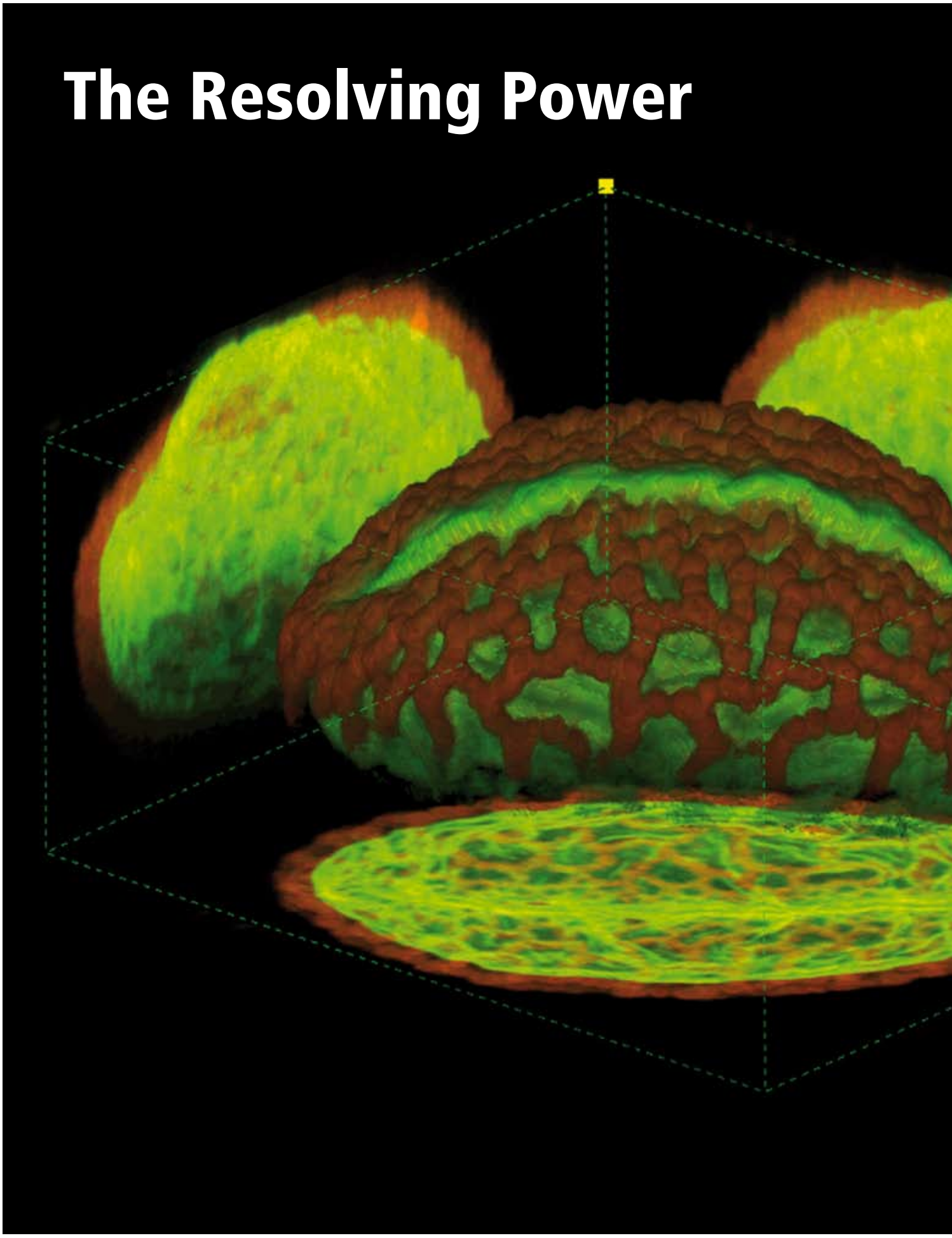
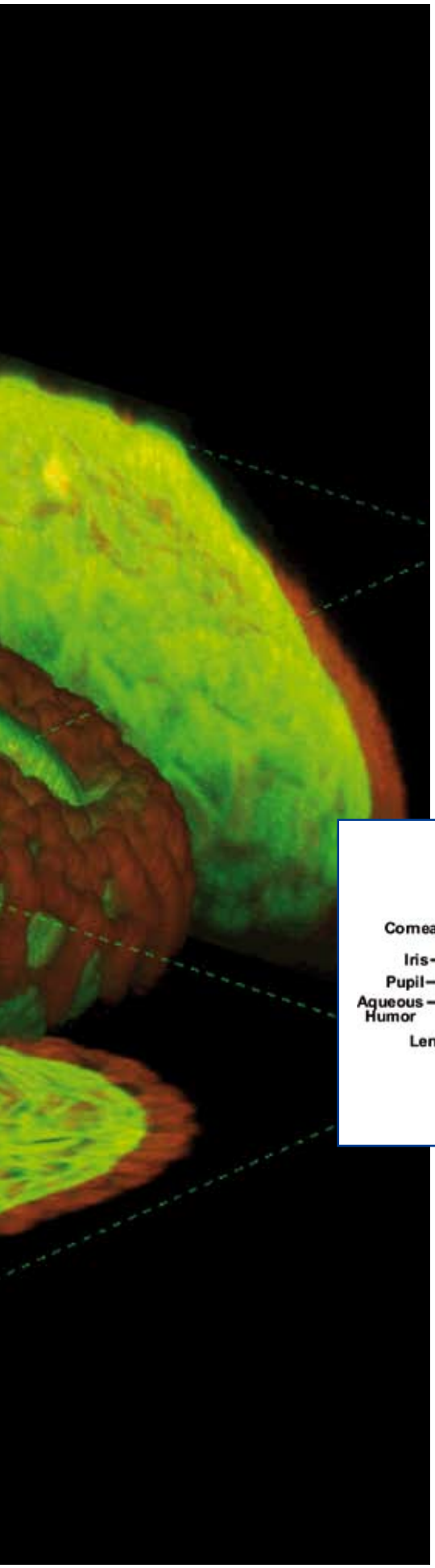
The Resolving Power





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

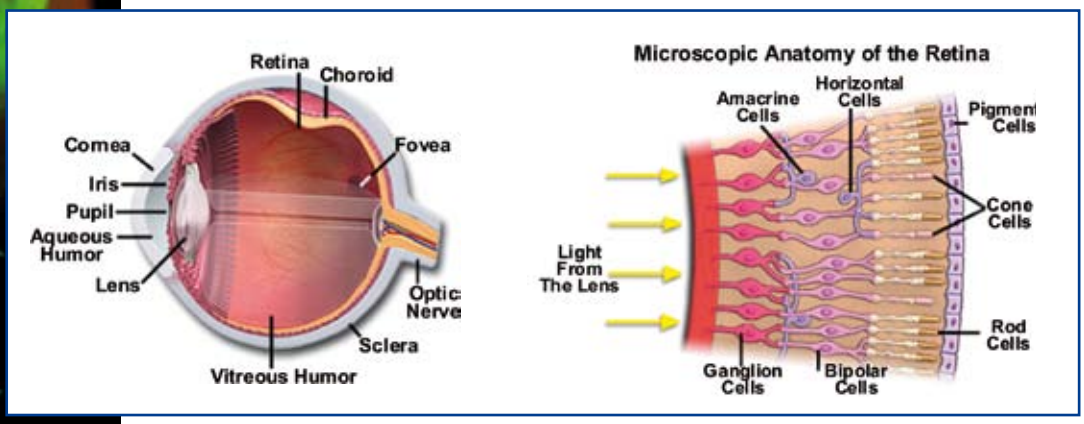


Fig. 12: Anatomy of the human eye and cross section of the retina.

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.

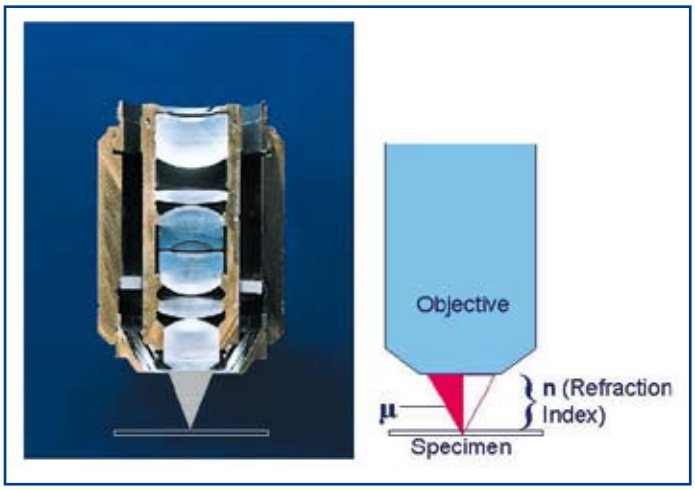


Fig. 13: Angular aperture of an objective.

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: 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).

Objective	Magnification	NA	Resolution	Lp/mm	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).

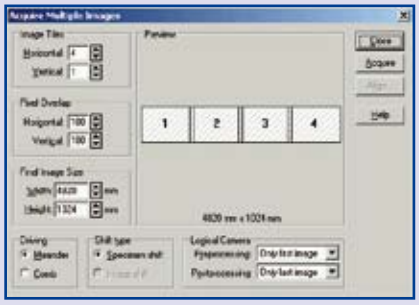
Box 3:

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.

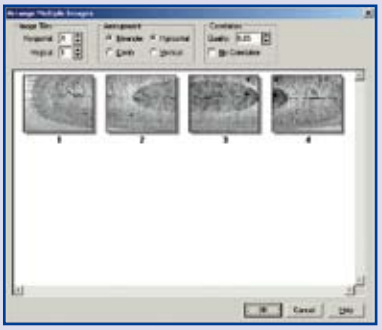
Here, the software takes control of the microscope, camera, motor stage, etc. All parameters are transferred to the imaging system through a remote interface. Using this data, the entire microscope and camera setups can be controlled and calibrated by the software. After defining the required image size 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-

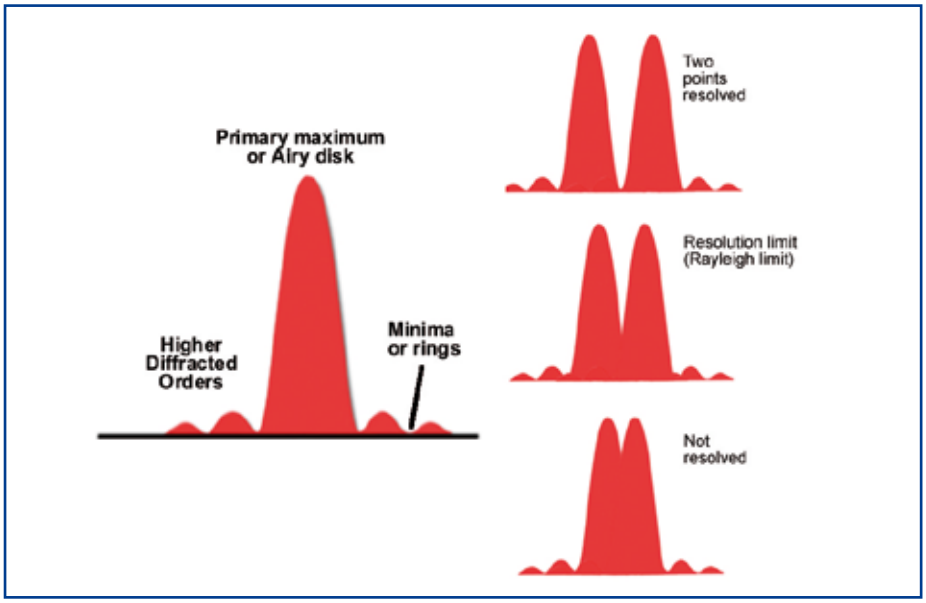


Fig. 15: Intensity profiles of the Airy disk patterns of one specimen detail and of two details at different distances.

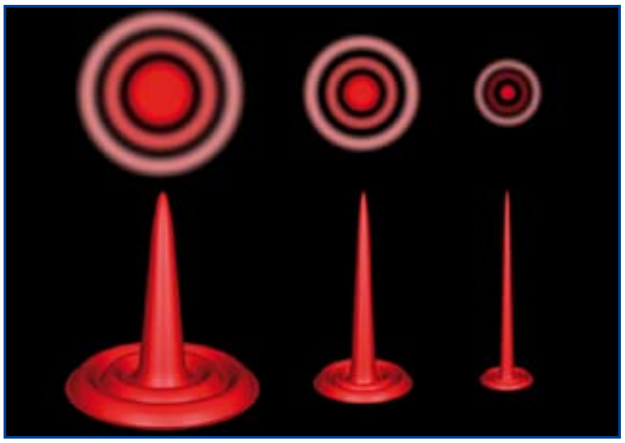


Fig. 16: Airy disk patterns of different size as an example of the resolving power for low NA (left) and high NA (right) objectives.

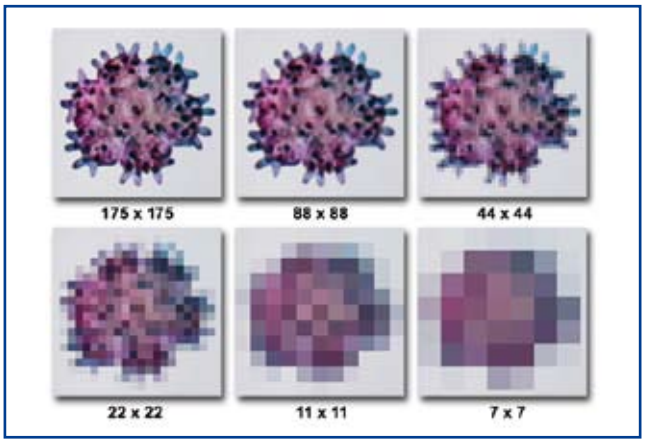


Fig. 17: Four representations of the same image, with different numbers of pixels used. The numbers of pixels is written below each image.

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

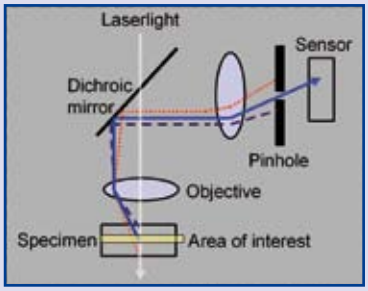
The xy-resolution desired is one aspect which makes an image 'high quality'. Another partially contradicting aspect is that we usually want to see the largest possible fraction of the sample – in best case the whole object under investigation. Here, the microscope's field of view becomes important. The 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).

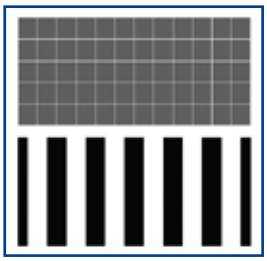


Fig. 18: Line pair pattern achieved with 2 pixels per line pair. Please refer to the text for the description.

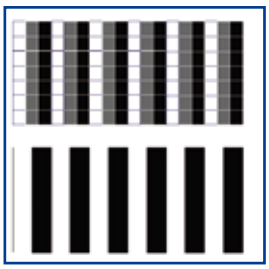


Fig. 19: Line pair pattern resolved with 3 pixels per line pair. Please refer to the text for the description.

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.

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-

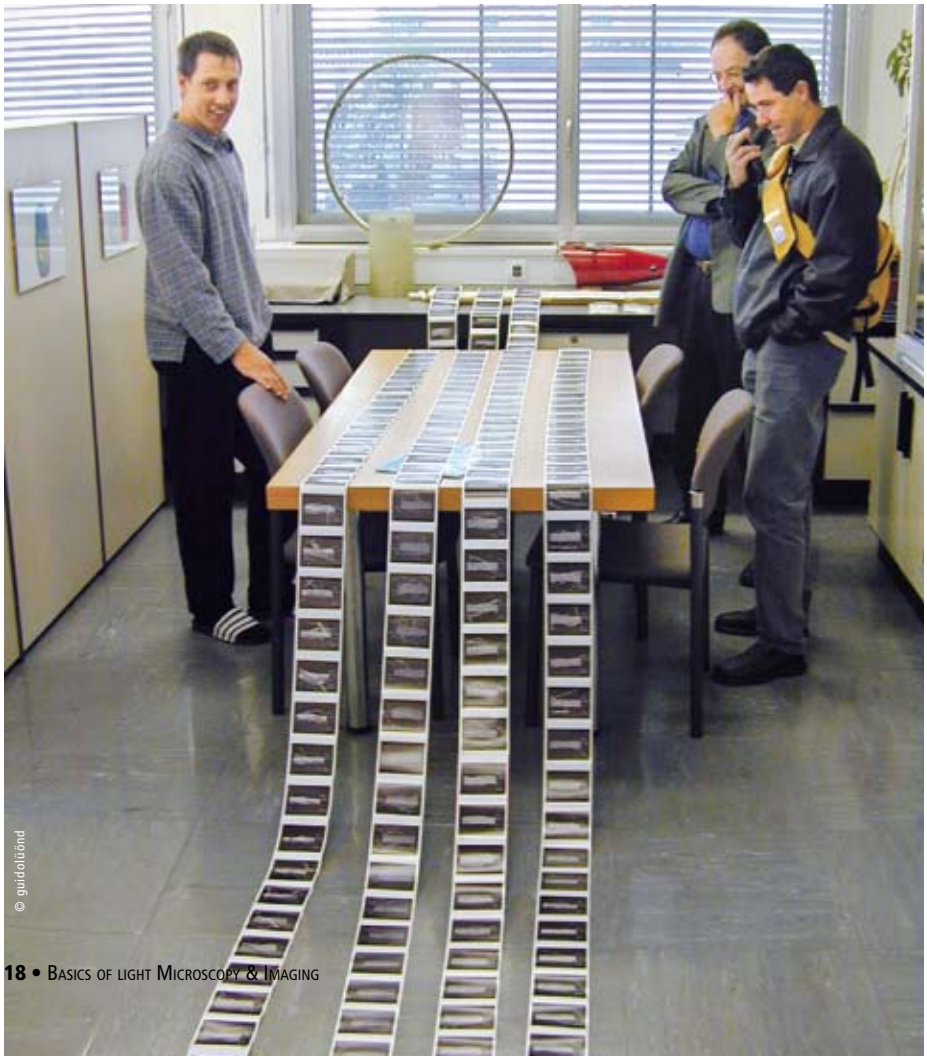


Fig 20: Mr. Guido Lüönd, Rieter AG, Department Werkstoff-Technik DTTAM, Winterthur, Switzerland, works on fibres using a microscope. For their investigations they often have to glue the single images into an overview image. Previously this was a considerable challenge. Now a piece of software takes over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

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).

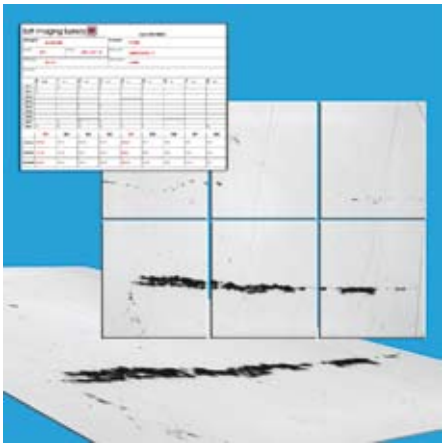


Fig. 21: The analysis of non-metallic inclusions according to DIN, ASTM and JIS requires the processing of areas up to 1000 mm² while the sulphide and oxide inclusions to be analysed are themselves are less than micrometres wide. No camera is available offering such a large CCD sensor. The image processing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

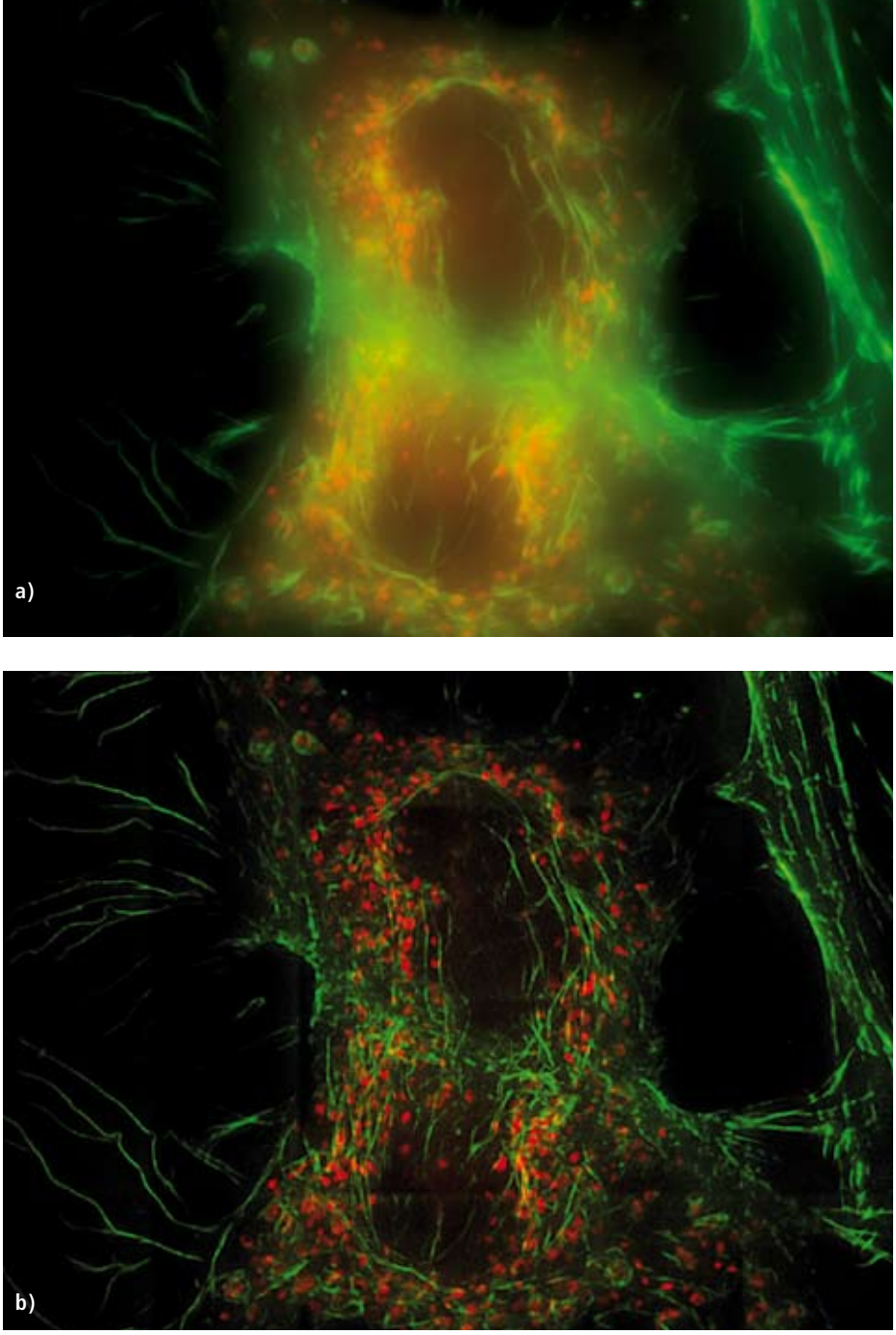


Fig. 22: Via deconvolution artefacts can be computed out of fluorescence images. a) These artefacts are caused by the stray light from non-focused areas above and below the focus level. These phenomena, referred to as convolution, result in glare, distortion and blurriness. b) Deconvolution is a recognised 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,

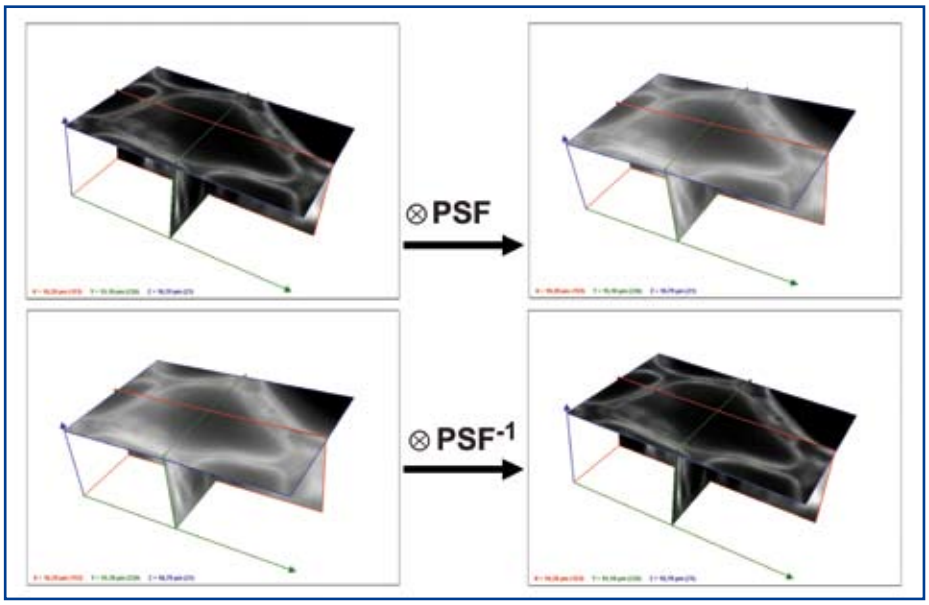


Fig. 23: Stray light originating from areas above and below the focal plane results in glare, distortion and blurriness (convolution) especially in fluorescence microscopy and histology. Deconvolution is a recognised mathematical method for correcting these artefacts. The degree to which an image is 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.

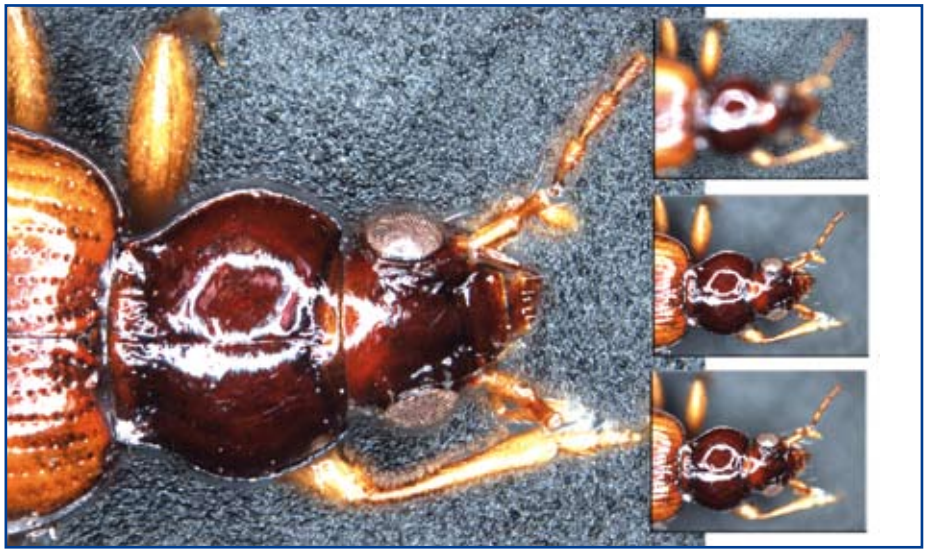


Fig. 24: The software extracts the focused areas from the component images of an image series and reassembles them into one infinitely sharp image. The example shown here is the resulting image computed automatically using 25 images of a *Bembidion tetracolum* sample.

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.