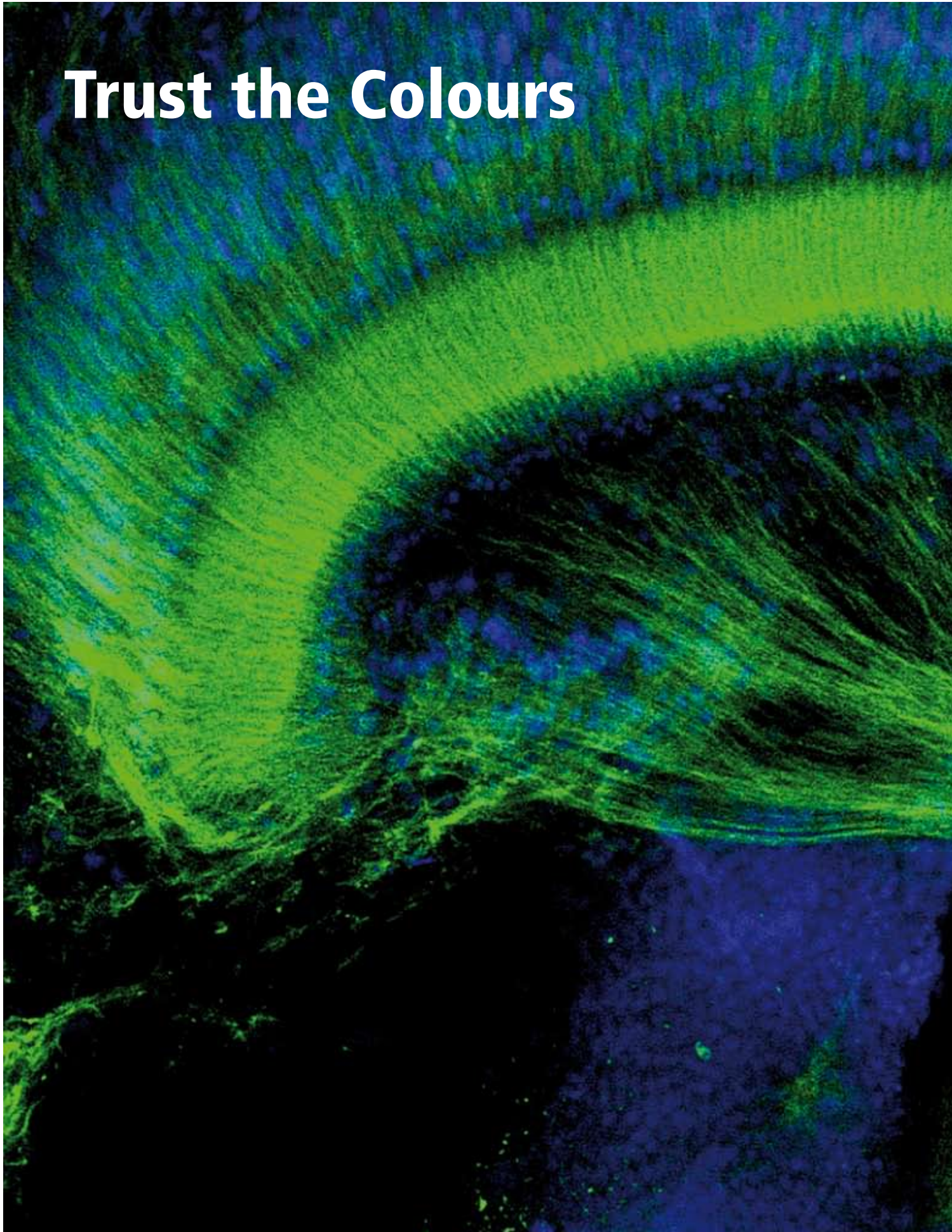
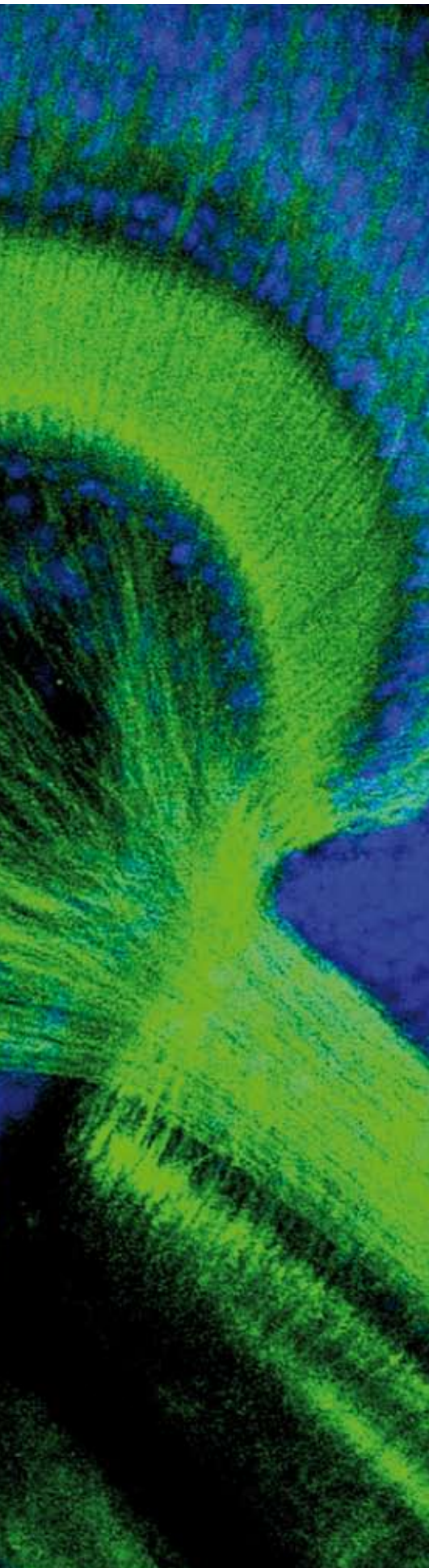


Trust the Colours





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 CMYK 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 col-

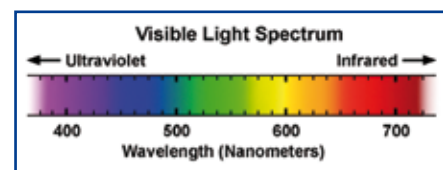


Fig. 1: Visible light spectrum.

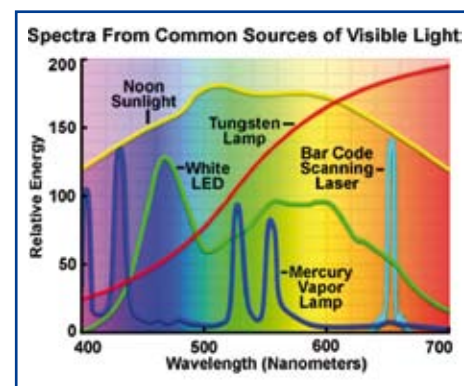


Fig. 2: Spectrum of the sun, and spectra of common sources of visible light.

our. 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.

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 per-

ceives 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 prac-

tical 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.

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

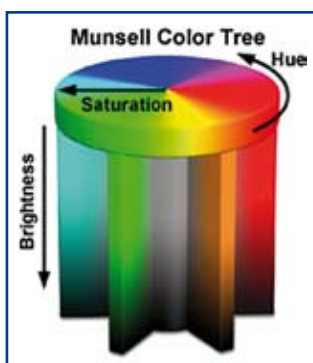


Fig. 3: Munsell Colour Tree.

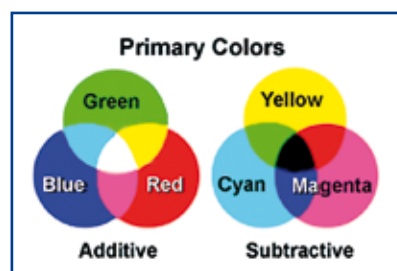


Fig. 4: Primary colours.

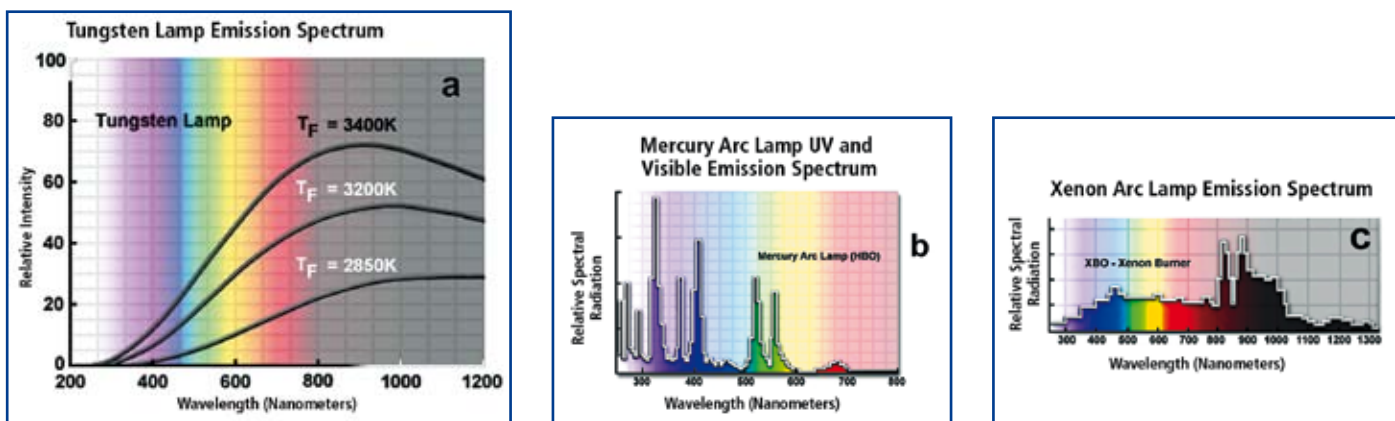


Fig. 5a-c is showing the emission spectra of three typically used light sources at a microscope: (a) the tungsten halogen bulb, T_F = colour temperature at different power settings, (b) the mercury burner, (c) the xenon burner.

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.

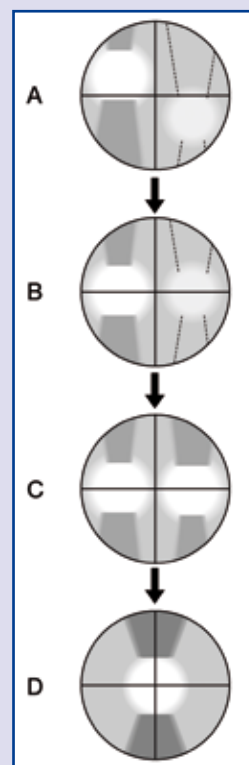
But here a problem arises: If the light intensity is to be kept fixed in the light microscope the light might be too bright for observation. In daily life if sunlight is too bright sunglasses help – but they

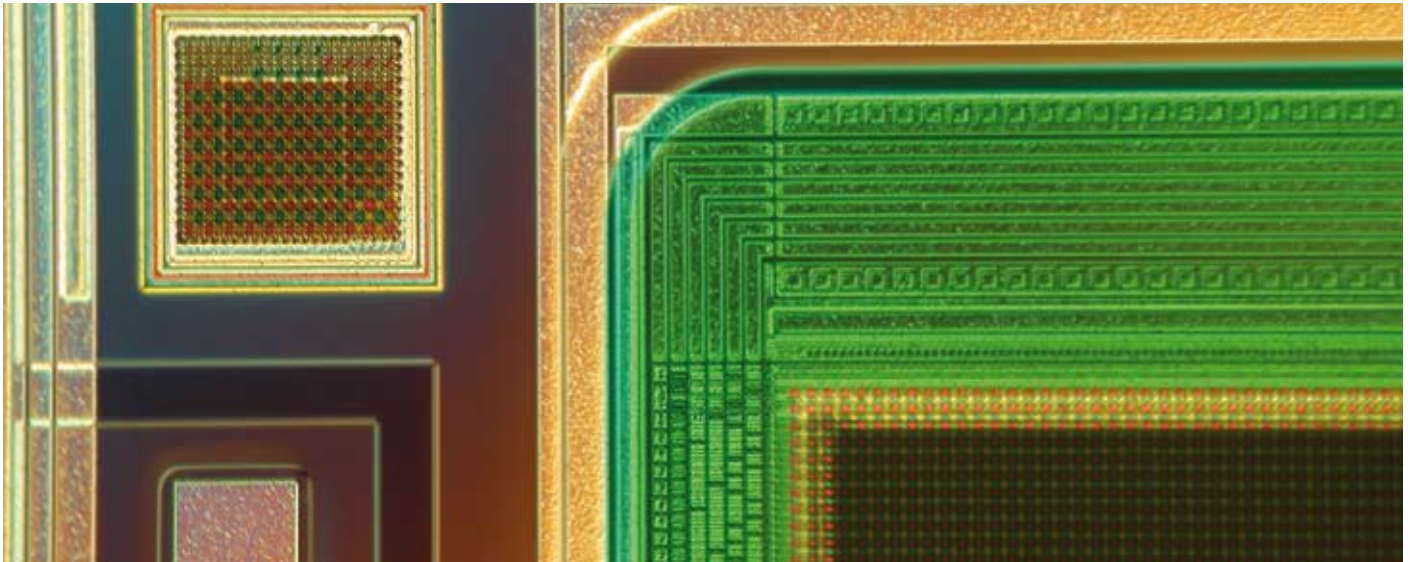
Box 1: Centring of a mercury burner

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

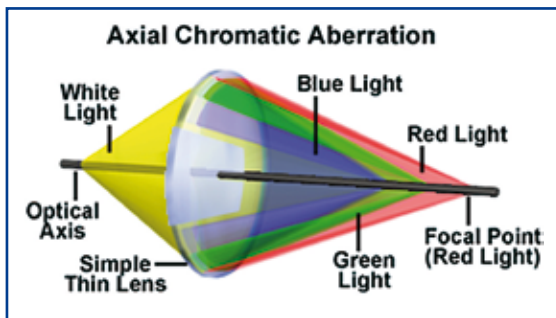


Fig. 6: Schematic of axial chromatic aberration.

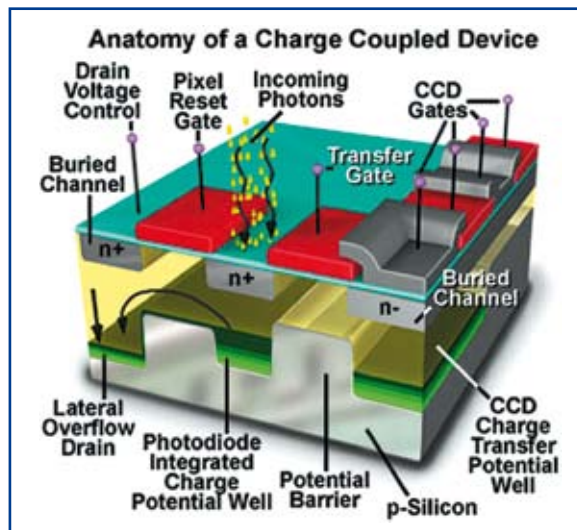


Fig. 7: The principle behind how CCD cameras function.

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 flint spar, 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, apochromat, 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 900nm 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

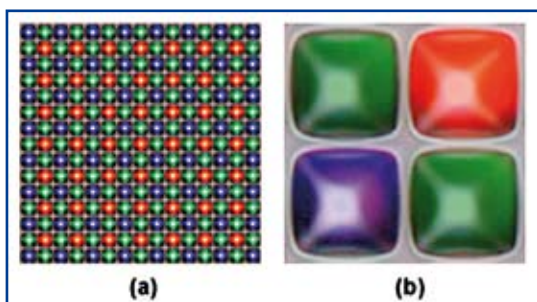


Fig. 8: Bayer colour filter mosaic array and underlying photodiodes.

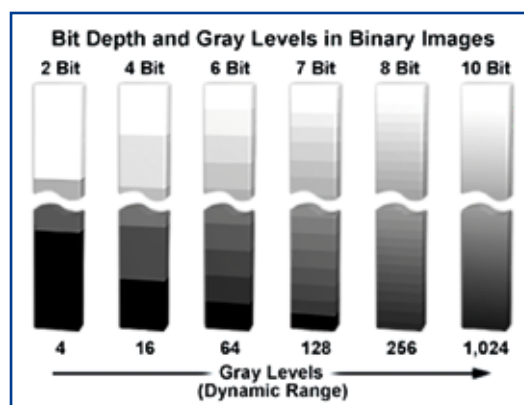


Fig. 9: Bit depth and grey levels in digital images.

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 (65536 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 $\langle F(R) \rangle$, $\langle F(G) \rangle$ and $\langle F(B) \rangle$ 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-

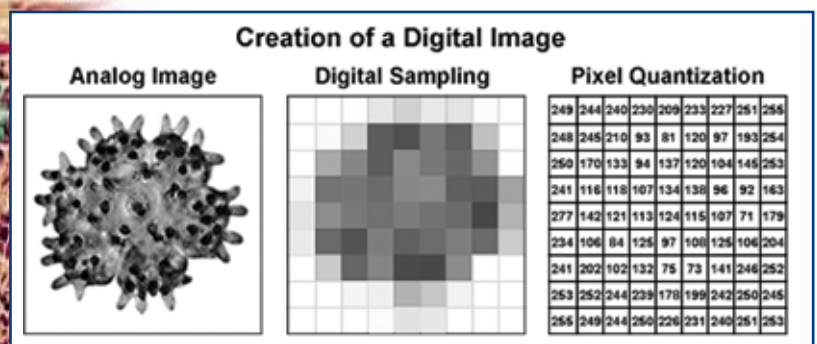
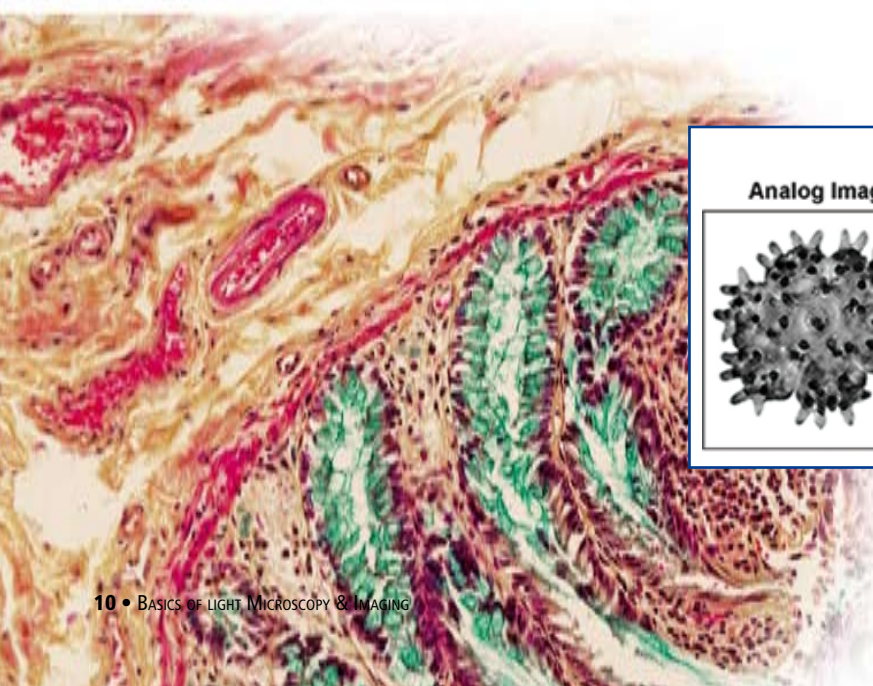


Fig. 10: Creation of a digital image.

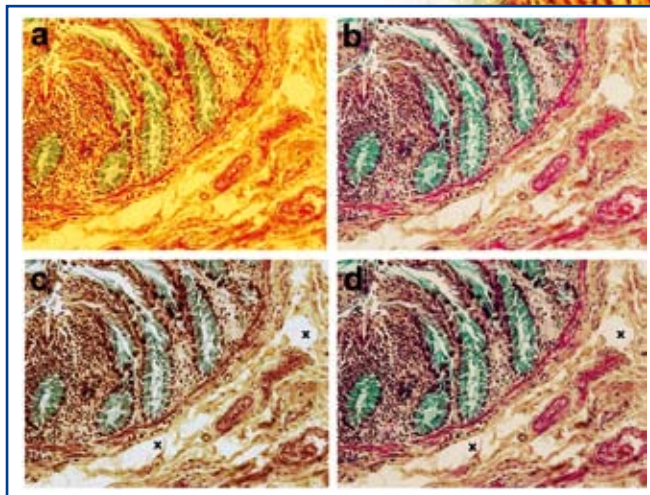


Fig. 11: White balance adjustment using microscope and software options on a histological specimen.
a: Image with wrong microscope settings (low power 3V, no LBD filter), note that areas that are not stained (background) show a yellowish colour.
b: image using optimised microscope settings (9V + LBD filter + Neutral density filter 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 extent 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.