Fourier Transform filters are global operations.

fraction differences at various parts of the specimen shape are used for contrast enhancement. It allows semi-transparent specimens structures to be analysed in a manner difficult to achieve using bright field microscopy. (See also

www.olympusmicro.com/primer/techniques/ hoffmanindex.html).

For this contrast technique a special condenser and objective are needed. In advanced systems the condenser is equipped with a polariser and a condenser slit plate (according to the objective in use, fig. 36 A and B area). The achromatic or fluorite objective contains a modulator insert at one side of the back focal plane (fig. 36 dark and grey area). The slit plate at the condenser has to be aligned according to the modulator within the objective (fig. 36). This can be done by visualising these elements via a focused centring telescope (remove one eyepiece and insert the telescope). The resulting contrast can be varied by rotating the polariser at the condenser.

The interpretation of DIC and relief contrast images is not intuitive. These techniques contrast different refractive indices within a specimen into a pseudo-three-dimensional image. This means that specimen details which look like holes or hills on the surface of a structure (see fig. 35 left and right side) may simply be areas of different refraction index but not necessarily different in height.

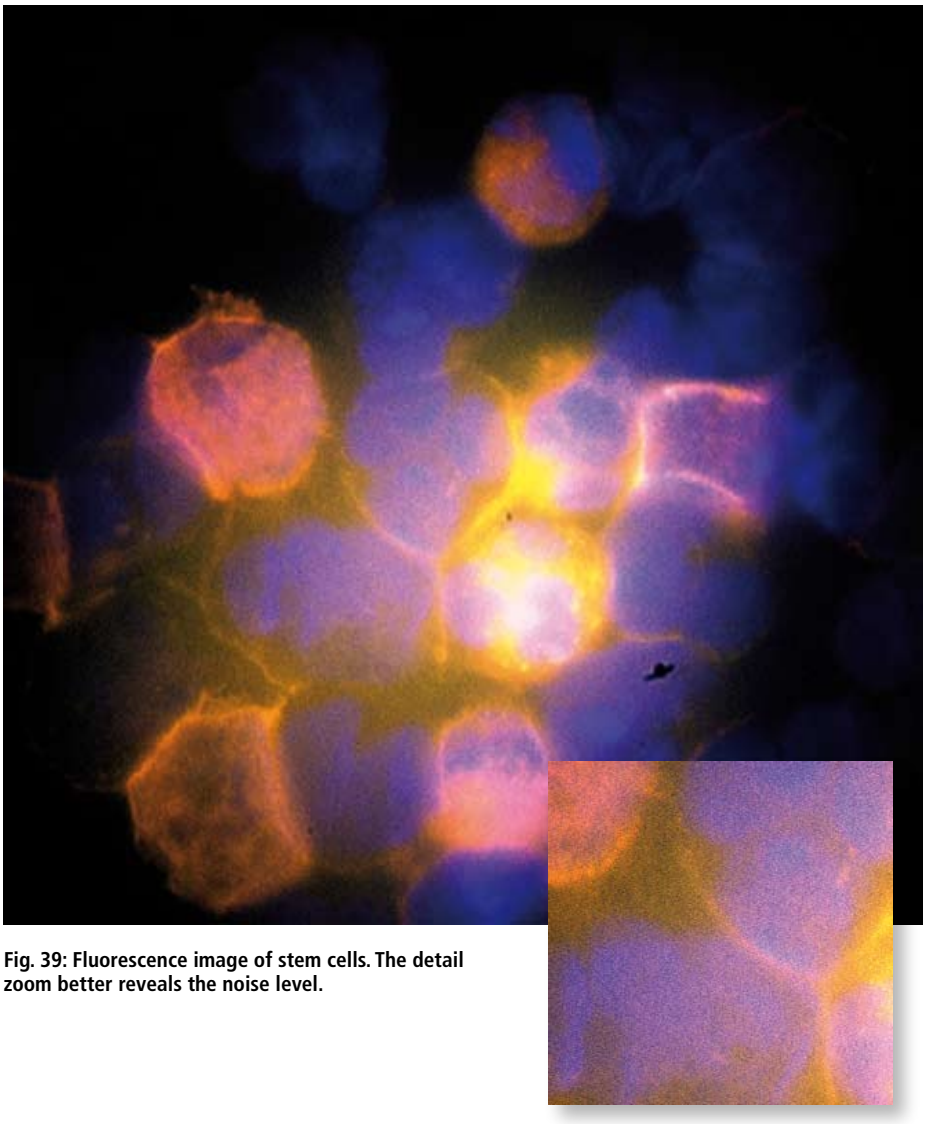


Fig. 39: Fluorescence image of stem cells. The detail zoom better reveals the noise level.

Get more then expected – Polarisation

DIC equipment on a microscope allows the user to employ a totally different microscopic method, simple polarisation. In this case no prisms are used and only the first settings of aligning polariser and analyser in crossed positioning (see box 11) are needed. This setting will generate a dark image because the vibration direction of light that is travelling through the specimen is exactly the vibration direction that is totally blocked by the analyser. However, if the specimen contains material that is able to turn the light, some light can pass the analyser and is observed as a bright detail on a dark background. Examples of such anisotropic materials are crystalline Vitamin C, emulsions of butter, skeletal muscles, urate crystals (Gout inspection), amyloid, rocks and minerals, as well as metal surfaces or the DIC prism itself. The list is very long and often the combination of simple polarising microscopy and DIC can offer a more detailed analysis of the specimen, simply by using equipment that is already available. Beside all the analytical advantages, polarising microscopy also offers images of high aesthetical value (fig. 37).

Revealing structures with imaging filter techniques

We are now at the point where the suitable microscope contrast method has been selected and the optimum camera settings have been made. So the field has been tilled and digital filter and image processing techniques come into play. This is explained in more detail below.

Along a thin line

Image processing can turn the photograph of an ordinary face into an unbelievable beauty or create images that have no counterpart in the real world. Knowing this, digital image processing is often compared with manipulation of results. It might be true for many microscopists that they enjoy the amazing beauty of hidden nature as being mirrored in the images they acquire, but the focus of the daily work lies upon the actual information within the image. Yet this information can be superimposed by artefacts which might be created by specimen preparation, illumination conditions, camera settings or display parameters. At this point, filter techniques come into their own right.

Depending on the reason for imaging, a wide variety of processing steps can be applied to reveal new information or to enhance image clarity for the details that are under observation. What kind of artefacts may appear in a digital image? Imperfect digital images may look weak in contrast, unevenly illuminated, wrongly coloured, diffuse, blurred, noisy, dirty etc. These distortions might make the image look unprofessional. But what is more, they might make any automatic measurement routine based on threshold values or edge detection difficult and sometimes even impossible to apply.

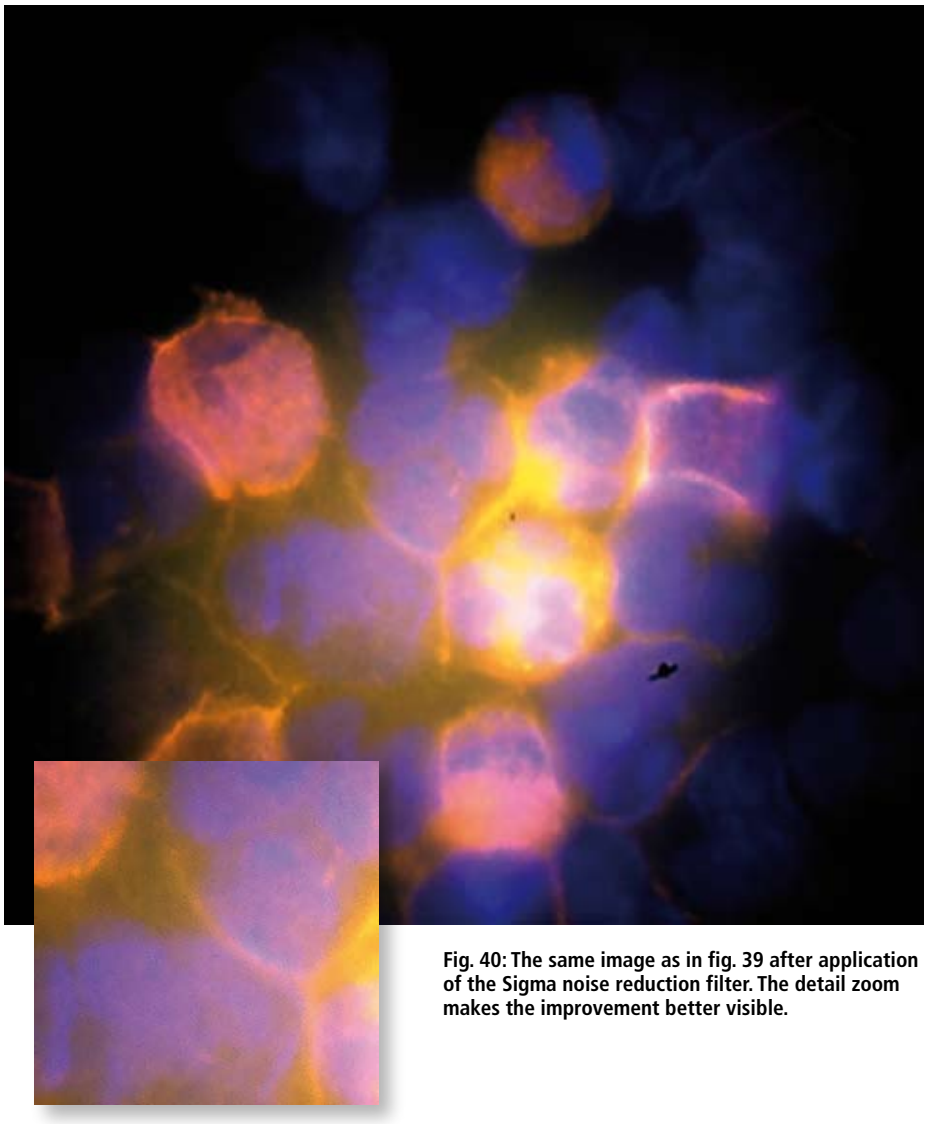


Fig. 40: The same image as in fig. 39 after application of the Sigma noise reduction filter. The detail zoom makes the improvement better visible.

Many of these artefacts can be reduced by using a suitable digital filtering technique. Often there exist several different filters to improve the image quality. It is not always easy to find the best method and parameter settings to suppress the image defects without disturbing the "real" image information. This is the highest standard which the optimum filter should meet: removing the image artefacts and keeping the specimen structures in the image. This goal cannot always be accomplished. When doing image processing, there is often a thin line between what needs to be done and what would be good to accomplish. In addition, there is a thin line between scientific virtue and creative composition!

Now or later – both are possible

Digital image processing is usually applied after image acquisition and image storage. But the latest image processing systems support "Live Digital Image Processing", which means that numerous real-time functions can be executed during image acquisition. These include the online histogram, which is used to monitor the image brightness and contrast. Additionally, the histogram function itself can be used to have the image contrast improved automatically or to set it in the live mode by hand. (How to use the histogram to improve image contrast see box 8.) Another real-time function is the white balance, which corrects colour shifts at the moment of acquisition. Non-uniform specimen illumination can also be immediately corrected in the live image using the online shading correction. This is described below. The operations of contrast enhancement, white balance and shading correction can be applied during or after image acquisition. They are point operations, mathematically speaking (refer to box 12). This makes them suitable for reducing image artefacts without distorting the image content itself.

Where there is light, there is also shade

One major source of distortion in a light microscopic image is brightness fluctuation, which may arise from out-of-axis illumination conditions or when the microscope is not aligned optimally. Uneven illumination artefacts may also appear under regular observation conditions using low objective magnification and/or in combination with low magnifying camera adapters. Then the shading artefacts are manifested as a ring shaped shade structure which becomes even darker near the edges of the image (fig. 38 left side). The minor shading within the background needs to be corrected. The operation applied here is called background correction, flat-field correction or shading correction. (A simple tool for background correction can be downloaded at www.mic-d.com.)

The best method is to record a background image and a dark image in addition to the "raw" specimen image. The background illumination profile is simulated in the background image, whereas the noise level of the camera system is apparent in the dark image. The advantage here is that these images are gathered independently from the specimen image.

The background image is usually obtained by removing the specimen and leaving an area of mounting medium and cover slip in place. If this is not possible, the same result can also be achieved by leaving the specimen in place but to defocus the microscope. It is important that the background image does not show any debris. The dark image is acquired by closing the camera shutter. It should use the same exposure time as the specimen image.

The shading correction algorithm first subtracts the dark image (d) from the specimen image (s) as well as from the background image (b). Then it divides the corrected background image through the corrected specimen image: (s–d)/(b-d). This procedure can also be performed automatically with the live image. Here, the background and the dark images have to be acquired only once for each microscope objective using a standard sample and standard acquisition parameters. This is how the image in fig. 38 (right side) was taken.

If the background and dark images are not available, the shading correction can also be derived from the specimen image itself. For example, a very strong smoothing filter averages out all structures and reveals the background. Or a

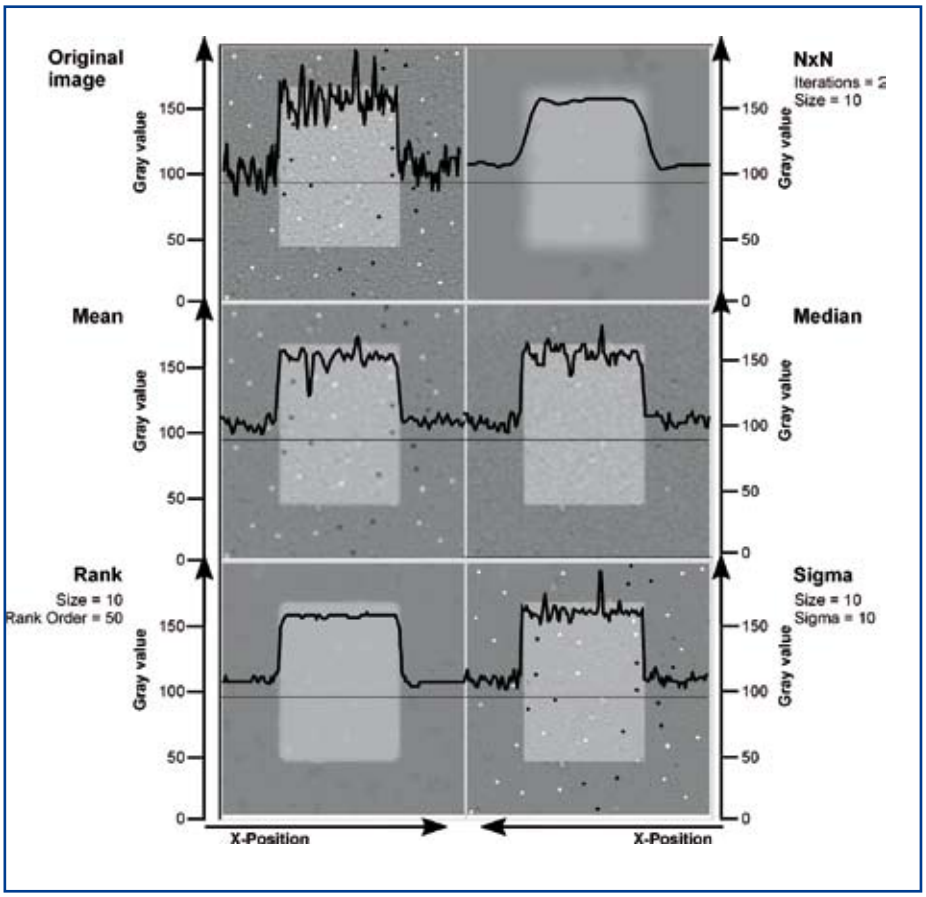


Fig. 41: Comparison of different smoothing filters. See text for further explanation.

multi-dimensional surface function can be applied to fit the background illumination profile.

Just too much noise

Random noise can be an annoying phenomenon which is encountered when the specimen signal is low and/or was enhanced with a high gain factor. An example from fluorescence microscopy is shown in fig. 39. In regular brightfield light microscopy images random noise is usually not visible. But it is often unsheathed when a sharpen filter is applied to the image. See fig. 42, first and second image as an example. There are different smoothing filters which can be used to reduce the noise. Smoothing filters are also applied to suppress artefacts deriving from small structures like dirt, dust, debris or scratches. Yet the challenge is to find a filter which eliminates the noise and the artefacts without smoothing the object edges too much.

Fig. 41 shows an artificially created image having only one real structure: a simple rectangle of light shading upon a dark background. This structure is superimposed by two different kinds of distortion: strong statistical noise interference on one hand and so-called hot pixels or shot noise on the other hand. The shot noise is individual bright and dark pixels and can come from a defective camera. These pixels also mirror artefacts from dust particles, dirt, debris or small scratches.

The noise reduction filters applied to the image are local or neighbouring operations, i.e., the neighbouring pixels are taken into account to calculate the new pixel value of the resulting image (see also box 12). The Mean and NxN filters just average everything and thus also broaden the structure of the rectangle (fig. 41). The more powerful the smoothing effect, the more noticeable the morphology of the object within the image will be altered. Shot noise will not be removed. Whereas the Mean filter applies fixed settings, the extent to which the NxN filter averages depends on the parameters set.

The Sigma or Gaussian blur filter is a special average filter which does not affect any pixels deviating greatly from their surrounding area (fig. 41). So "real" structures and edges like the rectangle are not touched whereas the random noise disappears. The Sigma filter is the method of choice to reduce selectively statistical noise without greatly broadening the specimen structures (see also example in fig. 40).

The Median and Rank filters eliminate the shot noise widely (fig. 41). They are especially suited to suppress dust and dirt, in general all small structures which stand out the underground. Comparing the effects the different smoothing filters have on the artificial sample image (fig. 41), the image's two different distortions could be eliminated best by applying two filters successively: first the Sigma filter against the statistical noise, and then the Rank filter against the shot noise. This leads to the somewhat philosophical rule which says that it is best not to strike everything all at once but to cut the problem into pieces and find the appropriate measure to deal with each piece separately.

NxN-, Sigma- and Rank filters have user-definable controls which make it possible to adjust the smoothing effect optimally to the image. There is another option to reduce statistical noise, usually with fixed parameters, which gives quite good results: the lowpass filter. This filter is a global operation (refer to box 12) which filters out high frequency and periodic distortions. Yet here, the edges become somewhat broadened. An even better global operation is the bandpass filter which reduces the noise and preserves the steepness of edges.

Revealing the details

Another class of filters seen from the application point of view are the sharpen filters. Sharpen filters can be used to enhance fine image details. After processing an image with a sharpen filter, the image appears to look clearer. But sharpen filters have to be applied somewhat carefully because they can create artefacts themselves if overdone. The established sharpen filters are local neighbouring operations like the convolution filters (see box 12). The newer filters use the so-called unsharp mask algorithms. (Please have a look at fig. 42.) Sharpening the first, original image, gives the second image. Unfortunately, this enhances not only the structure but also the noise present in the original image. So the sharpen operation makes the noise visible. Additionally, the filter parameters have been set too aggressively so that the "lamellae" structures of the diatom look artificial. This context has to be kept in mind when applying a sharpen filter: Before the sharpen filter can be applied to an image, a noise reduction filter must often be applied first. Any

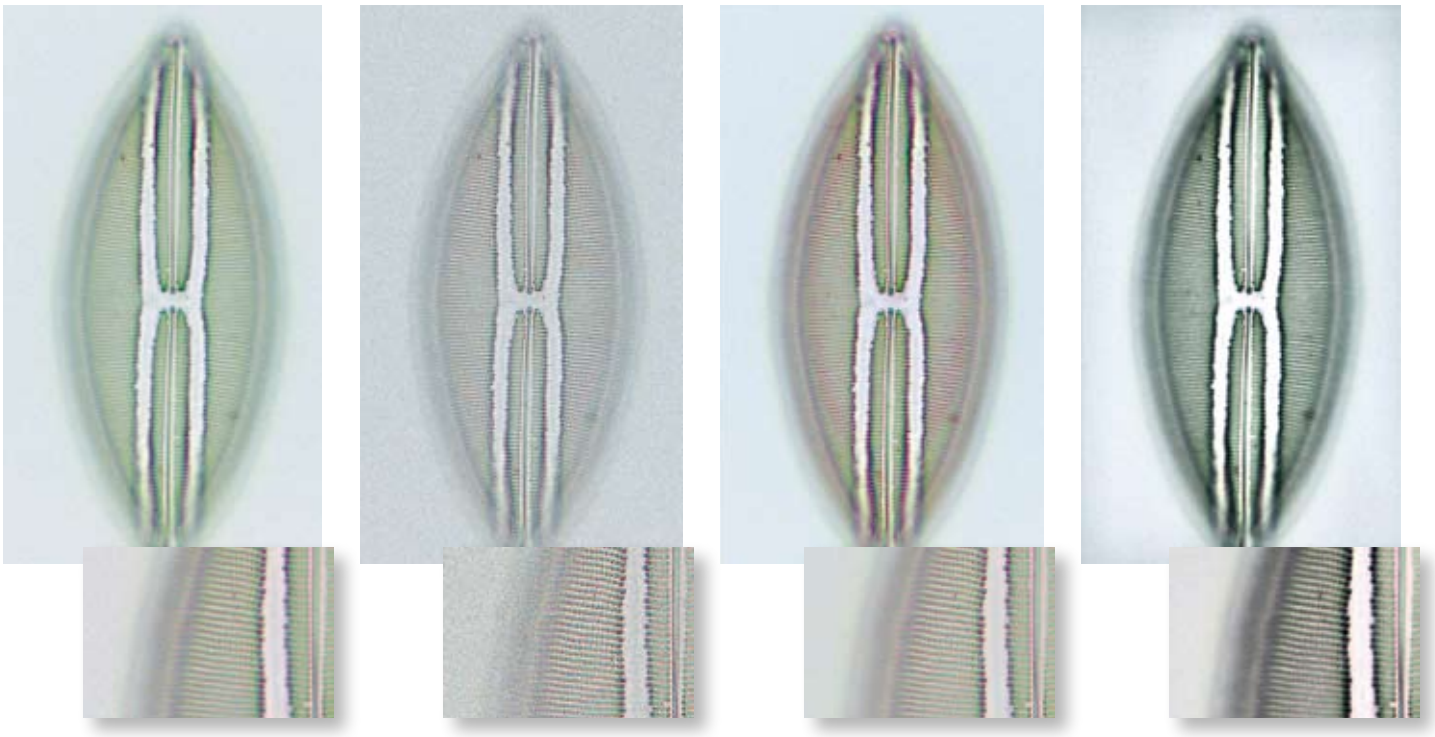


Fig. 42: Brightfield image of a diatom. (a): original image. (b): after application of a strong sharpen filter. This increases the noise. (c): after application of the Sigma noise reduction filter and a moderate sharpen filter. (d): after application of the Sigma noise reduction filter and the DCE sharpen filter.

sharpen filter with user controls should be applied conservatively. Here, less is often more. The third image in fig. 42 shows the result after noise removal via a Sigma filter and successive moderate sharpening.

The DCE filter is a specially designed sharpen filter and is part of the Olympus image processing software solutions. The letters DCE stand for Differential Contrast Enhancement. The DCE filter enhances weak differences in contrast. It selectively takes the lesser intensity modulations between neighbouring pixels and enhances them, while greater intensity modulations remain as they are. So the DCE filter renders image structures visible which are barely distinguishable in the original image. The filter works the better the more fine structures the image has. Resulting images are more detailed and appear more focused. See fig. 42, last image, and both images in fig. 43 as examples.

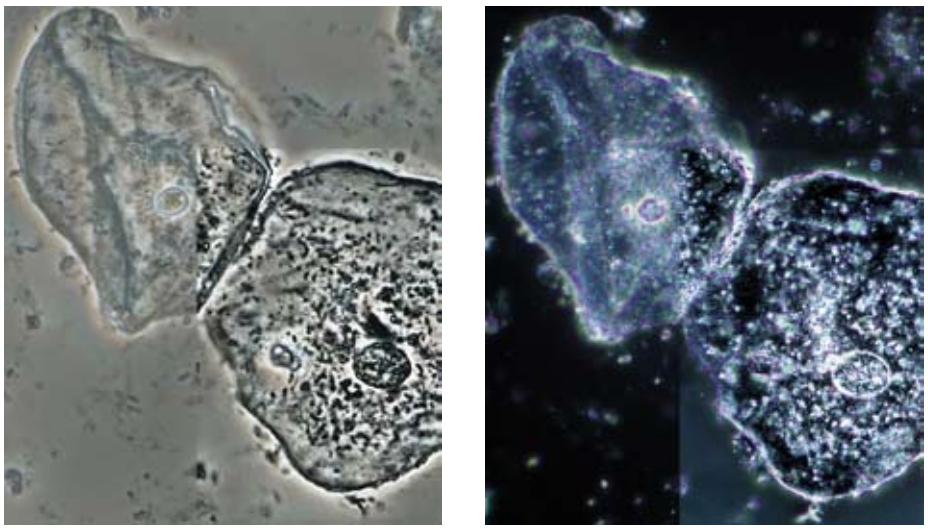


Fig. 43: Epithelial cells viewed with phase contrast (left side) and transmitted darkfield contrast (right side). The original images are acquired with optimised microscope settings. These have already been shown in box 8 and fig. 30. The lower right parts of the images are each filtered with the DCE filter.

Putting everything together

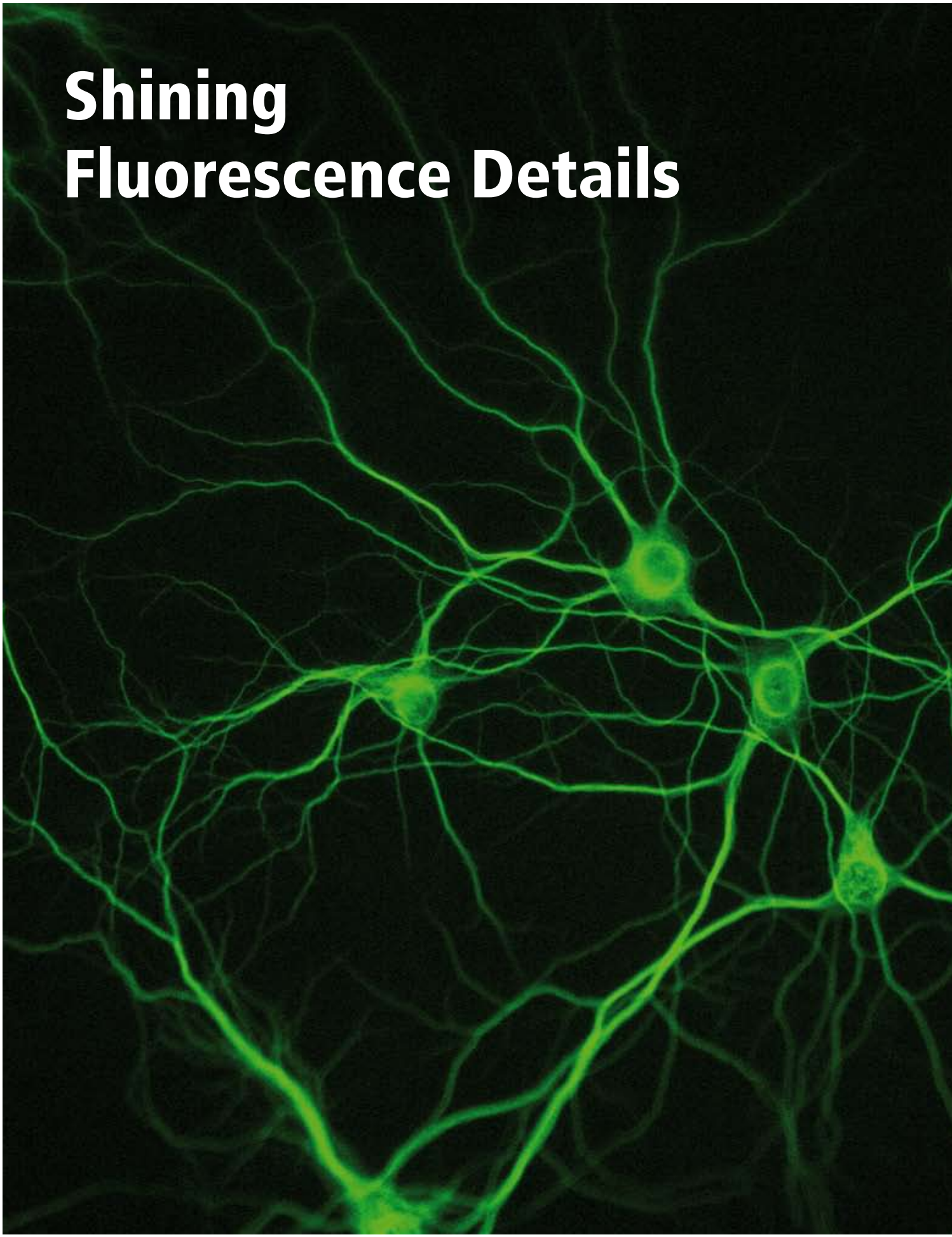
To integrate what has been said above, there are some image processing operations which are meaningful or even necessary to apply to an image, either in real time or after acquisition. These operations can reduce image distortions, prepare the image for automatic measurements or just make the image look better. The following steps suggest a possible strategy to proceed with digital image operations when acquiring an image (see also:

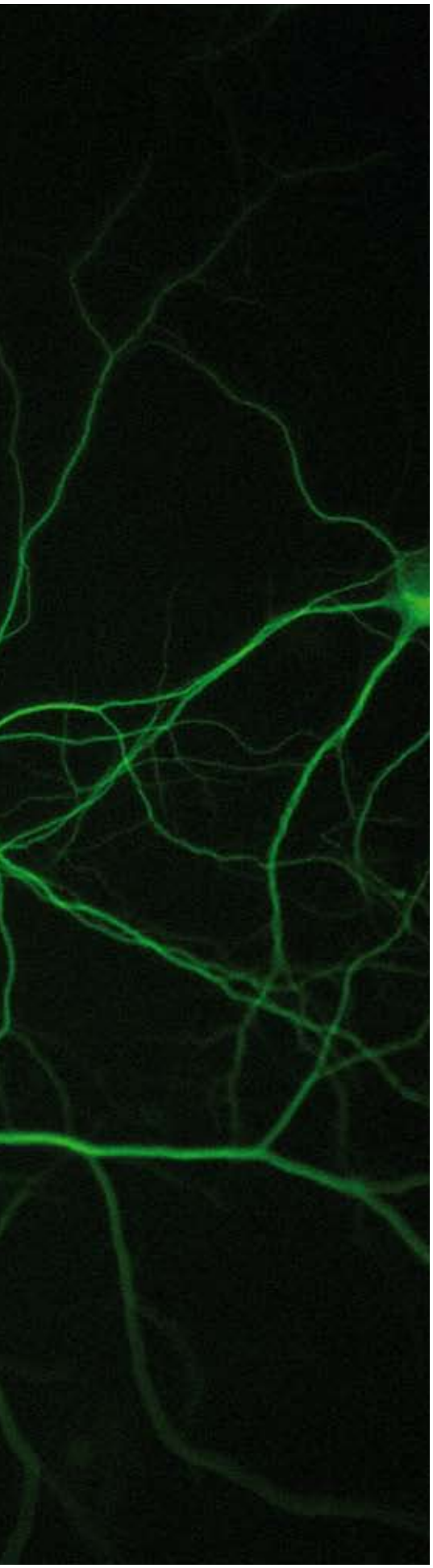
www.olympusmicro.com/primer/digitalimaging/imageprocessingsteps.html)

1. Shading correction is to equalise uneven background illumination.
2. Contrast enhancement is to optimise brightness and contrast.
3. White balance is to adjust colour shifts.
4. Smoothing is to reduce random noise and suppress shot noise and small artefacts like dust and dirt.
5. Sharpening is to enhance fine edge detail.

Here, we have covered a few of the wide range of possible filter operations. There are many more which go beyond scientific applications and transcend the pure picturing of the visible reality towards creative, funny, intelligent and beautiful compositions.

Shining Fluorescence Details





A first introduction

Most of the newly developed microscopic techniques make use of fluorescence. Fluorescence microscopy is more than 'just making colourful images in one, two, three or even more colours', it is an enormously powerful tool for investigations in the biological field. Fluorescence techniques place numerous benefits in the hands of researchers wishing to exploit the upper limits of sensitivity and resolution in microscopy. Beyond the scientific benefits, just studying the fluorescence images can sometimes offer a new insight into a reality which is usually hidden from the view of the world.

Fundamentals – a development both remarkable and ongoing

Within the last few decades numerous new techniques such as confocal, deconvolution, ratio-imaging, total internal reflection and applications such as the use of fluorescent proteins (e.g. GFP) have initiated a real renaissance in the microscopy field. All of these techniques make use of fluorescence, a phenomenon first observed by Sir George Gabriel Stokes in 1852 and physically described by Alexander Jablonski in 1935 (see box 13). Compared with today, the number of widely used fluorochromes was restricted to just a few in the 1990's. For example, nowadays the fluorochrome FITC filter set for fluorescence microscopy can also be used for a wide range of different fluorochromes with green emission spectra.

specific questions regarding life science or materials science specimens and to visualise the result in a specific colour. For example, to identify the distribution of a specific protein within a tissue, a fluorochrome can be used to mark the protein via an antibody (immunohistochemistry).

Histological staining procedures for transmission light microscopy have a long history in microscopy. One essential advantage of fluorescence microscopy, however, is the presence of fluorescent molecules themselves. Even if a structure is too small to be resolved by a light microscope, the emission light remains visible.

Fluorescent molecules act like light sources that are located within specific areas of a specimen, indicating their location with light of a specific colour. These indicators require energy to emit light and this is given to the fluorochrome by the excitation light, provided by the microscope light source. A specific range of wavelengths is needed to excite a specific fluorochrome. For example, a range of blue wavelengths around 480 nm can excite the FITC fluorochrome. This involves using two different light beams and having to separate them. On the one hand, we need to direct the light of the microscope light source onto the specimen and on the other hand we have to observe the light that is originating from the fluorochromes. This separation is possible due to the "Stokes shift", which describes the fact that the wavelength of fluorescent light (emission) is always longer than that of the excitation. Using a blue excitation light will thus result in a green emission for the FITC fluorochrome. Every fluorochrome has its own excitation and emission spectra. The microscope must be perfectly equipped to visualise this fluorescence accordingly.

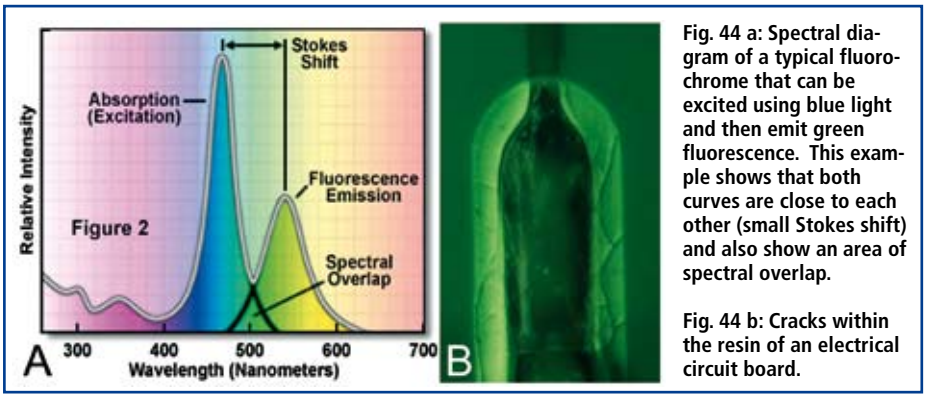


Fig. 44 a: Spectral diagram of a typical fluorochrome that can be excited using blue light and then emit green fluorescence. This example shows that both curves are close to each other (small Stokes shift) and also show an area of spectral overlap.

Fig. 44 b: Cracks within the resin of an electrical circuit board.

Why use fluorescence?

Using fluorescence can be compared to the situation where a teacher asks if the students have done their homework. The rapidly changing facial colours of the "guilty" students provide conclusive "results". However, fluorescence techniques are not really for answering questions such as the above. They help to address

Fluorescent molecules

There are two options for using fluorescent microscopy, depending on what is

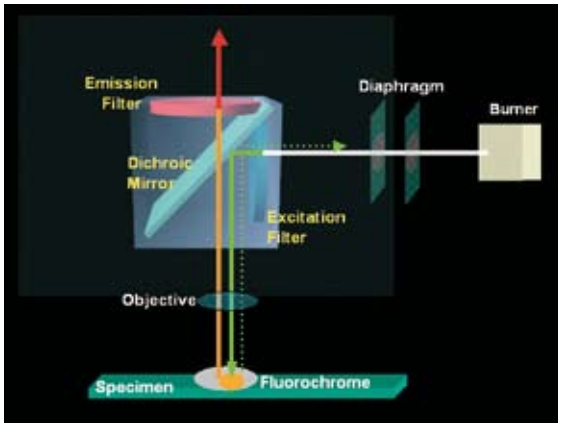


Fig. 45: Light path on a microscope equipped for fluorescence.

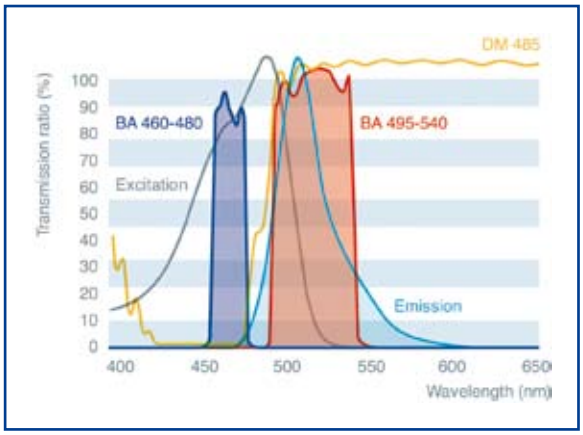


Fig. 46: Characteristics of an Olympus HQ filter set optimised for GFP. By using up to 100 layers of an ion deposition technique with new substrates and coating materials, filters can be created with high transmission and exceptionally sharp cut-off (tolerances < 2 nm). Autofluorescence is significantly reduced and the filters are equipped with a stray light noise destructor to enhance the signal-to-noise ratio.

being investigated: either the specimen itself already contains molecules that show fluorescence; or specific fluorochromes have to be added to the specimen. Autofluorescence is widely found in materials such as plant sections or electrical circuits, for example. The resin on circuits is fluorescent and can easily be inspected under blue excitation (fig. 44b). The green emission of the resin enables the detection of the tiniest cracks which may influence material quality.

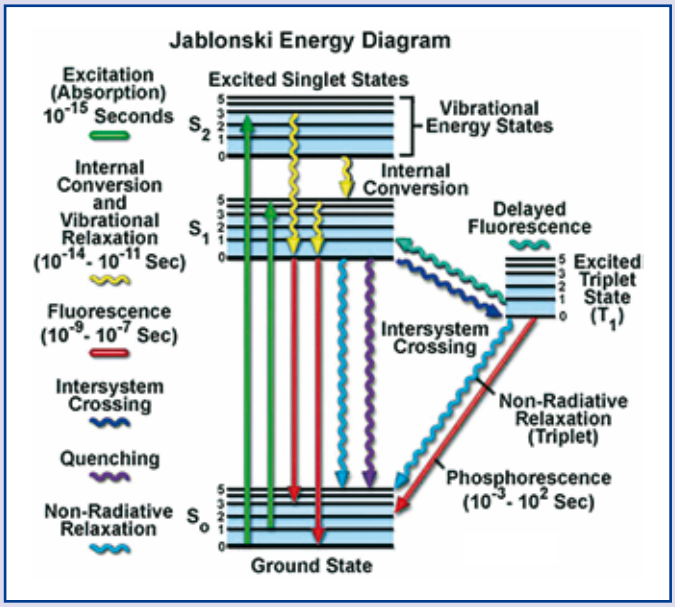
Fluorochromes themselves can be divided into at least three groups. The first are fluorochromes that require other molecules, such as antibodies or lectins, to bind to specific targets. This rapidly growing group of fluorochromes includes longstanding ones such as FITC and TRITC. Most of these fluorochromes are sold together with the specific target-finding molecule (e.g. a goat anti-mouse IgG antibody Cy5 labelled). Quantum dots are also partial members of this group but different in structure and theory. They are nanometre-sized crystals of purified semiconductors and exhibit long-term photo stability, as well as bright emission. The main difference feature-wise is their capacity to be excited by wavelengths up to the blue range and having different emission colours depending on their size. Due to their flexible capabilities they can also be used for direct staining of cells (e.g. cell viability).

The second group contains fluorochromes that have inherent binding capacities, such as the DAPI nucleic acid stain or the DiI anterograde neuron stain. This group also contains fluorochromes that change their fluorescent properties when bound to different amounts of molecules such as calcium (e.g. Fura-2). This means that these fluorochromes are used directly and do not necessarily require a transportation system such as an antibody.

The third group contains fluorescent proteins produced by organisms themselves such as GFP. This makes it possible to set up experiments in an entirely different way. It is most often used for live cell imaging or developmental studies and molecular biology. All fluorochromes show distinct spectral properties (fig. 44a) and can often be combined for a multicolour specimen analysis.

Box 13: What is fluorescence exactly?

Fluorescence activity can be schematically illustrated using the familiar Jablonski diagram, where absorbed light energy excites electrons to a higher energetic level in the fluorochrome. These lose a proportion of their energy by vibration and rotation and the rest is then given off as fluorescent light as they return to their original state after only about 10 ns.



Requirements for a fluorescence microscopy system

The Light Source

To excite the fluorescence of a fluorochrome, an intense light source is needed that provides the necessary excitation wavelengths to excite the particular fluorochrome. In the first chapter we described the most frequently used light sources for light microscopy and their alignment. A correctly aligned burner plays an essential role in creating good fluorescent images. If the burner is not correctly aligned, the signal from the fluorochrome may be excited in a very weak way and the field of view will not be homogeneously illuminated, or can show a bad signal to noise ratio.

Due to the very different specimens and applications that can be analysed using fluorescent techniques, there is no one-size-fits-all strategy. All fluorochromes are subject to the process of photo-bleaching, which is the chemical destruction which takes place during excitation. Living cells, too, may be damaged by the intense light. This makes it of supreme importance to restrict the excitation brightness and duration to the ex-

act amount needed. The amount of light can be efficiently modified with neutral density filters or a motorised attenuator. When the light is not needed for excitation, the shutter is closed. These features can be predefined in motorised microscopes and help to optimise experiments.

The Filter Sets

In addition to the special light path within a fluorescence microscope (fig. 45), another necessity is having a filter which only permits the required range of excitation wavelengths to pass through. This is achieved using an exciter filter with what are referred to as bandpass filter characteristics (box 14). After restricting the light to the particular colour that is needed to excite the fluorochrome, the light is directed to the specimen via a dichromatic mirror (fig. 45).

As indicated by its name, the dichromatic mirror treats different colours differently. It reflects light below a given wavelength and is able to let longer wavelengths pass through. The excitation light travels through the objective to the specimen, acting like a condenser. This is where the fluorescence phenomenon takes place. Excited by the light, the fluorochromes emit the fluorescence light of longer wavelengths. This is captured by the objective, moving on to the dichromatic mirror, now letting the longer wavelengths pass through. The last step of filtering is undertaken by the emission filter (also called a barrier filter, see fig. 46). This filter restricts the light colour to best fit the fluorochrome emission and the question being investigated. It ensures that no unwanted wavelengths are observed and analysed. The emission filter can be designed as a bandpass filter (precisely restricted to one spectrum) or as a longpass filter (brighter in effect, but less optimal due to a reduction in restricted wavelengths). To help find the best filter combination for the fluorochromes in use and the analysis in mind, a variety of web pages is available (e.g. www.olympusmicro.com/primer/java/fluorescence/matchingfilters/index.html). A straightforward example (below) will demonstrate how the combination of filters may differ depending on the context.

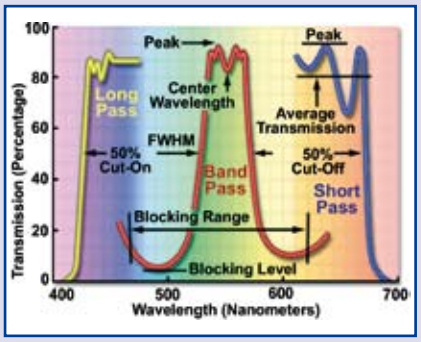
Using the correct filter set – an example

If you wish to make a vital analysis of a cell culture you may choose a Fluorescein-diacetate (FDA) to stain vital cells. This fluorochrome is excited by blue light and will have a green emission. The vital cells will be the ones appearing green. The filter set could thus be chosen as follows: an exciter with BP460-490 bandpath characteristics, a dichromatic mirror with DM505 characteristics and an emission filter with LP510 long path characteristics. This will result in a bright green image of all green fluorescent molecules. So far, so good. The vital cells are stained. To verify that non-labelled cells are in fact dead, propidumiodide (PI) dye may be used. This dye cannot pass through intact cell membranes. The DNA of dead cells only will be labelled and appear red. This means it can be used along with the FDA. When doing so, however, the excitation of the FDA with the filter mentioned will cause some problems. PI will already be excited by the blue light and the red emission is also visible. This is caused by the emission filter because in this set up, all wavelengths above 510 nm are allowed to pass through. Both dyes are thus excited and visible. A definite separation of both signals is not possible and as we will see later on, this can cause problems during imaging.

To separately identify both signals from the cell culture, the emission filter required for FDA is a bandpass filter

Box 14: How are filters described?

Filters for fluorescence microscopy are described using letters and numbers: e.g. BA 460-480. In this case, BA stands for bandpass filter and the numbers indicate the 50 % cut on and the 50 % cut off (fig. 46 and see boxfigure below). For a longpass filter, just the number for the 50 % cut on is indicated. Some companies use a different description for the same overall filter characteristics (e.g. 470/20), 470 indicating the central wavelength and 20 indicating the range of the full width at half maximum (FWHK). The correct transmission characteristics of the filter can only be provided using a transmission/wavelength diagram (see boxfigure below).



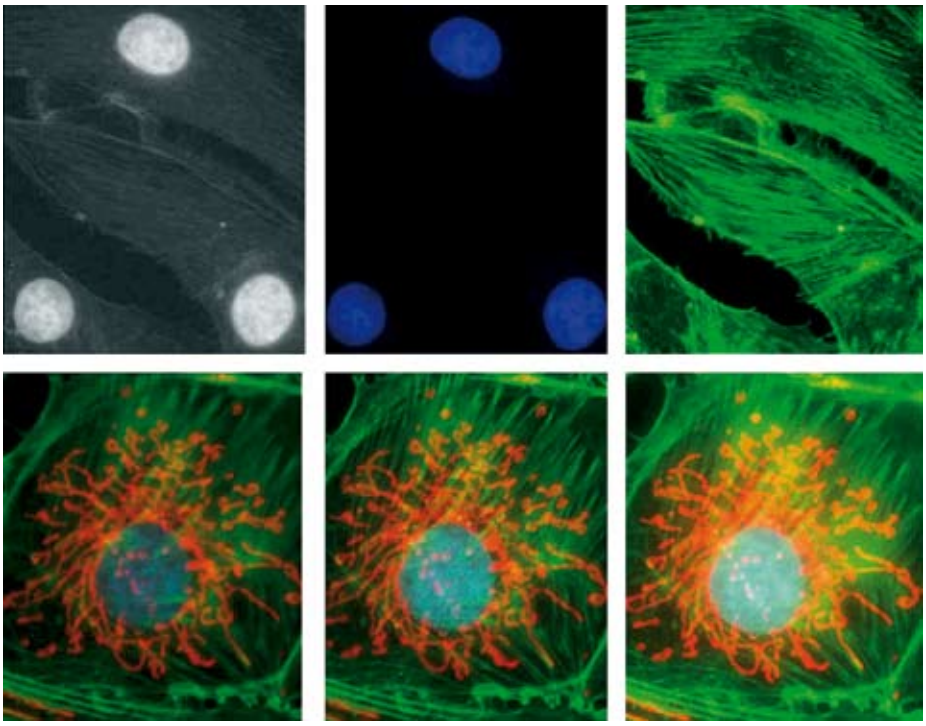
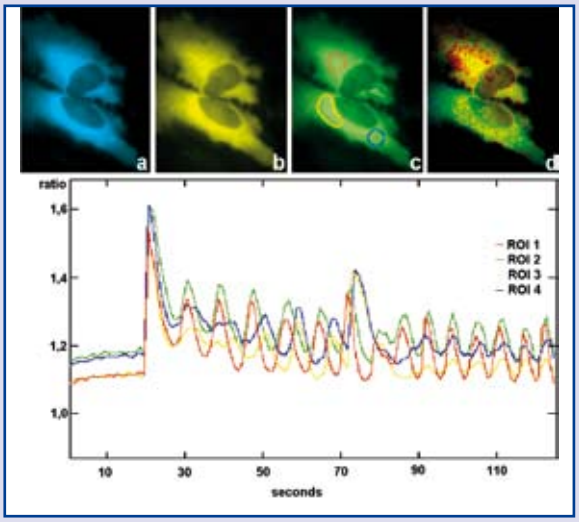


Fig. 47: Upper row: Images of a double-labelled specimen (DAPI for DNA staining and Alexa 488-labelled antibody for F-Actin staining). The first image shows the detection of the fluorescence using a filter set with the appropriate excitation filter for DAPI and with a long pass emission filter. Analysed with a monochrome camera, even the Alexa-488-labelled structures are visible and prevent effective analysis. The blue and the green image show the same specimen with optimised emission filters, allowing the separation of each signal.
The lower row shows an example of an image area analysed with an achromat (left), a fluorite (centre) and an apochromat objective (right) at the same magnification.
Note that the signal intensity and colour transmission increases from achromat to apochromat due to the increasing NA and quality of the objectives, whereas the background intensity in the upper left corner remains unchanged.

Box 15: How to measure FRET.

Ratio measurement

An epifluorescence microscope equipped with highly specific fluorescence filter sets, a digital camera system and image analysis software is required. The ratio between the intensity of the sensitised acceptor emission (= FRET signal) and the intensity of the donor emission is calculated (see figure). The results have to be corrected for spectral cross-talk by spectral unmixing software.



Acceptor photo-bleaching.

This effect occurs when the acceptor is irreversibly destroyed by extensive illumination from excitation light. After bleaching of the acceptor fluorophore, FRET is stopped. Excitation of the donor will result in donor fluorescence emission. After the acceptor photo-bleaching, the donor shows more intensity than before.

Donor-bleaching.

The sample is irradiated with the specific excitation wavelength of the donor. Images of the donor are continuously acquired and the intensity decay is quantified. FRET decreases the mean lifetime of the donor and protects the donor from photo damage. As a result, when using FRET, the rate of photo-bleaching is lower; without FRET, the rate of photo-bleaching is higher.

(e.g. BP510-550). This filter will only allow the green emission of the FDA to pass through and the emission of the PI will be blocked. A second filter set can then be used to analyse the PI signal efficiently (e.g. BP530-550 excitation filter, LP575 emission filter, DM570 dichromatic mirror). This principle also applies to other multicolour staining procedures. Best results are achieved when the emission filter for the detection of the fluorochrome with the shorter wavelength is a band pass filter (fig. 47 upper row).

The objectives

The light gathering capacity of the objective plays an essential role in fluorescence microscopy. For optimal signal strength, a high-numerical aperture (high NA) and the lowest useable magnification should be employed. For example, using a 1.4 NA aperture lens instead of a 1.3 NA lens of the same magnification will result in a 35 % increase in light intensity, assuming that all other factors are the same. Furthermore, the type of glass that is used requires good transmission for the wavelengths used. Fluorite or apochromat objectives are used for that reason. Further enhancement can be achieved by selecting objectives with extremely low autofluorescence of the glass material used (Olympus UIS2 Objectives). When all of these factors are provided for – high NA, good transmission and low autofluorescence – this ensures a perfect signal-to-noise ratio (i.e. a strong signal with low background intensity (fig. 47 lower row)). Background noise can also be introduced by the specimen itself due to fixation, autofluorescence of the specimen or non-optimised staining procedures.

Type of camera

The imaging device is one of the most critical components in fluorescence microscopy analysis. This is because the imaging device used determines at what level specimen fluorescence may be detected, the relevant structures resolved and/or the dynamics of a process visualised and recorded. Numerous properties are required to use fluorescence microscopy effectively. These include: high resolution, extreme sensitivity, cooling, variable exposure times and an external trigger function. Generally no single detector will meet all these requirements in fluorescence microscopy. Consequently, the fluorescence microscopist frequently has to compromise. However, the cameras used in fluorescence microscopy should at the very least offer high signal sensitivity, low noise and the ability to quantify intensity of intensity distribution.

Colour cameras are less sensitive than their monochrome counterparts because of the additional beam-splitting and wavelength selection components. Therefore, monochrome cameras are preferable. They image the fluorescence intensity of each fluorochrome separately and can handle the respective images later on within a specific colour space using the appropriate software. The resulting multicolour images can be displayed, printed out and analysed or further processed. Every channel of colour represents the result of one fluorochrome. This result can be obtained if the fluorescence filters are chosen correctly. Returning to our example of the FDA and PI double labelling: when using the longpass emission filter to detect the FDA signal, the red emission of the PI will also contribute to the resulting digital image. A monochrome camera would not differentiate between red and green or between blue and green as shown in fig. 47 (first image) – it will only show intensity in grey values. Therefore, the image will represent the resulting distribution of both fluorochromes within the same colour. The use of the bandpass emission filter as described above will help in this respect.

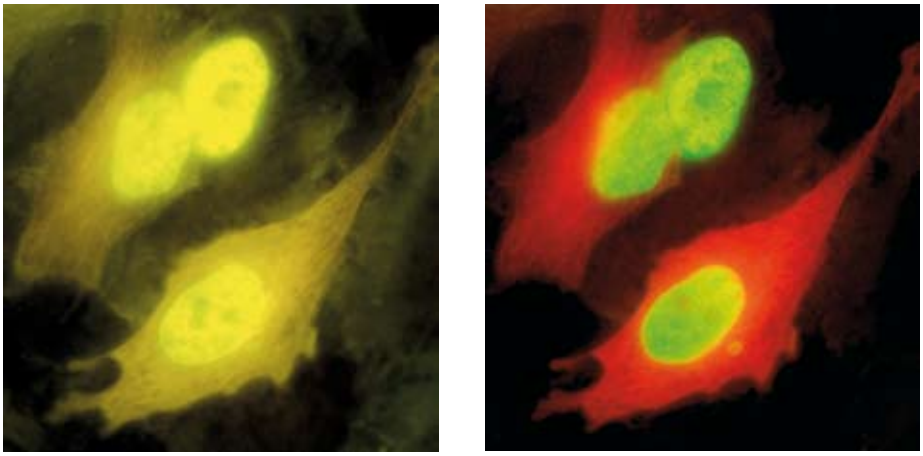


Fig. 48: Multi-colour image of a GFP/YFP double-labelled sample, before (left) and after spectral unmixing (right). In this sample, GFP was fused with the H2B histone protein and YFP with tubulin. The visible result is a pronounced chromatic resolution of both structures now displayed in green and red. The tubulin on top of the bright green nuclei is even detectable.

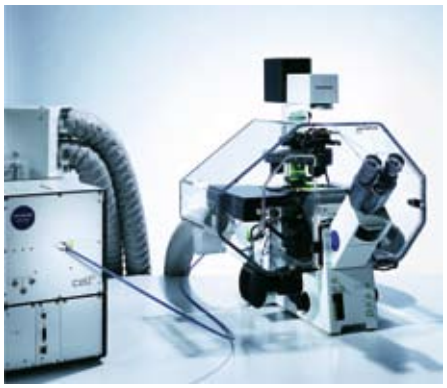


Fig. 49: A climate chamber offers features like control of temperature, humidity and CO_2 content of the atmosphere.

Software-tools: Spectral unmixing

A further problem can occur when using different fluorochromes with overlapping spectra within one sample. The considerable overlap of excitation and emission spectra of different fluorochromes is exemplified by the spectra of enhanced green fluorescence protein (eGFP) and enhanced yellow fluorescent protein (eYFP), two commonly used variants of the green fluorescent protein (fig. 48 left side). Even with the use of high quality optical filters it is not possible to separate the spectral information satisfactorily. This means that YFP-labelled structures are visible with a GFP filter set and vice versa, affecting the resulting images significantly and detrimentally. This phenomenon, known as "bleed-through", strongly reduces colour resolution and makes it difficult to draw accurate conclusions. The solution to overcome this effect is called spectral imaging and linear unmixing.

Spectral imaging and linear unmixing is a technique adapted from satellite imaging to wide-field fluorescence microscopy. Using this highly effective method, it becomes possible to ascertain the specific emission of the different fluorochromes to the total signal and to restore a clear signal for each colour channel, unaffected by emission from the other fluorochrome. This is achieved by redistribution of the intensity (fig. 48 right side). It is important to note that original data is not lost during linear unmixing nor is any additional data added to the image. The original image information is all that is used in this procedure. The overall intensity of pixels is maintained. Thus, the technique does not result in artificially embellished images. After unmixing, quantification analysis not only remains possible, but also becomes more precise.

How to use light as a tool

After having laid the fundamental principles and the microscopic requirements we can now go deeper into different state-of-the-art qualitative and quantitative imaging methods for fluorescence microscopy. These approaches require advanced microscopic and imaging equipment which varies from case to case. So only general statements can be made here about the hardware and software used, along with a description of the applications and methods.

Life under the microscope

Wherever there is life there are dynamic processes – observation of living cells and organisms is a tremendous challenge in microscopy. Microscopy offers different techniques to reveal the dynamic processes and movements of living cells. The use of fluorescent proteins and live fluorescent stains enable the highly specific labelling of different organelles or molecules within a living cell. The intensity of the emission light of these markers can be used to image the cells. In addition to the application protocol and the fluorescent technique to be used there are further general considerations to be aware of.

First of all, there is the definition of the needs for the specific environmental conditions and controlling these with regard to specimen handling. Assuming you are using a cell line and would like to analyse processes over time, it may be necessary to provide appropriate environmental conditions for these cells. Dynamic processes in single cells can occur within the millisecond range – such as shifts in ion concentrations. Or they may take minutes – such as the active or passive transport of proteins or vesicles. Microscopes can be equipped with heating stages and/or minute chambers, or with complete cultivation chambers to ensure cultivation of living cells with all the appropriate parameters on the microscope while observation is conducted for hours or days (fig. 49).

Where do all the ions go?

Fluorescent dyes such as FURA, INDO or Fluo show a spectral response upon binding Ca^{2+} ions and are a well established tool to investigate changes in intracellular Ca^{2+} concentration. The cells can be loaded with a salt or dextran conjugate form of the dye – e.g. by microinjection, electroporation or ATP-induced permeabilisation. Furthermore, the acetoxymethylester of the dyes can be added to the medium, loaded passively into the cells and cleaved enzymatically to produce cell-impermeable compounds.

For the analysis of a typical two channel FURA experiment it is necessary to switch between the excitation of 340nm and 380nm. When observing FURA loaded cells without any stimulus, Ca^{2+} is bound in cell compartments. The FURA molecules show strong fluorescence at an emission of 510nm when excited with 380nm, and weak fluorescence when excited with 340nm. As the cell releases Ca^{2+} from storage compartments due to a reaction to a stimulus, FURA molecules form complexes with these released Ca^{2+} ions. The fluorescence signal in the emission channel of 510nm increases when excited with 340nm, and decreases when excited with 380nm. The ratio between the signals of the two excitation channels is used to quantify the change of intensity.

Why use a ratio? Lamp fluctuations or other artificial intensity changes can cause a false signal which can be misinterpreted as a change in ion concentration when intensity is measured in one channel only. The second drawback of a single channel analysis is that it displays the amount of the fluorescence only. Therefore, thicker parts may look brighter than smaller parts of a cell; however, they simply contain more fluorochromes due to the larger volume. Physiologically relevant changes in ion concentration in small areas such as growth cones of a neuron may then not be visible over time because they are too dim in fluorescence compared to the bright centre of the cell. After background subtraction, the calculation of a ratio between

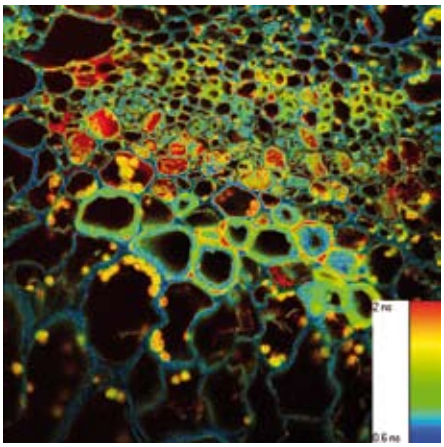


Fig. 50: Autofluorescence of an apple slice (Lieder). The image was taken with an Olympus FluoView FV1000 and the TCSPC by PicoQuant. The colour bar in the lower right corner is a key, showing the distribution of the various lifetimes within the image.

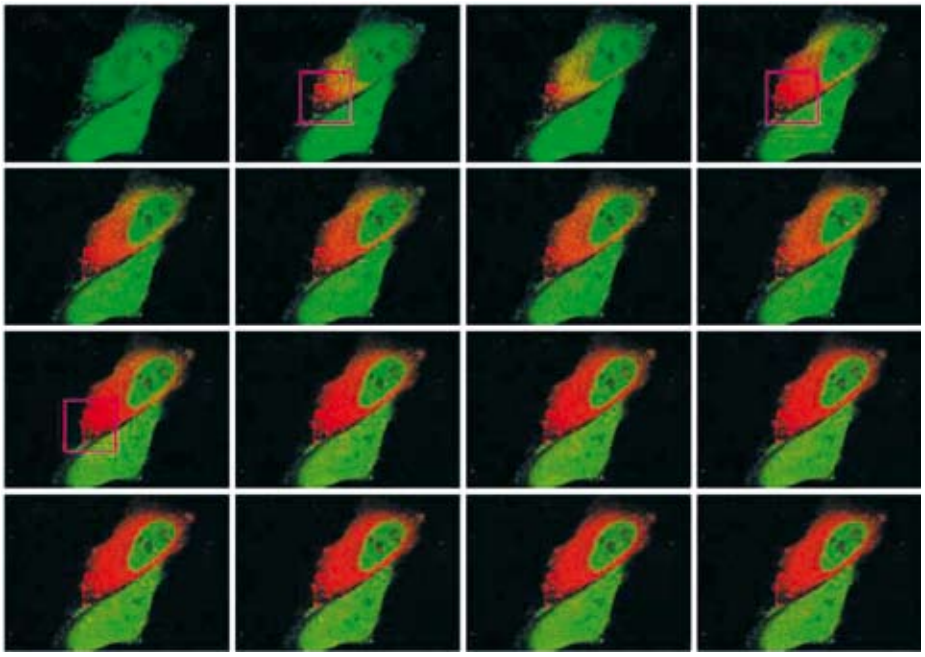


Fig. 51: Photoconversion: In Miyawaki's lab, Ando et al [1] succeeded in cloning the gene that encodes the Kaede fluorescence protein of a coral. By irradiation with UV light, the green fluorescence can be converted to red fluorescence. By using excitation wavelengths in the blue and green spectral range, the movement and distribution of the protein can be observed in a living cell. The figure shows the protein diffusion in Kaede-expressing HeLa cells. Stimulation via 405 nm laser in the region of interest converts the Kaede protein from green to red emission. Confocal images were recorded every three seconds using 488/543 nm laser excitation, showing the activated Kaede protein spreading throughout the HeLa cell. By using two synchronised scanners (one for photo-conversion, one for image acquisition), fluorescence changes that occur during stimulation can be observed.
Data courtesy of: R. Ando, Dr A. Miyawaki, RIKEN Brain Science Institute Laboratory for Cell Function Dynamics.

two channels corrects the result for overall, artificial fluctuations and specimen thickness. Following a calibration procedure, even quantitative results are obtainable. There is a range of fluorochromes, with different spectral properties for numerous different ions, available on the market. Microscopical systems with fast switching filter wheels and real time control permit investigations even in the millisecond range.

Light as a ruler

Many processes in a cell are controlled by inter- and intra-actions of molecules: e.g. receptor-ligand interactions, enzyme-substrate reactions, folding/unfolding of molecules. Receptor-ligand interactions, for example, occur in the very close proximity of two proteins in the Angstrøm range. Colocalisation studies do not reveal interactions of molecules in the Angstrøm range because the spatial resolution of a light microscope is limited to 200 nm. When using a light microscope, how can the proximity of two molecules in the Angstrøm range be proven beyond the physical limits of light microscopy? Fluorescence Resonance Energy Transfer (FRET) helps to find an answer to this question. FRET is a non-radiative energy transfer between two different fluorophores. The first fluorophore (the donor) is excited by light. The donor transfers its energy to the second fluorophore (the acceptor) without radiation, meaning without any emission of photons. As a result, the acceptor is excited by the donor and shows fluorescence ("sensitised emission"). The donor is quenched and does not show any fluorescence. This radiation-free energy transfer occurs within the very limited range of 1–10 nm distances between the donor and the acceptor. A positive FRET signal provides information about the distance between the FRET partners and can be quantified as FRET efficiency. When no FRET signal is achieved, there may be many reasons for that: e.g. too much distance between the FRET partners, insufficient steric orientation, insufficient dipole orientation, insufficient spectral overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor. See box 15.

How long does a fluorochrome live? – count the photons!

When a fluorochrome is excited, it is shifted to a higher energy level. The lifetime of a fluorophore is the average amount of time (in the nanosecond/picosecond range) that it remains at the higher energy level before it returns to the ground state. A fluorochrome's lifetime is a highly specific parameter for that particular fluorochrome. It can be influenced easily by changes of environmental parameters (e.g. pH, ion concentration, etc.), by the rate of energy transfer (FRET) or by interaction of the fluorochrome with quenching agents. Fluorochromes often have similar or identical spectral properties, therefore, analysing a fluorochrome's lifetime is critical to distinguishing the localisation of those fluorochromes in a cell (fig. 50). Here, the different experimental setups are referred to as FLIM – Fluorescence Lifetime Imaging Microscopy.

Fluorescence Lifetime Imaging Microscopy – FLIM.

There are different techniques for fluorescence lifetime imaging microscopy available on the market, for both wide-field and confocal microscopy. Here we focus on Time-Correlated Single Photon Counting (TCSPC). The fluorochrome is excited by a laser pulse emitted by a pulsed laser diode or femto-second pulsed Ti:Sa laser. A photon-counting photo- multiplier or single photon avalanche diode detects the photons emitted from the fluorophore. The time between the laser pulse and the detection of a photon is measured. A histogram accumulates the photons corresponding to the relative time between laser pulse and detection signal. Every pixel of the FLIM image contains the information of a complete fluorescence decay curve. If an image is composed of three fluorochromes with different lifetimes, distribution of all

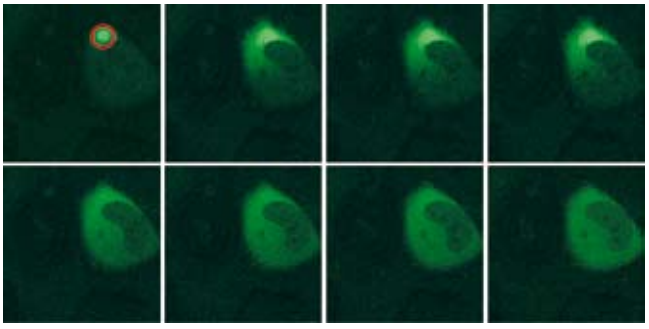


Fig. 52: Photoactivation of PA-GFP expressing HeLa cells. The PA-GFP was activated by 405 diode laser (ROI) and images of the PA-GFP distribution within the cell were acquired at 1 second intervals using the 488nm laser.
Data courtesy: A. Miyawaki, T. Nagai, T. Miyauchi, RIKEN Brain Science Institute Laboratory for Cell Function Dynamics.

dyes can be shown as three different colours (fig. 50).

Time-resolved FRET microscopy

With FRET, the lifetime of the donor depends on the presence or absence of radiation-free energy transfer (see above). Therefore, time-resolved FRET microscopy is a technical approach for quantitative measurement of the FRET efficiency.

FRAP, FLIP and FLAP

FRAP, FLIP and FLAP are all photobleaching techniques. By using the laser scanner of a confocal microscope, fluorochromes (which are bound to a specific protein, for example) in a selected region of a stained cell can be bleached (destroyed). As a result, the fluorochrome does not show any appropriate fluorescence. Other proteins that are also labelled, but where the fluorescence was not bleached, can now be observed during movement into the previously bleached area. Dynamic processes, such as active transport or passive diffusion in a living cell cause this movement. Therefore the intensity of the fluorochrome recovers in the bleached area of the cell.

FRAP = Fluorescence Recovery After Photobleaching

After bleaching of the fluorochrome in a selected region of a cell, a series of images of this cell is acquired over time. New unbleached fluorochromes diffuse or are transported to the selected area. As a result, a recovery of the fluorescence signal can be observed and measured. After correction for the overall bleaching by image acquisition, a curve of the fluorescence recovery is obtained.

FLIP = Fluorescence Loss In Photobleaching

The fluorophore in a small region of a cell is continuously bleached. By movement of unbleached fluorophores from outside of the selected region into the bleaching area, the concentration of the fluorophore decreases in other parts of the cell. Measuring the intensity loss, outside the bleached area results in a decay curve. A confocal laser scanning microscope which incorporates one scanner for observation and one scanner for light stimulation is especially appropriate for this technique. A simultaneous scanner system allows image acquisition during continuous bleaching.

FLAP = Fluorescence Localization After Photobleaching

The protein of interest is labelled with two different fluorochromes. One is bleached, the other remains undamaged and acts as a reference label. The population of bleached fluorochromes can be identified by subtraction of the image of the bleached dye from the image of the unbleached dye.

FAUD = Fluorescence Application Under Development!

We are looking for inventors of new fluorescence techniques – please keep in touch!

From black to green, from green to red …

If we sunbathe for too long, the colour of our skin changes from white to red and is referred to as sunburn. If we take a UV laser and irradiate a cell with Kaede protein, the colour of the Kaede protein changes from green to red – and we call this photo-conversion (fig. 51). A confocal microscope can be used to stimulate fluorochromes in selected parts of the cell. The Olympus FluoView FV1000 even offers stimulation of one area with one laser whilst observing the result in a different channel with a second laser simultaneously. A recently developed photoactivatable GFP mutant, PA-GFP, can be activated by irradiation using a 405 nm diode laser (fig. 52). The intense irradiation enhances the intensity of the fluorescence signal of PA-GFP by 100 times. PA-GFP is a powerful tool for tracking protein dynamics within a living cell (fig. 52).

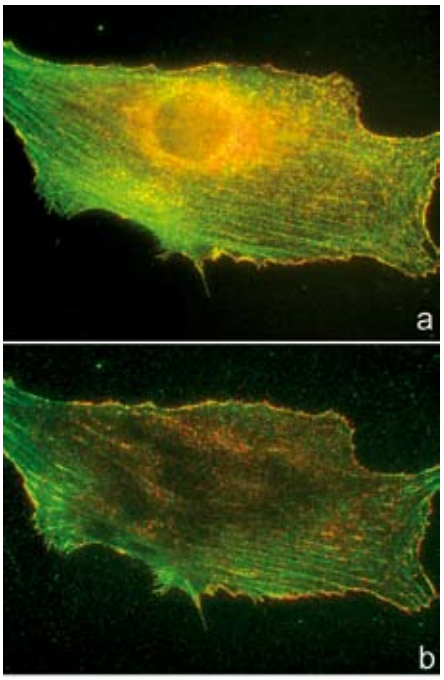


Fig. 53: Imaging at the Outer Limits of Resolution.
(a) Dual emission widefield
(b) TIRF images acquired with a 488nm laser
green Fitc-Actin
red DyeMer 605-EPS8.

Courtesy of M. Faretta, Eup. Inst. Oncology, Dept. of Experimental Oncology, Flow Cytometry and Imaging Core, Milan, Italy.

Imaging at the upper limits of resolution

The bleaching and photoactivation techniques described above can be undertaken easily using a suitable confocal laser scanning microscope. The laser is a much more powerful light source than a fluorescence burner based on mercury, xenon or metal halide. The galvano mirror scanners, in combination with acousto-optically tuneable filters (AOTF), permit concentration of the laser onto one or more selected regions of the cell without exciting any other areas in that cell. This makes the laser scanning microscope the preferred tool for techniques which use light as a tool. Additionally, the confocal principle removes out-of-focus blur from the image, which results in an optical section specifically for that focal plane. This means that high resolution along the x, y and z axes can be obtained. A confocal microscope is an ideal system solution for researchers who want to use light not only for imaging, but also as a tool for manipulating fluorochromes.

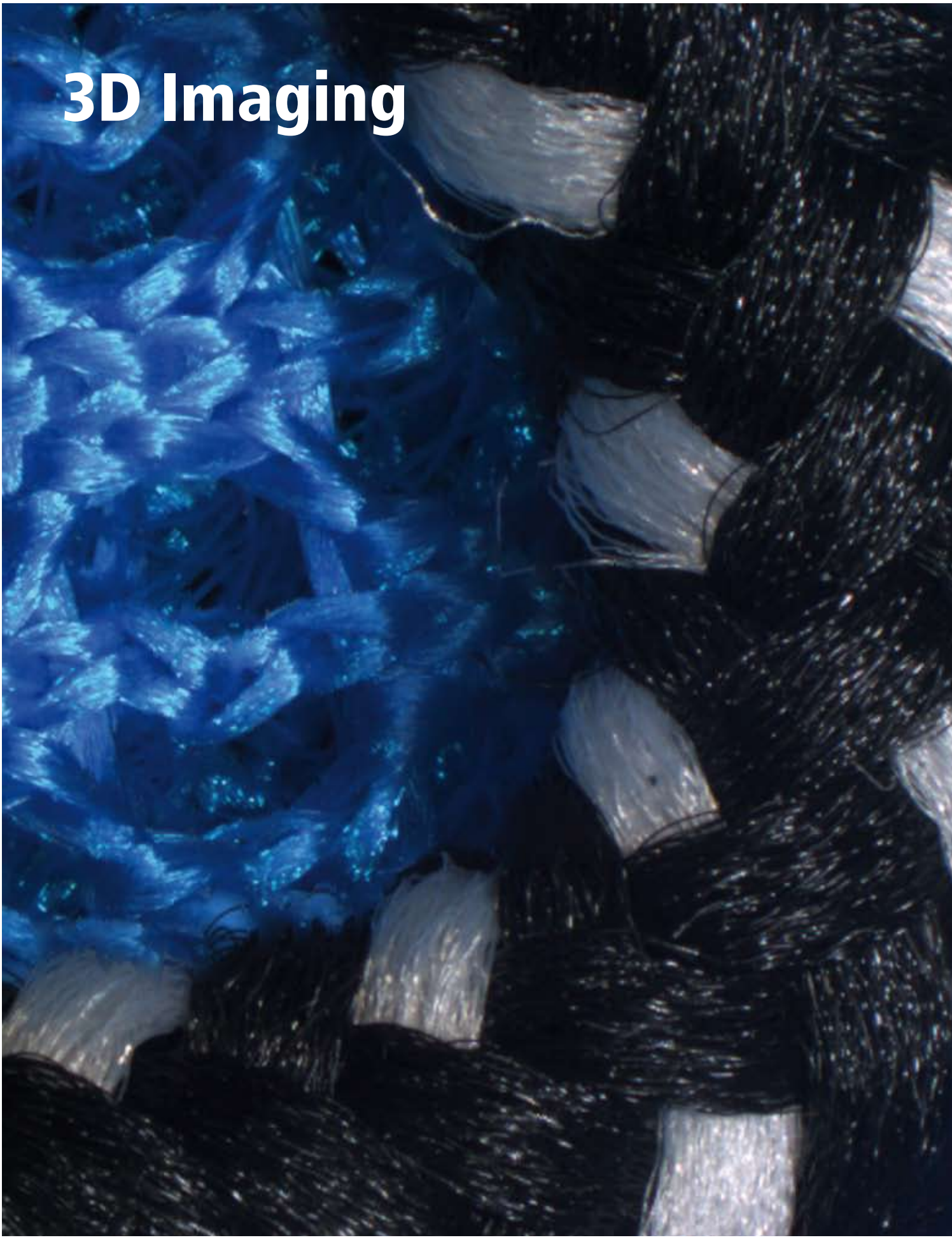
To achieve an even thinner optical section (for a single thin section only and not a 3-D stack), Total Internal Reflection Microscopy (TIRFM) may be the technique of choice (for more information on TIRFM, see box 4 or at www.olympusmicro.com/primer/techniques/fluorescence/tirf/tirfhome.html). The evanescent field used for this technique excites only fluorochromes which are located very closely to the coverslip (approx. 100–200 nm). Fluorochromes located more deeply within the cell are not excited (fig. 53). This means that images of labelled structures of membranes, fusion of labelled vesicles with the plasma-membrane or single molecule interactions can be achieved with high z resolution (200 nm or better, depending on the evanescent field).

References

[1] R. Ando, H. Hama, M. Yamamoto-Hino, H. Mizuno, A. Miyawaki , PNAS Vol. 99, No. 20, 12651–12656 (2002)

[2] G. H. Patterson, J. Lippincott-Schwartz, Science, Vol 297, Issue 5588, 1873–1877 (2002)

3D Imaging



Reality is multi-dimensional

What do we see when we observe a sample through a light microscope? What information do we perceive? Most light microscopes give us a two-dimensional view of physical reality. What we usually observe in the widefield microscope is the projection of a three-dimensional physical structure down to a two-dimensional image. This means one dimension is lost which significantly restricts our perception of the physical reality viewed.

When looking at two dimensional images we often reverse the process and, based on experience, more or less unconsciously extend the two dimensional view into the third dimension. But how can we be sure that this interpretation is accurate? While watching the night sky we cannot tell whether two stars are close to each other or are in fact hundreds of light years away in space. Painting techniques, however, offer numerous examples of how to create a distinct impression of a third dimension using different lighting and shading of contrast, as well as well-placed usage of objects with familiar proportions. The fact that we are accustomed to recognising different lighting on a structure in a three dimensional way is also how relief is shown – such as in contrast methods used in microscopy (e.g. Differential Interference Contrast (DIC)).

The stereo view

Our eyes always view objects from a slight angle defined by the distance of our eyes and the distance between the object and us. Together with the interpretation that our brain provides us with, this creates a three-dimensional view with these two sources of perception. To see objects within a microscope in a similar way, both eyes have to each be provided with a separate light path to enable observation of the specimen at an angle similar to the one our eyes see from naturally. This is exactly what stereo microscopy is all about. These microscope types, often called "binos", provide a view of a specimen that looks natural, including a high depth of field and working distance. With a convergence angle of 10–12°, the left eye and right eye receive views of the same object from a slightly different perspective (fig. 54).

For viewing surfaces, the stereo microscope provides a reliable three-dimensional impression of the object. We get an idea of how the surface is shaped and which structures are higher or lower. What we are investigating here is a curved, two-dimensional surface expanded into three dimensional space. However, one general restriction is still the limited depth of field which makes it often impossible to obtain a completely sharp image. Additionally, stereo microscopes are restricted in magnification and resolving power due to their general design when compared with upright or inverted widefield microscopes. Furthermore, when using a stereo microscope for documenting images, only one of the microscope pathways will be used for the camera in most cases. This means the resulting digital image is only two-dimensional – there is no third dimension.

Fig. 54: The stereomicroscopic (Galileo type) light path – two microscopes in one – offers observation of specimens at the natural conversion angle of our eyes. This makes the 3D topography visible. However, when the specimen is documented the image will nevertheless remain a two-dimensional view.

Thus, when we want to have a sharp view of a topographic structure in light or stereo microscopes – or in general, when we want to know more about the three-dimensional internal set-up of structures, several sections or slices have to be acquired. All at a different z position or on a different focal plane. Have a look at an image stack containing several such sections of the human head acquired via Magnetic Resonance Imaging (MRI) (fig. 55).

Not too few – Not too many – just enough

Using the light microscope, optical sections are usually acquired using a motorised focus. First the maximum and the minimum position along the z axis of the stage or the nosepiece are defined. Then you either set the distance of two z-positions (i.e. the focal change (Δz) or z spacing), or you set the total number of sections to acquire. Depending on the application, a minimum number of sec-

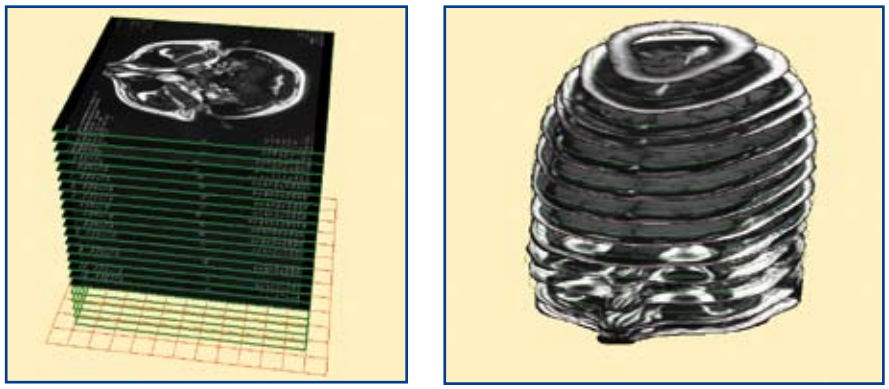


Fig. 55:
Left: Stack of images taken via MRI (Magnetic Resonance Imaging).
Right: The head structures are extracted from the individual image sections.

tions with the optimum z spacing (Δz) will be necessary.

One application in light and stereo microscopy is the acquisition of z stacks for overcoming the limited depths of field of a single frame. This is where the focal change (Δz) between two sections should be the same or somewhat smaller than the depth of field. As described above, the depth of field depends mainly on the numerical aperture of the objective. In practice, a usable Δz value can be easily derived by lifting the stage slowly. At the same time, you observe at which focal change a sharp structure goes out of focus. You apply this value to all the sections over the whole height range of your sample (fig. 56). With digital imaging, the focus parameters can be entered; the software takes control of the motorised focus and acquires the z stack. The focused areas through the sections can then be composed to generate an image which has an unlimited depth of field in principle (fig. 56). The method referred to is called EFI (Extended Focal Imaging). (See also box 6)

In addition to the resulting image (fig. 56) with an unlimited depth of field, a height map can be generated (fig. 57). The software algorithm knows which section to obtain the sharp pixels from to compose the resulting image. This information is used to create an image where each grey value represents a specific height. This height map contains as many grey value levels as sections have been acquired. This means that the smaller the depth of field of your objective and the more sections actually acquired, the more exact the height information will be. This is significant when the height information is represented in a three-dimensional view (fig. 57). When a sufficient number of sections are acquired, the viewer receives a clear spatial impression of the surface. To smoothen the height map, peaks and steps may be flattened somewhat. The three-dimensional view becomes quite a realistic representation of the actual object structure when the sharp, composite image is placed over the surface as a texture (fig. 57).

Just the right number

In life science applications and fluorescence microscopy, the appropriate number of sections for three-dimensional imaging can also be derived from the experimental set-up. It is well known that out-of-focus haze can significantly diminish fluorescence images' scientific value, as well as the apparent clarity in fluorescence images acquired in standard fluorescence widefield microscopes. There are two main techniques used to remove this out-of-focus blur and to restore the images: the deconvolution computational technique and confocal microscopy. One of these approaches is desirable to create a reasonable three-dimensional representation of the fluorescence signals.

Deconvolution requires a three-dimensional image stack of the observed sample, having a minimum focal change (Δz) for obtaining maximum effect. The focal change (Δz) depends on the numerical aperture (NA) of the objective, the sample fluorochrome's emission wavelength (λ) and the refractive index (n) of the immersion medium, and can be calculated using the following equation:

$$\Delta z \sim (1.4*\lambda*n)/NA^2 \qquad (7)$$

To get a better idea of the actual numbers, an example will be calculated. A Plan Fluorite 40x objective has an NA value of 0.75. The refractive index of air is 1. At 540 nm emission wavelength, this yields a minimum required focal change, i.e. z spacing (Δz) of about 1.3 µm. The numerical aperture mostly influences the z spacing. When using a higher quality Plan Apochromat objective with a numerical aperture (NA) of 0.9, a z spacing (Δz) of about 0.9 µm is required. When observing cells, a height range of 5 to 10 µm must be covered depending on cell type and preparation. So when using a Plan Apochromat 40x objective, up to 10 sections or frames should be acquired. Fig. 58 shows an example.

Software solutions available today offer either definition of the numerical aperture and the refractive index. With fully motorised systems, the software also reads out the numerical aperture and the refractive index of the current objective. When the fluorochrome is selected, the z spacing is automatically calculated. You define the maximum and

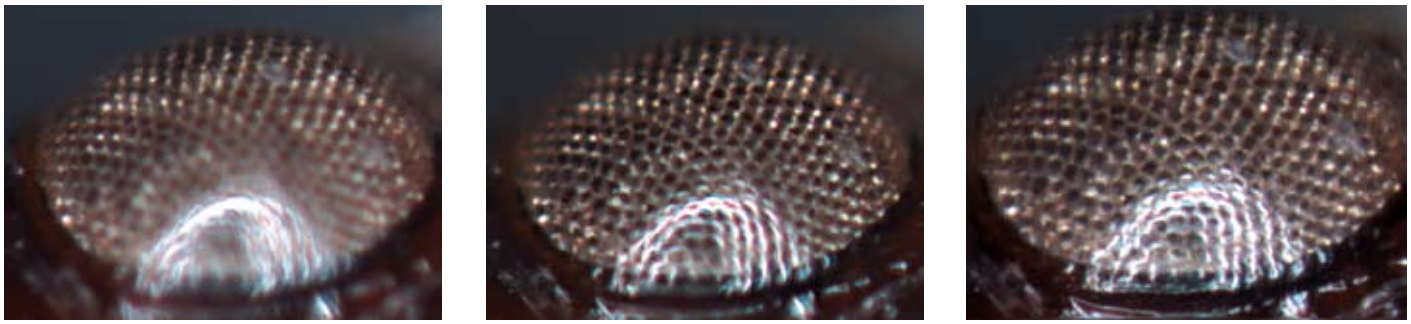


Fig. 56:
Left and middle: This is an eye of a beetle acquired at two different focal planes. The focal change (Δz) is such that no structure between these focal planes is not sharply in focus.
Right: Seven optical sections acquired at different focal planes are combined to create one sharp image with unlimited depth of field.

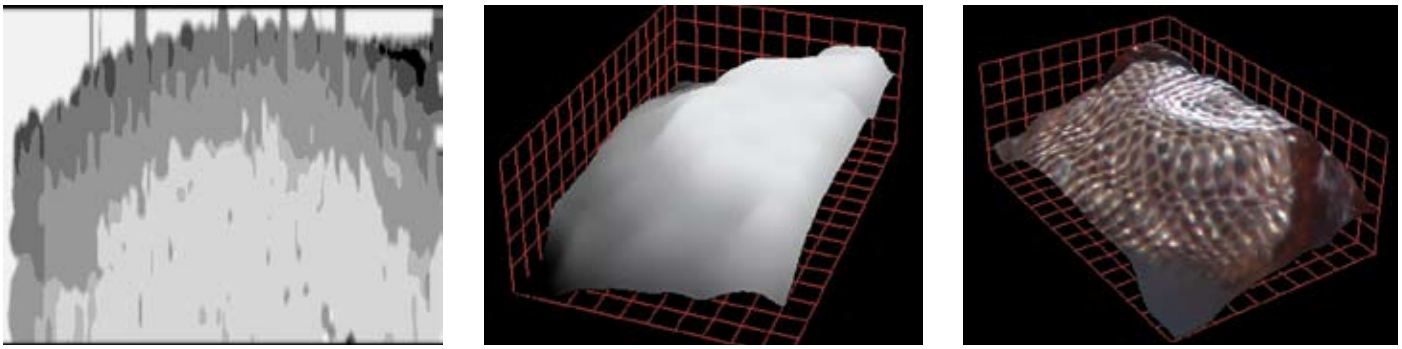


Fig. 57: Left: Height map. Middle and right: Three-dimensional view of the surface. The surface shape can be textured with the composite image.

the minimum lift of the stage and the z stack is acquired.

The deconvolution filter can be directly applied to the z stack image object. The most powerful deconvolution filter is called blind deconvolution. It does not assume any prior knowledge of the system or sample. Instead, it uses elaborate and time consuming mathematical iterative approaches. These include the constrained maximum likelihood estimation method to adapt to the actual three-dimensional point spread function (PSF) of the system (for a more detailed description see the paragraph „What exactly is a point spread function“ in the chapter „The Resolving Power“). Knowing the three-dimensional PSF of a system, the blind deconvolution takes the blurred z stack and essentially provides a three-dimensional image restoration of the sample (fig. 58, 60, 62). Deconvolution algorithms are applied to a lesser degree in confocal microscopy image stacks and to images from transmitted light widefield microscopy as well.

The confocal view

The second way to create optical sections in a fast and high resolution manner is via confocal microscopy. Here, a light beam, usually a laser, is concentrated on a small area of the specimen. The light originating from this spot (emission light of the fluorescence or reflected light of opaque materials) is captured by a sensitive detector. Within the confocal optics, an aperture (usually a pin hole) is placed at a position which is optically conjugated with the focusing position. This conjugated focal position set-up is why the term “confocal” is used (see also Box 4). The system is meant to eliminate light from all areas other than that on the focal plane. Therefore, while scanning along x, y axes, an optical section of the focused area can be obtained without requiring software algorithms.

One for all

Today‘s software generally handles the z stack as one object. All sections and frames are saved in a single file. The information about the single frames’ x-y resolution and calibration, as well as the z spacing and the other meta-data such as the channel (fluorochrome and wavelength), point in time acquired, numerical aperture and refractive index are saved in the same file as well. The single layers can be viewed separately or extracted. A quick way to display all the significant structures through the whole stack is the maximum intensity projection. This is where the brightest pixel value at each x-y position throughout all layers is shown in the projection image (fig. 58, 60, 62). Other projection methods are mean intensity and minimum intensity projections.

Projection methods have to be used with care because the lack of out-of-focus blur shows all details from the various z-levels as if they were all located on the same horizontal plane – which is obviously not the case. For readers viewing such images in a scientific journal, a seemingly satisfactory multicolour confocal two-dimensional image will in fact reveal less valid information than a “normal” widefield fluorescent image. Another way of presenting stacks is by animating them – like watching a movie. Image objects are truly multi-dimensional. In addition to the third axis in space, they may also contain different colour channels of multiply labelled fluorescent specimens – the fourth dimension (fig. 59, 60 and 62). Image objects may also be comprised of a fifth dimension – time: where slices are acquired at different points in time.

It‘s all spatial

Z stacks and multidimensional images can be visualised as three-dimensional objects via three-dimensional volume rendering, or voxel viewing. Voxel stands for Volume Element. Fig. 61 shows the correspondence between pixel and voxel. A voxel is a pixel which has a height value additionally assigned to it. The height assigned corresponds to the z spacing of the frames. In this way, voxels are reconstructed from the frames of the z stack.

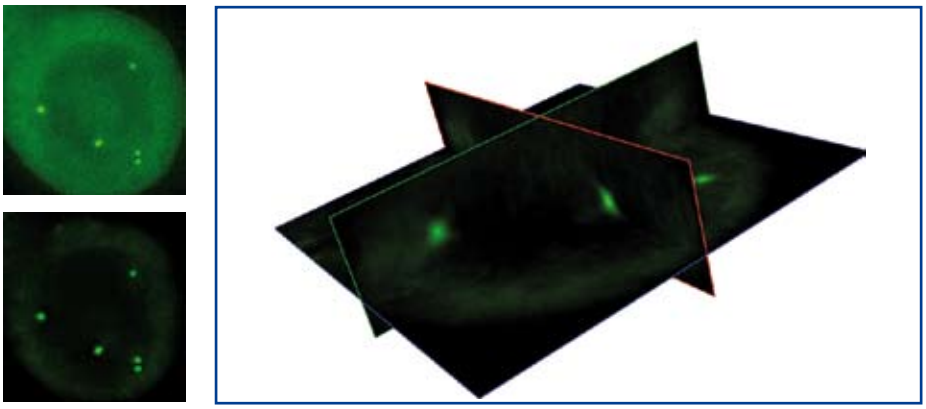


Fig. 58: Human breast cancer tissue labelled with a probe against the HER2-gene (Texas Red – not shown here, see fig. 62) and Chromosome 17 (FITC).
Uppler left: Maximum intensity projection of 25 frames, λ(FITC) = 518 nm, numerical aperture NA = 1, z spacing (Δz) = 0.18.
Lower Left: The same z stack after applying the blind deconvolution algorithm. Right: The three-dimensional slice view gives a good impression of the proportions and helps to spatially locate the fluorescence signals.

Each voxel of a three-dimensional object has a colour. There are several well-known methods for volume rendering, such as projection and ray casting. These approaches project the three-dimensional object onto the two-dimensional viewing plane. The correct volume generating method guarantees that more than just the outer surface is shown and that the three-dimensional object is not displayed simply as a massive, contourless block. Inner structures within a three-dimensional object, such as fluorescence signals can be visualised. The maximum intensity projection method looks for the brightest voxel along the projection line and displays only this voxel in the two-dimensional view. Fig. 62 shows the maximum intensity projection of two colour channels of 25 frames each when seen from two different angles. Changing the perspective helps to clearly locate the fluorescence signals spatially. The three-dimensional structure will appear more realistic when the structure rendered into three-dimensions is rotated smoothly.

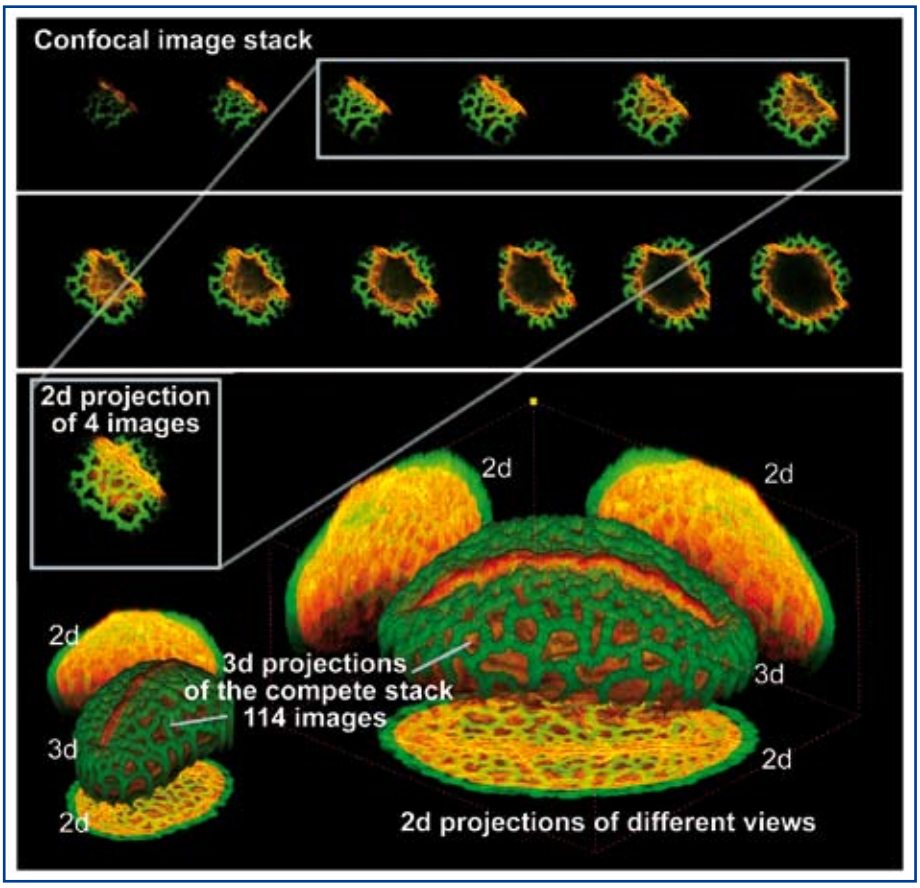


Fig. 59: Confocal images of pollen. The upper rows show the first 12 images of a series of 114, that can be used to create either two-dimensional projections of parts of the pollen or create a 3D view of the surface structure. This three-dimensional projection shown here is accompanied by two-dimensional projections as if the pollen was being viewed from different perspectives. Images were created using the Olympus Fluoview 1000.

How many are there – One or Two?

Managing several colour channels (each having many z layers) within one image object can be deceptive. The question is whether two structures appearing to overlap are actually located at the same position spatially or whether one is in fact behind the other. The thing is, two fluorescence signals may overlap – because their mutual positions are close to each other within the specimen. This phenomenon is called colocalisation. It is encountered when multi-labelled molecules bind to targets that are found in very close or spatially identical locations.

Volume rendering makes it possible to locate colocalised structures in space visually. For better representation and in order to measure colocalisation in digital image objects, the different fluorescence signals are first distinguished from the background. This may be done via threshold setting in each of the colour channels. A new image object can be created with only those structures that were highlighted by the thresholds. The colocalised fluorescence signals are displayed in a different colour. Rendering these image objects clearly reveals the colocalised signals without any background disturbance. To obtain quantitative results, the area fraction of the colocalised signals compared to the total area of each of the two fluorescence signals can be calculated throughout all image sections (see table 4).

Table 4:

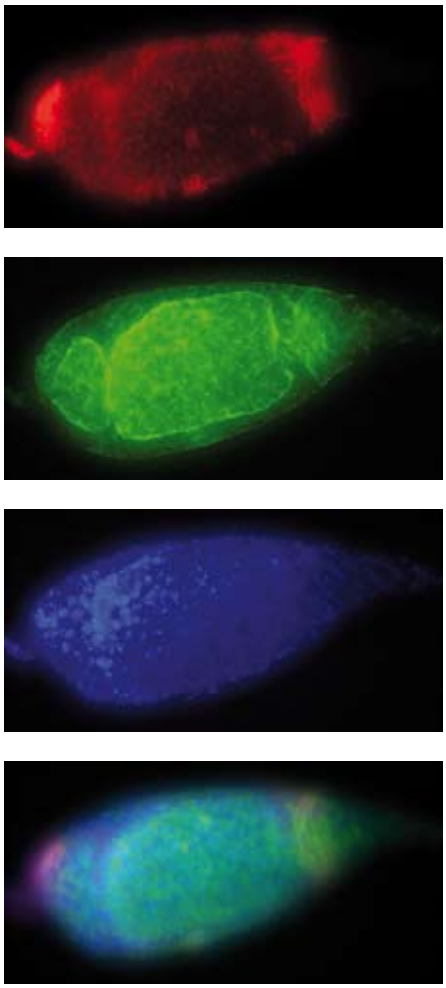
Layer	Red Area[%]	Green Area[%]	Colocalisation Area[%]	Colocalisation/Red Ratio[%]	Colocalisation/Green Ratio[%]
1.00	0.11	0.20	0.04	39.60	21.22
2.00	0.15	0.26	0.07	47.76	27.92
3.00	0.20	0.36	0.10	49.93	28.08
4.00	0.26	0.53	0.13	51.84	25.32
5.00	0.35	1.34	0.20	57.39	14.87
6.00	0.43	2.07	0.26	61.00	12.81
7.00	0.38	1.96	0.24	61.66	12.07

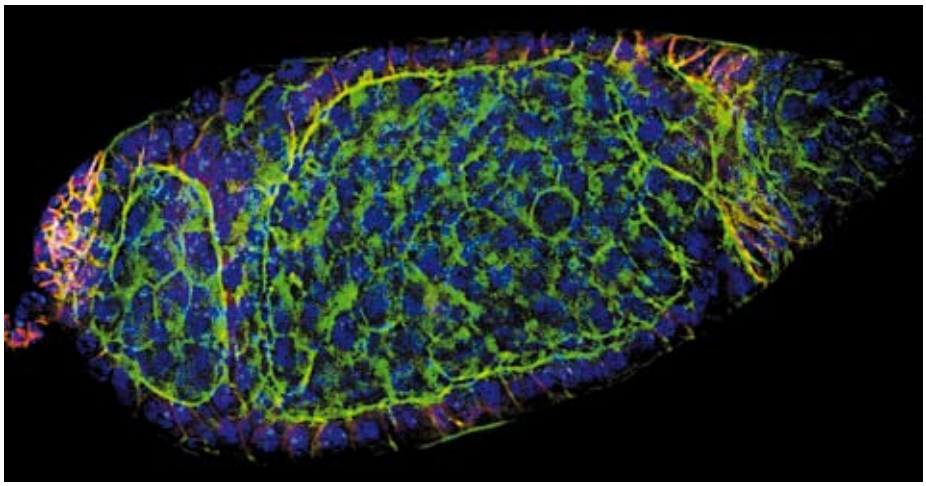
Colocalisation sheet: A ratio value of about 52 % in the Colocalisation/Red column in layer 4 means that 52 % of red fluorescence signals are colocalised with green fluorescent structures here.

3-D reconstructions

Now let us draw the net wider and move for a minute beyond the light microscopy approaches in imaging three-dimensional objects. In general. determining three-dimensional structures from macro to atomic level is a key focus for current biochemical research. Basic biological processes, such as DNA metabolism, photosynthesis or protein synthesis require the coordinated actions of a large number of components. Understanding the three-dimensional organisation of these components, as well as their detailed atomic structures, is crucial to being able to interpret what their function is.

In the materials science field, devices have to actually „see“ to be able to measure three-dimensional features within materials, no matter how this is done: whether structurally, mechanically, electrically or via performance. However, as the relevant structural units have now moved to dimensions less than a few hundred nanometres, obtaining this three-dimensional data means new methods have had to be developed in order to be able to display and analyse





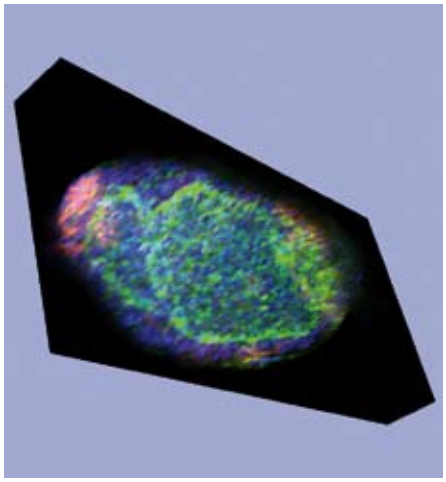


Fig. 60: Ovariole from Drosophila melanogaster mutant bgcn. Multicolour channel and z stack images can be managed in one image object. This image object contains three colour channels each consisting of a 41 frame z stack. This image is a maximum intensity projection.
Red: Fascilin III, Green: F-Actin, Blue: Nuclei.
Above right: Single z layer after applying the blind deconvolution algorithm to all three channels of the image object. Left: The three-dimensional, volume-rendered structure.
Image courtesy of: Dr. Ralph Rübsam, Institute for Biology, University of Erlangen-Nürnberg, Erlangen, Germany.

three-dimensional structures. Among these methods, different 3-D reconstruction approaches have become a useful tool in various applications in both the life and materials sciences. They result in three-dimensional displays which are particularly illustrative and instructive. Therefore, we now take a closer look at two of these 3-D reconstruction methods, one using an image series of specimen sections acquired in a light microscope, the second using electron tomography in a transmission electron microscope.

3-D Reconstruction using an image series of specimen sections

One commonly used method when dealing with 3-D reconstruction is to make a series of ultra-thin slices of the specimen being investigated. These slices are then dyed. The dye makes structures more easily recognisable and/or highlights specific parts. The slices are viewed one at a time beneath the microscope and drawn. These drawings are then enlarged to scale. In the past, a 3-D model was reconstructed layer by layer from pieces of cardboard cut according to the drawings. Alternatively, wax or plastic was used to make the model. Today, this is greatly simplified by the computer assembling the virtual model. Special 3-D programmes calculate the three-dimensional model based on digitised image slices. This model is referred to as a 3-D reconstruction.

Today's powerful software offers 3-D processing, display and evaluation of two-dimensional image information. Ergonomic functions and flexible project management provide the user with the ability to reconstruct even the most complex models, and allow easy access to spatial views of the interiors of such structures. Up until now, these interior views were extremely difficult to generate. Box 16 shows the necessary steps of 3-D reconstruction, such as those used for displaying embryonic growth processes in three dimensions. This approach is attractive for research conducted in other areas as well – such as the materials sciences.

3-D reconstruction by tilting the specimen

Tomography is another way of reconstructing the three dimensional (3-D) structure of a specimen from a series of two dimensional (2-D) micrographs. Some sample tomography applications include not only those for electron microscopy (called electron tomography) but also

* eucentric is derived from the Latin for well-centered. It implies, e.g., in a goniometer and especially in a specimen stage, that the specimen can be tilted without moving the field of view. This capability is highly desirable for microscope stages.

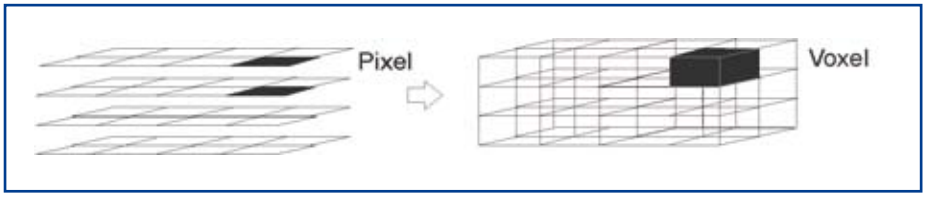


Fig. 61: This is how a two-dimensional pixel (picture element) is expanded to a three-dimensional voxel (volume element).

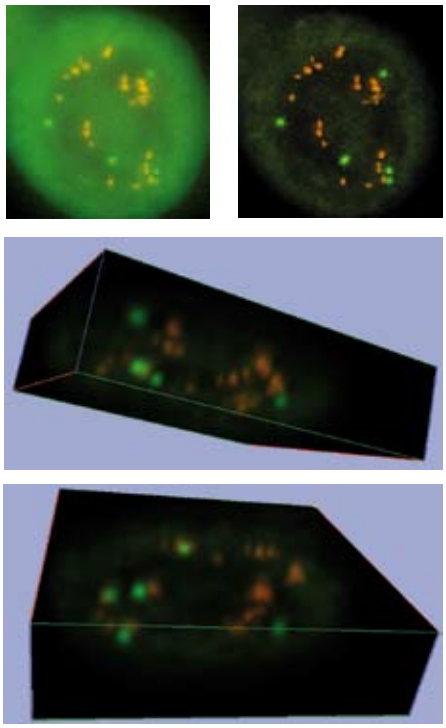
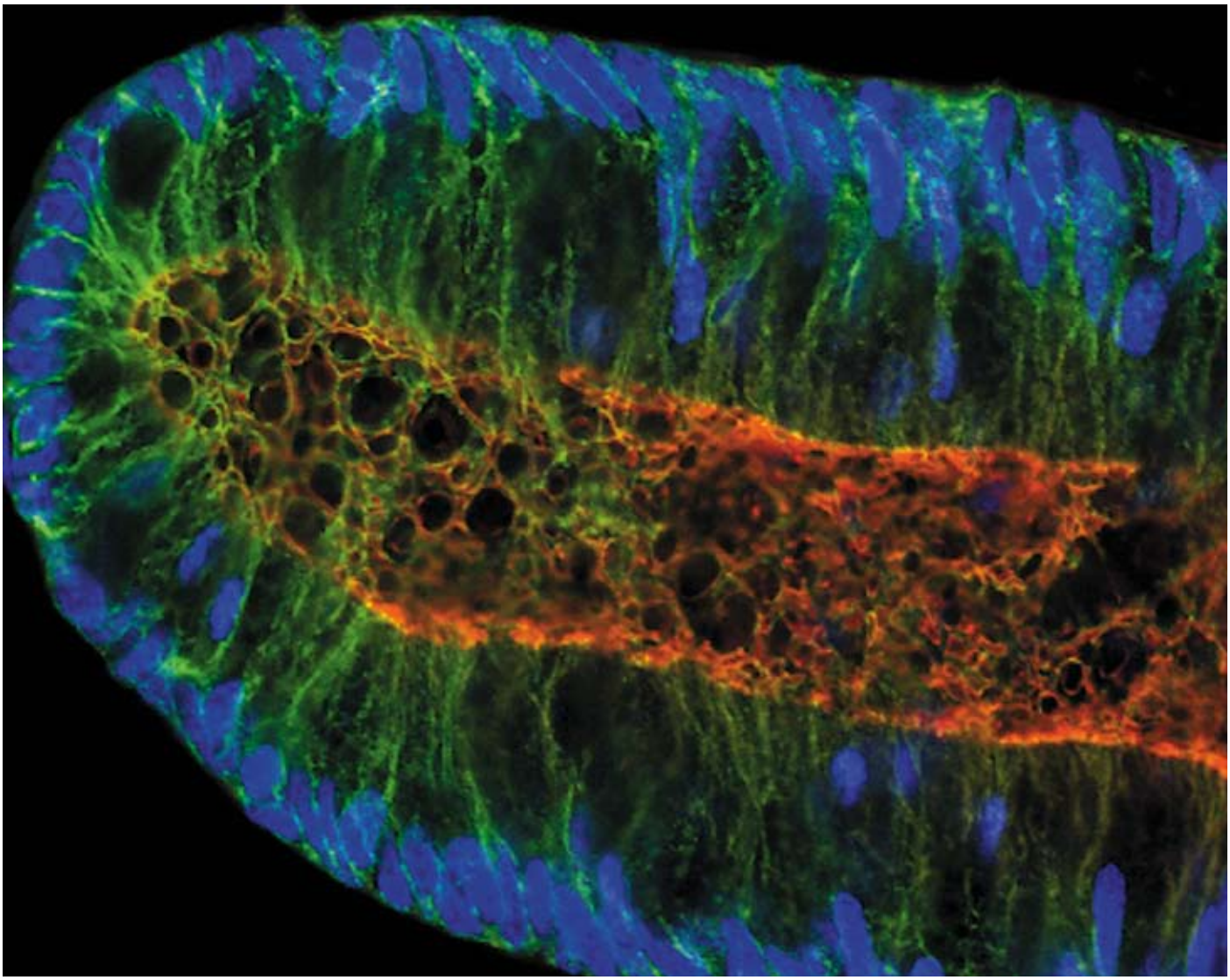


Fig. 62: Human breast cancer tissue labelled with a probe against the HER2-gene (Texas Red) and Chromosome 17 (FITC).
Upper left: Maximum intensity projection of 25 frames, λ(FITC) = 518 nm, numerical aperture (NA) = 1, z spacing (Δz) = 0.18.
Upper right: Blind deconvolution applied to both channels. Left: Maximum intensity projection. When the volume-rendered structure is seen from two different angles, the spatial composition of the fluorescence signals is shown.

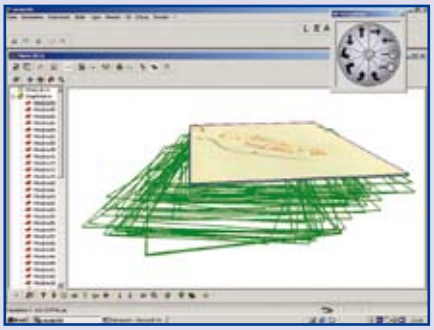
computerised axial tomography, (CAT) – familiar to many in medical applications as Computerised Tomography (CT).

Computer tomography has long been a part of the standard repertoire for routine medical applications. Transmission electron tomography, however, has grown increasingly important as well in the last few years. Transmission electron tomography made possible what had seemed impossible in biology: 3-D imaging of interior cellular structures, just nanometres in size. This has become a reality due to a combination of new technologies – suitably equipped electron microscopes, high resolving and highly sensitive CCD cameras, increased computer processor performance and improved software.

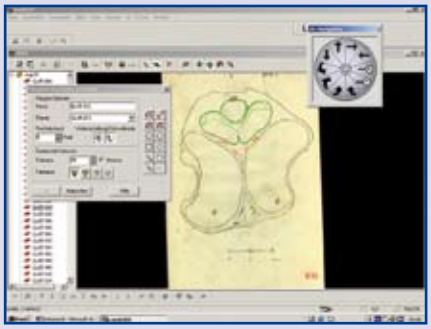
The way in which electron tomography works is actually quite straightforward (see box 17). The specimen holder is tilted along an eucentric* axis (if possible) and in the process, images are acquired from a wide range of angles. This guarantees that the 3-D structure will have adequate resolution along the three axes. Usually the specimen is tilted along a single axis from at least −60° to +60° with images (projections) recorded at equidistant increments of 1° or 2°. The images are acquired using a CCD camera and stored as a tilt stack. Due to effects such as sample shift, tilt of axis position or thermal expansion, the tilt stack has to be aligned before further processing can begin. Once the stack has been aligned, it is ready to be used for reconstruction. Reconstruction can be performed using different approaches. Two



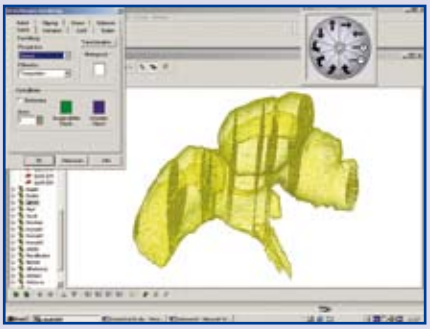
Box 16: The steps involved in 3-D reconstruction using an image series of specimen sections



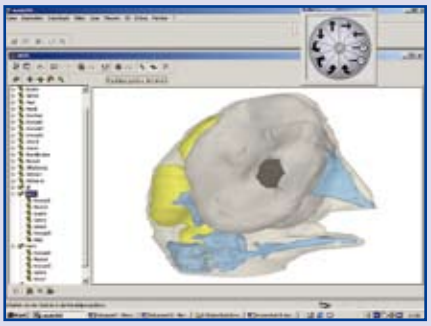
In the past, pieces of cardboard cut out by hand had to be glued together to make a 3-D model. Today this is all taken care of by the computer. First, the software digitally acquires microscope images of the series of image slices (or drawings are scanned into the computer, as in this case). The software then stacks these digitised slices. Any displacements or misalignments can be corrected interactively or semi-automatically.

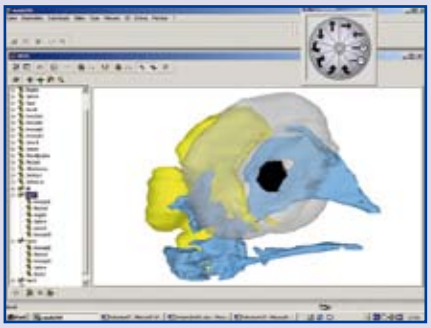


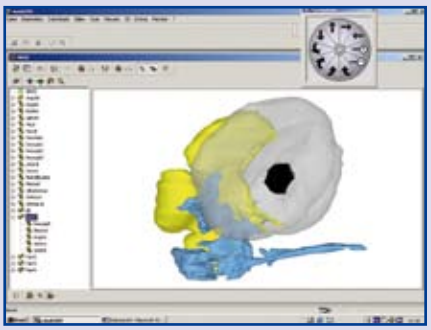
The contours of a tissue structure can be traced with the cursor or are automatically detected following a click of the ‚magic wand' tool (a local threshold approach). The software then reconstructs the contours as polygons. This is a scanned drawing done at a stereo microscope. The procedure is exactly the same when using digital microscope images.

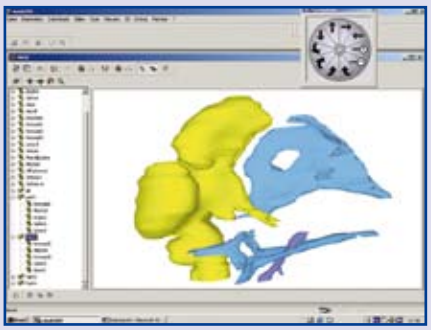


The software joins the two-dimensional polygons of the component layers to form spatial, three-dimensional structures. This is undertaken automatically. If necessary, manual corrections can be made. Using navigation tools, the object can be enlarged, reduced and rotated spatially in any direction.

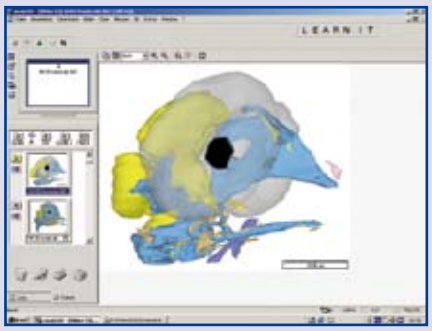




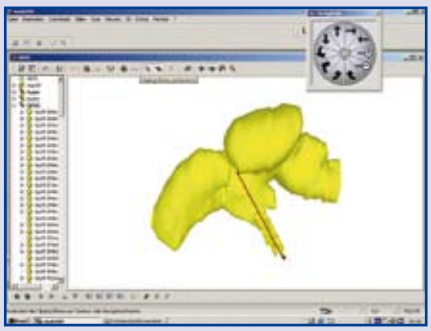




Virtual specimen preparation: the digitally reconstructed skull of a quail embryo can be reduced to its component parts at any time. Virtual slices can also be generated in any direction.



This is a complete reconstruction of the skull of a quail embryo. To be able to investigate and measure precisely it is extremely important to be able to look inside the model. This is achieved by making individual parts transparent or fading them from view entirely.



Distance, polygonal length, area, volume and absolute position can all be measured directly within the 3-D view.

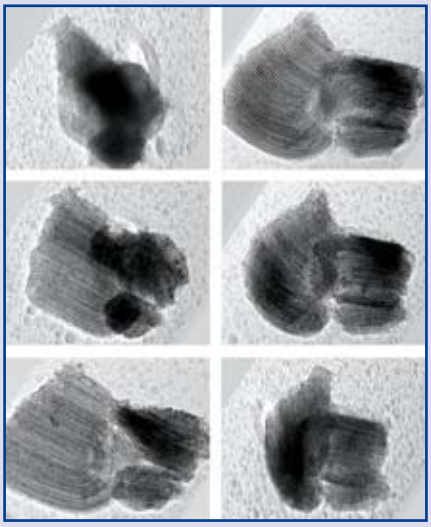
approaches available are called |w| filtering and (un-)weighted back projection via FFT. The resulting reconstructed image is stored and visualised as a z stack. Box 17 explains the steps necessary for the electron tomography process.

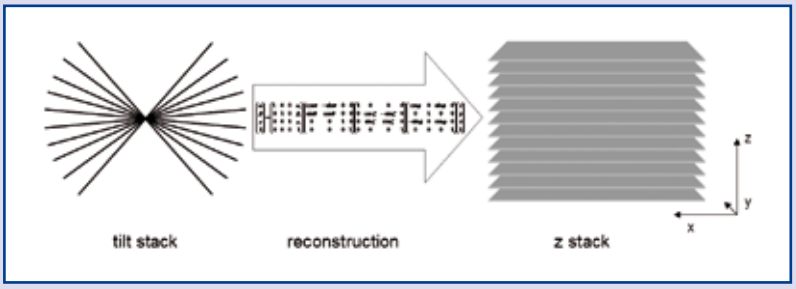
The more dimensions – the more there is to see

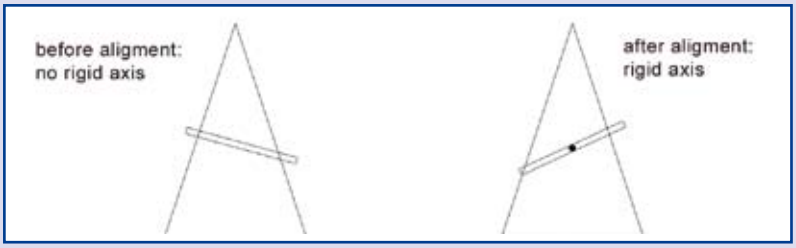
Many image-analysis methods, especially two- and three-dimensional approaches, have become well established and are applied on a daily basis in both industrial and research applications. Innovative procedures involving even more dimensions with applications in the Life Sciences have also become available and are certain to become just as well established in the foreseeable future.

Wavelength is viewed as a fourth dimension in the field of fluorescence microscopy, as described above. Being able to differentiate wavelengths provides additional structural information. We have seen many new applications using this technique. How the object being investigated changes over time is also of decisive importance in the Life Sciences. One particular reason for this is that this information may be crucial for assessing cell culture growth or for assessing how a fluorescent-labelled molecule behaves over time (see the paragraph „Fluorescence Lifetime Imaging Microscopy" in the chapter „Shining Fluorescence Details"). In addition, there is further information which can be combined with x, y information efficiently. One example is

Box 17: The steps involved in 3-D reconstruction via Electron Tomography (TEM).







Data collection for automated electron tomography involves a complex setup of TEM, CCD camera and a data gathering and processing system. Usually assembling the data would require a tilt of +/–90°. However, due to the geometry of the sample holder, or the thickness of the sample itself, the range of rotations is limited. This means that in practice, tilt angles ranging from –70° to +70° are recorded at equidistant increments. The image shows only 6 of at least 144 acquired images of the complete image stack. (Image courtesy by Oleg Abrosimov, University of Nowosibirsk, Russia.)

A key feature offered by automated electron tomography is automatic alignment of the tilt series. Various algorithms can be used. The rigid axis is calculated using information such as magnification, tilt range and increment. The images are then stored in an aligned tilt stack.

Special reconstruction routines are used to generate a z-stack based on the data contained by the tilt stack.

Using visualising tools, 3-dimensional views inside the specimen becomes possible. The sequences can be animated and stored as files in .AVI format. The image shows 4 different views clipped from one .avi file.

energy loss. Energy loss plays a key role in fluorescence applications such as FRET (see the paragraph „Light as a Ruler“ in the chapter „Shining Fluorescence Details“). It is also used in several electron microscopy methods. Let's have a look at two of these methods: X-Ray Microanalysis and Energy-Filtering Transmission Electron Microscopy (EFTEM).

Combining morphological and chemical data

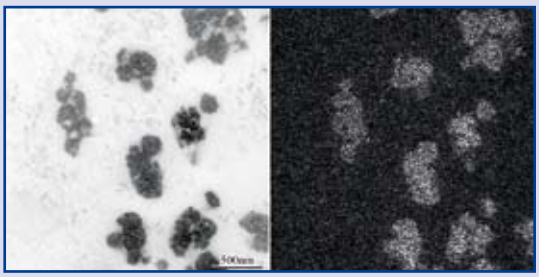
X-rays can be described as electromagnetic radiation with wavelengths ranging from 10^{-9} to 10^{-11} m or energy in the 10 eV – 100 keV range, respectively. X-rays are employed for element analysis in the field of biology, as well as in materials research. Characteristic X-rays and radiative deceleration of electrons within matter are recorded using special detectors. After correction for absorption, atomic weight and fluorescence, each sample section shows a characteristic spectrum. By scanning the sample and simultaneous recording of the X-ray signals, an element distribution image can be recorded and visualised. Using image evaluation of these acquisitions, additional information becomes available which has previously been unattainable. This technique is in widespread use and referred to as EDX (Energy Dispersive X-ray Analysis) (fig. 63).

Combining digital image analysis with X-ray microanalysis makes it feasible to study and characterise the properties of new materials. This involves examining macroscopic material properties in relation to the microstructure. One application where this is essential is for improving and developing aluminium alloys, products and fabrication processes. To obtain an analysis that is statistically-reliable and representative requires a large number of samples. A TEM (Transmission Electron Microscope) with an integrated scanning mode can be used for analysing particles within the 10–300 nm range. This ensures that local resolution along with sufficient electron intensity is as required. The software controls and automates the entire acquisition and analysis process. This includes simultaneous TEM and EDX analyser control, as well as analysis of the images acquired. The morphological data obtained within the context of the analysis is saved and displayed using tables and graphs. Subsequently, the automatic acquisition of the EDX spectra – at the centre of the particles being investigated – takes place. Data is also saved and quantified automatically. The proportion of the exudation volume can then be determined in conjunction with a layer-thickness measurement. These results can provide researchers with important micro-structural information (fig. 64).

Combining local data with energy loss data

The capacity for analysing multidimensional images is becoming more and more relevant. This includes image series of energy-filtering electron microscopy. EFTEM (energy-filtering TEM) image contrast occurs primarily due to the electron scattering within the sample – as is the case with the conventional TEM (box 18). Whereas conventional TEM

Box 18: How is an elemental map of iron generated?



EFTEM stands for Energy-Filtering Transmission Electron Microscope. The electron beam of the EFTEM is transmitted through the sample (here, an ultra-thin section of brain tissue) and generates a magnified image of the sample in the imaging plane. The built-in energy filter makes it possible to generate electron-spectroscopic images (ESI). If the energy filter is set to 725 eV, for example, only those electrons reach the imaging plane that have an energy loss of 725 eV (while travelling through the sample). All other electrons are blocked by the energy filter.

725 eV is the characteristic energy loss of an electron from the beam when it ‚shoots past' an iron atom at close proximity and causes a specific kind of inner-shell transition. The energy filter allows such electrons to pass through. These electrons contribute to image generation in the imaging plane. Unfortunately, other electrons manage to make it through as well. These also contribute to the image. These electrons have, however, coincidentally lost 725 eV – due to getting ‚knocked about', i.e., multiple impacts occurring while the electrons pass through the sample. Without these coincidental electrons, the ESI image at 725 eV would be a straightforward elemental map of iron. This means that the image would show how iron is distributed throughout the sample.

The elemental map can be calculated using multiple ESI images. Using what is known as the 3-window method, 3 ESI images are acquired. The first one is at 725 eV, the second and third ones at somewhat lower energy losses. The second and third images are used to compute a ‚background image'. This is then subtracted from the first image. This is how any background interference is removed from image number one (made up of all the contrast components caused by the coincidental electrons referred to above). The result is the desired elemental map of iron. This can now be used for making quantitative measurements.

Iron apparently plays a significant role in Parkinson's disease. Using an electron microscope, it can be shown how iron concentration has increased tremendously in the diseased part of the midbrain. The image on the left is an electron-spectroscopical image of an ultra-thin section taken from the substantia nigra** of a Parkinson's patient (inverted display). The dark areas represent the neuromelanin. The image on the right is the elemental map of iron computed using the 3-window method. The light areas show where iron has accumulated. The quantitative evaluation in the example shown above indicates a concentration of iron that is three times as high as what it would be in the brain of a healthy person. (A LEO 912 AB EFTEM was used (acceleration voltage of 120 kV, electron energy loss of 725 eV) along with analySIS TEM EsiVision image-analytical software. (by Prof. N. Nagai and Dr. N. Nagaoka))

**substantia nigra: is located in the midbrain and is a layer of large pigmented nerve cells. These cells produce dopamine and their destruction is associated with Parkinson's disease.

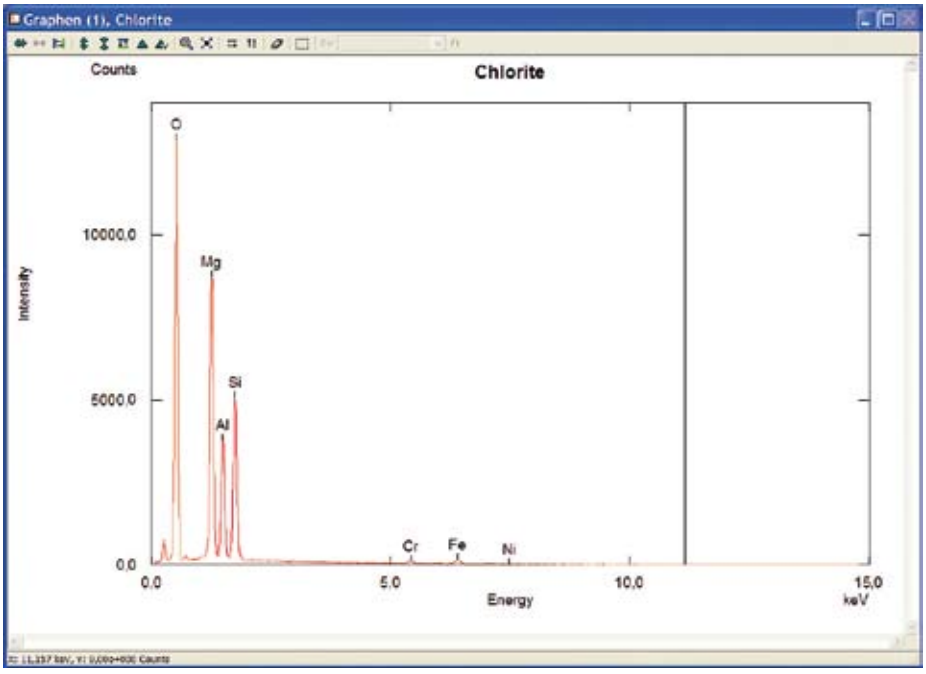


Fig. 63: Typical EDX spectrum.

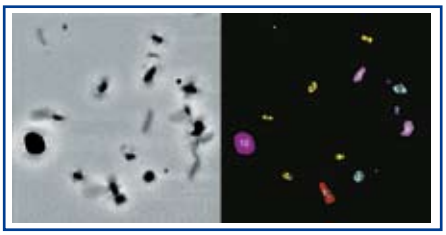


Fig. 64: a) Acquisition of an aluminium exudation, b) image following detection and classification. Chemical analysis is conducted following morphological evaluation.

contrast is caused by selecting the angles of the scattered electrons, the electrons within the EFTEM undergo an additional energy selection: using a spectrometer. Only electrons of a certain energy loss are selected (from the spectrum of the transmitted electrons) – the contrast-reducing portions are not visible. This means that only those electrons with a specific energy loss contribute to the acquisition. Thus the result offers enhanced contrast for all types of acquisition. Because this method does not affect local resolution, thin samples and those with no contrast, as well as frozen or unconventionally thick samples can be acquired with excellent contrast. Furthermore, this approach enables one to also select electrons with very specific scattering behaviour, thus yielding structural or element-specific contrast within the image. Images such as these provide new information on the sample that used to be inaccessible.

Control and remote control of the electron microscope for acquiring, displaying and analysing the images and spectrums is undertaken by special software. Much of the information can only be obtained via computer-supported processing and analysis. This method is used in the biomedical field, for example to demonstrate the presence of mineral particles in human lung tissue or when investigating diseases involving excessive accumulation of iron. This method has become greatly prevalent in the materials sciences as well: e.g., primary influences on the mechanical properties of steel – i.e., secondary phases, such as exudations or grain-boundary phases (carbide, nitride or also intermetallic phases). Determining the exact chemical composition, size, distribution and density is most important. This information can be obtained via the EFTEM method. Conventional methods are useless in this context.

Meet the Authors:

Dr. Rainer Wegerhoff

Born in 1964 in Hückeswagen near Cologne, Dr Wegerhoff studied Biology at Bonn University and completed his PhD dissertation in the field of developmental neurobiology in 1994. Working at the Biochemical Institute in Kiel from 1994 to 1999, he led a research group which focussed on the toxicological effects of insecticides on brain development.
Sience 1999, Dr Wegerhoff has worked for the European headquarters of Olympus in Hamburg, responsible for training (Olympus Akademie) and qualification in the field of microscopy. He is now Section Manager for Qualification – Olympus Life and Material Science Europa GmbH.

Dr. Olaf Weidlich

Born in 1964 in Hamm (Westfalen), Dr Weidlich studied Physics and Philosophy from 1983 to 1989 at the University of Münster (Westfalen), the focus of his diploma thesis was on electron microscopy. Studying for his PhD in Biophysics at the University of Freiburg (Breisgau) from 1991 to 1995, Dr Weidlich's thesis discussed FTIR spectroscopy on retinal proteins. Undertaking a postdoctoral position in 1996 at the University of Arizona in Tucson (USA), Dr Weidlich worked in the field of Raman spectroscopy on photosensitive proteins. He returned to Germany in 1997 to take up an intern lectureship in Physics and Chemistry in Essen (Nordrhein Westfalen).
In 1998, Dr Weidlich joined Soft Imaging System, now called Olympus Soft Imaging Solutions GmbH, as a Technical Documentation Expert and Trainer. He is now responsible for the customer courses (trainingCENTER) at the company.

Dr. Manfred Kässens

Born in 1964 in Haren (Niedersachsen), Dr Kässens studied Physics from 1986 to 1996 at the University in Münster (Westfalen). The focus of his diploma in Physics in 1993 was electron microscopy. Also undertaking his PhD in Physics at the University of Münster, he completed his PhD dissertation in the field of electron microscopy in 1996.
In 1996, Dr Kässens joined Soft Imaging System, now called Olympus Soft Imaging Solutions GmbH, as a Key Account Manager in Sales and Support. Since 1999, he has been Marketing Communications Manager at the company.

Acknowledgements:

We would like to thank the following people who have provided a significant contribution to this review:

Dr. Annett Burzlaff for co-authoring parts of the chapter on fluorescence.

Michael W. Davidson, (National High Magnetic Field Laboratory, Florida State University) for contributing many of the images. His websites have provided a huge pool of knowledge for the authors when drafting this review.

Esther Ahrent, (Section Manager Marketing Communication – Olympus Life and Material Science Europa) for her coordination during all steps of this project.

Pictures

Figures 1-10; 12; 17;44A; Box 13; Box 14: Data courtesy of Michael W. Davidson (National High Magnetic Field Laboratory, Florida State University).

Figure 48: Data courtesy of Dr Paulo Magalhaes and Prof. Dr Tullio Pozzan, University of Padua, Italy

Figure 51: Data courtesy of R. Ando, Dr A. Miyawaki, RIKEN Brain Science Institute Laboratory for Cell Function Dynamics, Wako, Saitama, Japan

Figure 52: Data courtesy of A. Miyawaki, T. Nagai, T. Miyauchi, RIKEN Brain Science Institute Laboratory for Cell Function Dynamics, Wako, Saitama, Japan

Figure 53: Data courtesy of M. Faretta, Eup. Inst. Oncology, Dept. of Experimental Oncology, Flow Cytometry and Imaging Core, Milan, Italy

Figure 60: Data courtesy of Dr. Ralph Rübsam, Institute for Biology, University of Erlangen-Nürnberg, Erlangen, Germany

Box 15, Figure 1: Data courtesy of H. Mizuno, A. Miyawaki, RIKEN Brain Science Institute Laboratory for Cell Function Dynamics, Wako, Saitama, Japan

Conclusion:

As is evident from this review, the combined fields of microscopy and imaging are extremely broad and there is much more that could have been discussed. For example, the subject of storage media has not been considered in great depth due to the rapid technology changes that occur within this area. Consequently, we have drawn on our expertise in microscopy and imaging to select the key basic information necessary to provide the reader with a solid foundation of knowledge within these ever expanding fields.

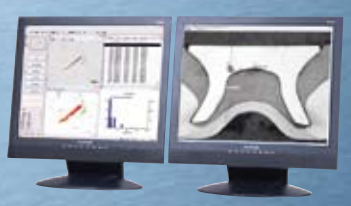
With the extraordinary rate of advance in technology in recent times, many new possibilities and opportunities are opening up for the application of microscopy and imaging. For example, the current escalation in use of digital microscopy has enabled the development of virtual microscopy which now allows entire slides be seen as one image from any location. As new techniques are developed, users are gaining an increasingly bigger picture which in turn delivers heightened understanding in many areas of application. Who knows exactly what the future will bring, but it's certainly looking bright for microscopy and imaging users ….

OLYMPUS

Your Vision, Our Future

INCREDIBLY SIMPLE.
YOU JUST HAVE TO THINK OF IT: OLYMPUS ANALYSIS IMAGING SOFTWARE.

At the end of the day, it's results that count. Anyone measuring and testing on a daily basis wants results directly, simply and quickly. And this is exactly what Olympus now offers with its analySIS imaging software for microscopic applications. Whether recording and analysing data or archiving and reporting, analySIS simplifies your daily procedures reliably, accurately and objectively. And the software is so user-friendly and intuitive that you feel at home with it straight away. Once configured for your application, analySIS delivers the results you need with just one mouse click. In fact, it's so simple that you will wonder why you didn't think of using it before. Olympus helps you solve problems easily.



For more information, contact:
Olympus Life and Material Science Europa GmbH
Phone: +49 40 2 37 73 54 26
E-mail: microscopy@olympus-europa.com
www.olympus-europa.com

O L Y M P U S M I C R O S C O P Y