1
Our Eyes and the Microscope

Outline

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Understanding Light Microscopy, First Edition. Jeremy Sanderson.
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1.1 Introduction

'Ah, I see!' demonstrates how dependent we are on our sight, and how it is linked inextricably with understanding. We are a visually oriented species, and our two eyes are our 'window on the world'. It is easy to forget that not all animals depend upon sight to the extent that humans do. Bats use echolocating sonar to navigate, and bees exploit ultraviolet (UV) light to discriminate foliage. At the other end of the visible spectrum, vipers sense infrared radiation to detect warm blooded prey. For those creatures whose habitat is underground (e.g. moles), vision necessarily cedes priority to other senses such as touch and smell. Other animals, chiefly birds, are more dependent upon their sight than humans, with a much greater proportion of their head devoted to large eyes.

Via the optic nerve, the eyes are a direct extension of the brain. The human eye is a wonderful device. If it were a camera, it might boast autofocus, wide-angle lens, auto-exposure, high sensitivity to light, automatic colour balancing, one-hundred megapixel resolution (each retina contains around 100 million sensitive cells) and 3D imaging (when used in pairs). We can see in bright daylight or discriminate a single light in the dark several miles away. The eye has naturally evolved to suit its principal function: helping its owner navigate the world.

Many modern microscopes are designed to be operated from a computer screen, rather than be used by viewing the image directly down the eyepieces. Nevertheless, all images must eventually be seen by our eyes, so in this chapter we shall discuss how our eyes function. We shall consider how well our eyes are adapted for their purpose but also the limitations and aberrations that they suffer from. These limitations are relevant to microscopy, affecting how we acquire and interpret scientific images (i.e. data) using the light microscope. We shall see how a magnifying glass works and why the microscope was developed to assist our vision.

1.2 How Our Eyes Work

Any image must ultimately be seen by the eye. Light is electromagnetic radiation that stimulates the eye. It is merely a fraction of the entire electromagnetic spectrum (Figure 1.1). Only a small proportion of solar radiation reaches the earth's surface. We depend on the ozone layer for protection; atmospheric dust, smoke, air molecules and water vapour also absorb a significant proportion of insolation, or incident solar radiation. Our eyes evolved from aquatic animals and contain a significant amount of water. Human sensitivity to electromagnetic radiation (Figure 1.2) corresponds closely to the wavelengths of minimum water absorbance, located away from harmful UV radiation and towards the infrared end of the spectrum.

Our eyes respond to the visible part of the electromagnetic spectrum from near UV at a wavelength of 380 nm to deep red at 710 nm. This stimulation depends on both the energy (frequency, expressed as wavelength) and the quantity (number of photons) of light. The wavelength of light is perceived as colour, and the quantity of light (expressed as the amplitude of the light wave) is seen as intensity[1] (see also Appendix A1.1.3 and Table A1.1, pages 45–46 in: Tilley, 2010). Suppose we have four LEDs: blue emitting at 490 nm; green at 555 nm; far red at 670 nm and infrared at 940 nm (commonly used for remote controls) all emitting the same radiant flux of 5 mW absolute power, measured in radiometric units. If we measure the respective light output of each of these LEDs in photometric units, the green LED will be the brightest (3.4 lm); the blue will be the second one (0.75 lm), and the far red will be the third (0.1 lm). The infrared LED will have a recorded emission of zero lumens (this example is taken from Tilley, 2010).

Further details about the electromagnetic spectrum and the nature of light are discussed in Chapter 2, Section 2.2 onwards. Light itself has no inherent colour; our perception of different hues is fundamentally a complex judgment experienced as a sensation by our brains. We discriminate colour very well, although brightness less so. This is why we choose paint in a range of colours, rather than different intensities.

The retina is the photo-sensitive tissue of the eye (Figure 1.3), within which there are two types of receptors. These are called rods and cones. Rods are more numerous (about 100 million per eye) and are better suited to night vision because they are about 100 times more sensitive to light than cones. Rods are thus adapted to low-intensity light, whereas cones (about 5 million per eye) give us our

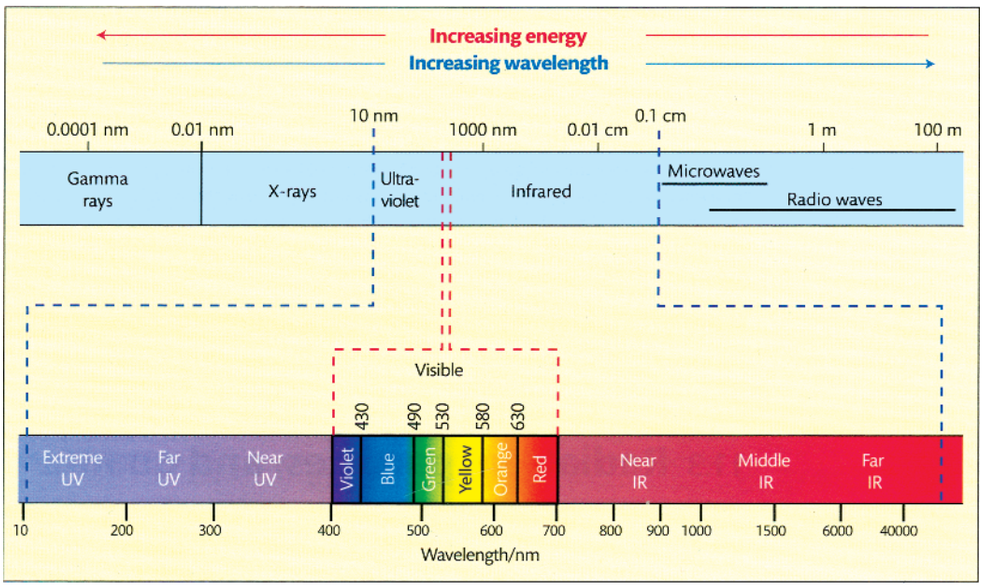

Figure 1.1 ***The Electromagnetic spectrum*** *The electromagnetic spectrum extends from beyond low frequencies used for modern radio to very high-frequency gamma radiation at the short-wavelength end of the spectrum, encompassing wavelengths from thousands of kilometres down to a fraction of a nanometre. Humans are able to discriminate the visible wavelengths from long wavelength, low energy, red at around 710 nm to short wavelength, high-energy violet radiation at around 380 nm. A very small number of people can see into the infrared, and people suffering from cataracts who have their lens removed (i.e. who are aphakic) can see into the UV, even if they can't focus well. Source: Glencross et al. 2011. Reproduced with permission from Oxford University Press.*

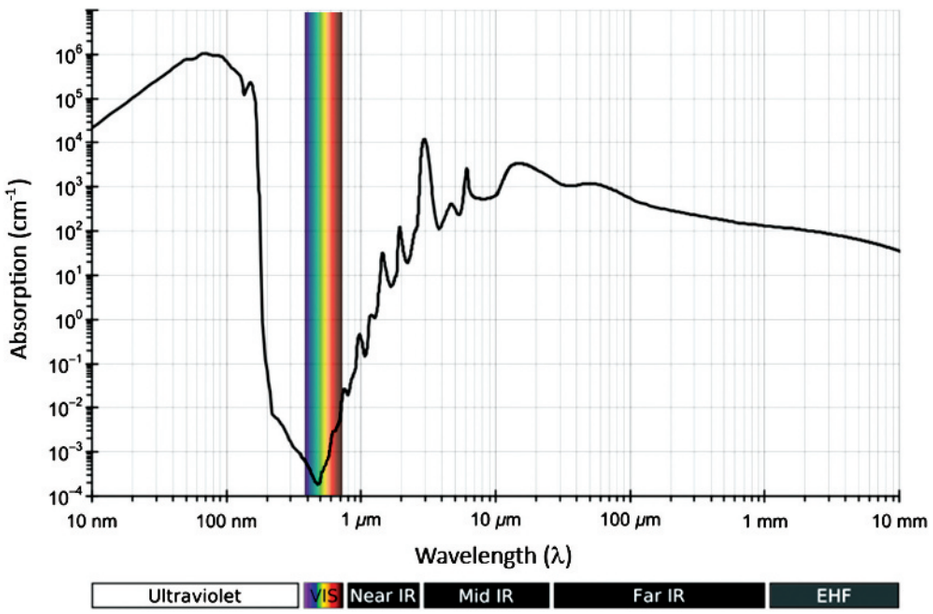

Figure 1.2 ***Human sensitivity to light*** *Visible light corresponds closely to the wavelengths of minimum water absorbance on the electromagnetic spectrum. See also Chapter 2, Section 2.5. Since our eyes are composed largely of water, it is advisable to use a heat filter (e.g. Schott KG5) in the light path to cut out any absorption of infrared light. Source: Adapted from Kebes at English Wikipedia under the terms of the Creative Commons Attribution licence, CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0).*

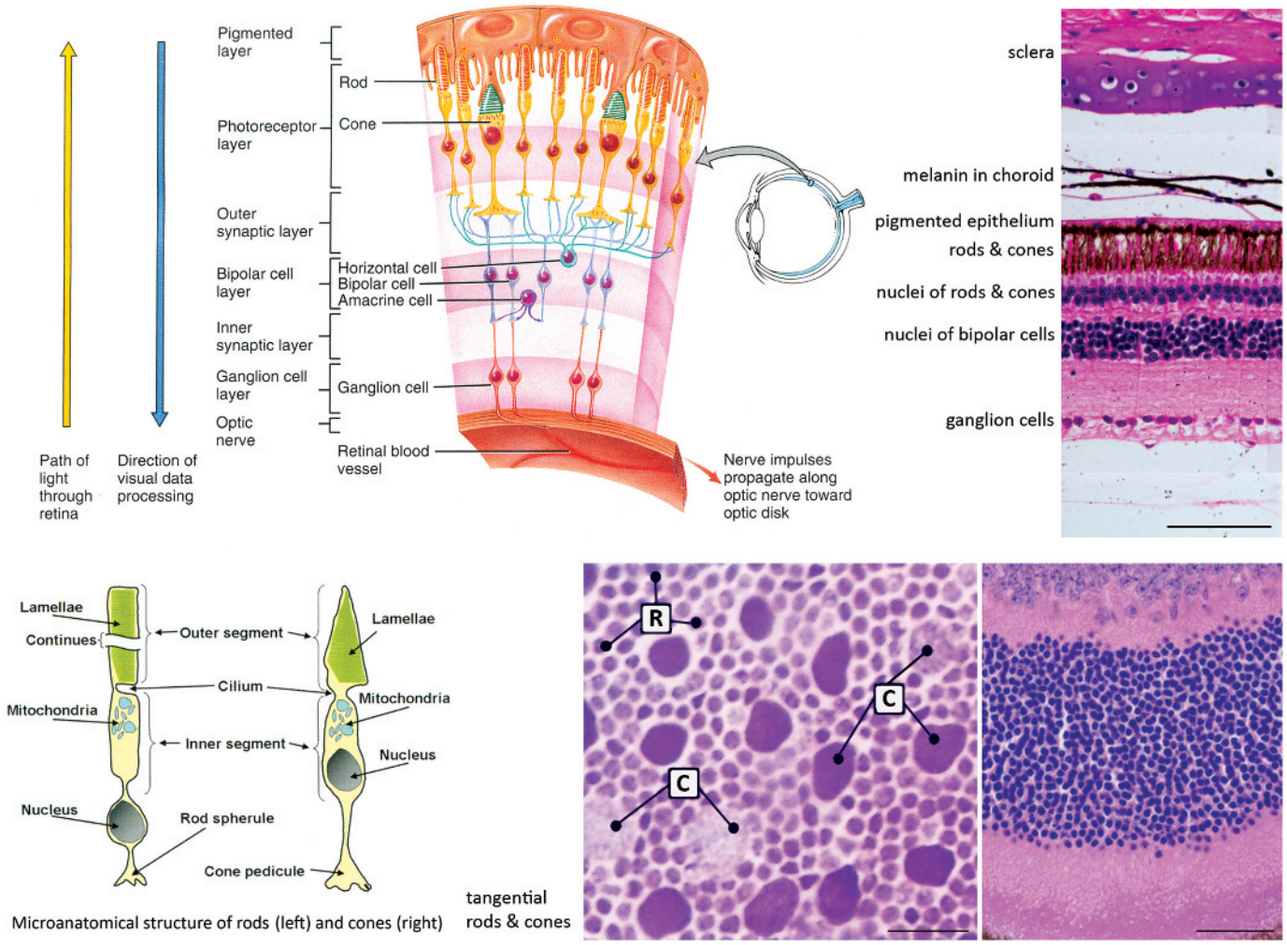

Figure 1.3 ***Structure of the retina*** *The upper row shows a photomicrograph of a section of primate retina, together with a cartoon diagram showing the organisation of the retina, which is between 200 and 250 mm thick in primates and is a delicate structure. Here the interface between the dense black choroid and the pigmented epithelial cells has separated during histological processing. The connective tissue of the sclera coat is seen at the top of the section. Section: haematoxylin & eosin; scale bar = 100 μm. Source: (top left) Tortora and Grabowski 2003. Reproduced with permission from John Wiley & Sons.*

The lower row shows the organisation of the rods and cones. The tangential section has been taken at the level of the inner segment of the photoreceptors. R = rods, C = cones; this area lies outside the fovea. Light passes through the layers of nerve cells to stimulate the photoreceptors. The retina lies on a layer of pigmented epithelial cells, which attach it to the choroid. Source: (bottom left) Allen and Triantaphillidou 2011. Reproduced with permission from Taylor & Francis Group. (bottom centre) Stevens and Lowe 1996. See also the relative distributions of rods and cones in Figure 1.12.

daylight vision in colour with high visual acuity (visual acuity refers to the sharpness and clarity of vision and our ability to resolve, or distinguish, fine detail with the naked eye).

Most humans have three colour pigments, or photopsins, giving trichromatic vision in red, green and blue hues. Some people lack one of the pigments, resulting in colour blindness or, more correctly, colour-deficiency. Other animals do not perceive colour as well as we do, though almost all mammals see colour to some extent. They may have only one or two pigments (e.g. dogs and cats), or may have different pigments from us altogether, relying more on motion, sound or the sense of smell for external awareness. Tree-dwelling and fruit-eating mammals have a strong sense of colour. Discrimination of colour allows us to differentiate between objects whose surfaces have equal luminance but which differ in hue. Other species have sacrificed colour discrimination in order to see better in low-light conditions. The basis of our colour vision is discussed further in Chapter 11, which covers contrast in the microscope image, and also in Chapter 31, which discusses how best to display the recorded image for the benefit of the colour-blind.

Our eyes are very sensitive to brightness: rods can signal the absorption of a single photon. From full sunlight to starlight represents a luminance ratio of 10 million to 1. We don't see equal increments of luminance as equal increments of brightness; rather[2] we see *logarithmic* increments of luminance as equal brightness steps (Figure 1.4). Therefore, as luminance increases, we require larger changes to discriminate a noticeable difference. As the illuminance (i.e. intensity) of light around us falls, we switch from daylight, or photopic,

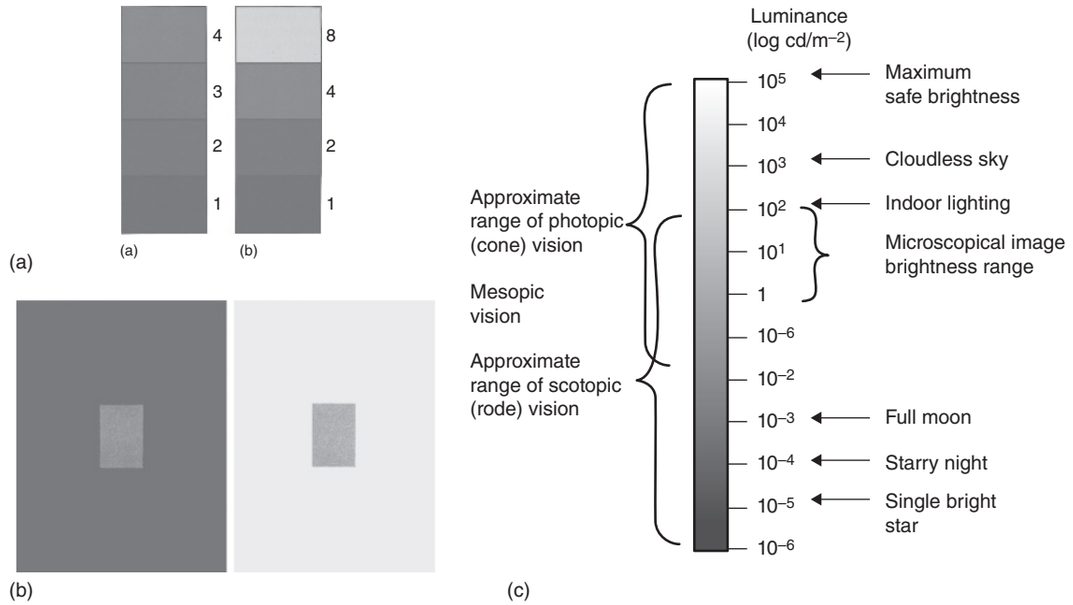

Figure 1.4 ***Response of the eye to luminance*** *Each greyscale luminance step of brightness on the left-hand scale differs by an equal arithmetic ratio, whereas those on the right vary logarithmically by the power of two. In the absence of a background, the eye sees the logarithmic scale as equal brightness steps, not so the arithmetic one. This is known as the Weber-Fechner law: the relationship between a stimulus and its perception is logarithmic. This is unlike a semiconductor CCD sensor, which discriminates brightness in a linear fashion. The eye responds logarithmically so that familiar objects tend to maintain their perceived reflectivity in changing light conditions, as our eyes adapt to the change. Otherwise (like exposure values using a camera) we would continually notice changing brightness and colours as the sun slipped behind, and emerged from, cloud. This also explains why we don't see stars in daytime, because the ratio of intensity of the stars to the sky is very small, whereas it changes at night as the intensity coming from the sky is small.*

However, if the backgrounds between areas of the same grey intensity are very different, this can alter our perception. In (b), the central grey rectangles are printed at the same intensity but are perceived differently, because of the contrasting adjacent backgrounds.

(c) shows the eleven orders of magnitude that the eye is able to perceive, although not all at once. At any given time, the eye is adapted to only 2–3 orders of magnitude on the scale shown.

Source: Falk et al. 1986. Reproduced with permission from John Wiley & Sons.

vision to night, or scotopic, vision. This occurs at luminance levels around 10^{-2} to 10^{-6} cd/m^2; below this light level we cannot read even large print text or recognise very small details, because the fovea[3] is inactive.

Since rhodopsin, contained in the rod cells, bleaches rapidly in bright light, this causes temporary blindness of the sensitive night vision. The practical consequence is that, although partial recovery may take 10 minutes, full recovery can take four times longer and affect visual acuity for viewing dimly-lit specimens (e.g. fluorescent samples). Daylight vision by the cone cells (Figure 1.5) adapts much more rapidly to changing light levels, adjusting well to a change such as coming indoors out of sunlight in just a few seconds. Rods are insensitive to light of wavelengths longer than about 640 nm. Red illumination in a darkened microscope room is useful[4] because red light bleaches rhodopsin inefficiently and thus maintains dark adaptation for high-quality microscopy.

Although rods can detect a photon as a single quantum of light, we are quite bad at seeing in the dark. This has implications when observing fluorescent specimens against a relatively dark background. Figure 1.6 explains why this is so. There is a statistical uncertainty associated with the detection of light by any photoreceptor (this is explained with respect to digital cameras in Chapter 30, Section 30.10). Although a rod can detect a single photon, a threshold exists to avoid spontaneous activation (and depletion) of rhodopsin: a minimum of six rods must be simultaneously stimulated with ≈10 photons in order to register a response at the threshold of vision. In Figure 1.6 there is a central dark area in the image. However, even at the visual threshold, the contrast is insufficient to discriminate this central area from the background. Only at 1000 times threshold is the central dark area surrounded by enough light areas to be visible with sufficient contrast. The converse is also true: small self-luminous fluorescent objects in the sample have to be sufficiently large to stimulate a group of rods in order to be seen against a large dark background.

We can see – at best – 60 shades of grey, particularly if there is an abrupt (i.e. straight edge) boundary between each shade and the next. The computer can record many more grey levels, shades, or intensities, irrespective of boundaries. This facet of digital imaging, and its consequences, is explained further in Chapter 18 explaining the operation of the confocal microscope in Box 18.1, and in Chapter 30, in Section 30.6, on recording the digital image. Suffice to say here that (a) computers can be used to enhance contrast so that details, and data, which we would otherwise miss, are made visible and (b) we need to use saturation look-up tables (LUTs) to ensure that all the dynamic range of electronic detectors (such as CCD and sCMOS cameras for widefield microscopy and photomultiplier (PMT) tubes for point-scanning confocal microscopes) is used effectively.

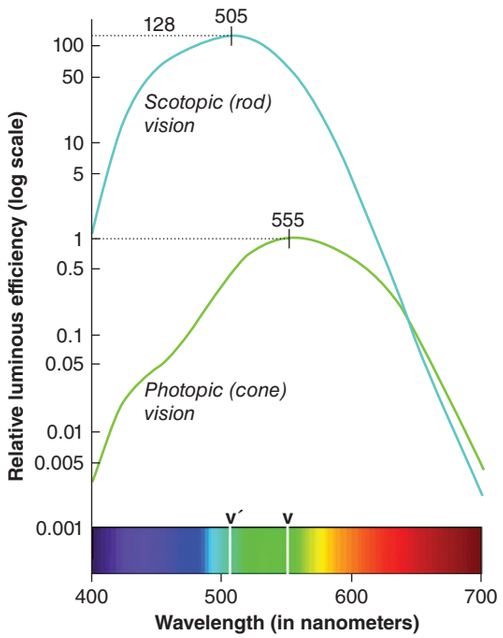

Figure 1.5 ***Photopic and scotopic vision*** *Daylight vision in bright light is called photopic vision. It occurs above 10 cd/m^2 when the rod photoreceptors become saturated, as illuminance rises. As the light levels around us fall, mesopic vision occurs when both rods and cones are active. At luminance less than 10^{-2} cd/m^2, photopic vision occurs. Sensitivity is plotted relative to peak photopic sensitivity on a log vertical scale.*

At light levels where the cones function, the eye is more sensitive to yellowish-green light (555 nm) than other colours because this stimulates the two most common (M and L) of the three kinds of cones almost equally. At lower light levels, where only the rods function, sensitivity is greatest at 505 nm, a blueish-green wavelength. Source: Handprint.com 2009. Reproduced with permission from B. MacEvoy.

1.3 The Anatomy of the Eye

The eyeball is a sphere approximately 24 mm in diameter with the cornea bulging slightly outwards. Figure 1.7 shows the features of the human eye that are important for our purposes. Essentially three transparent structures are held in position by three coats of tissue. The sclera is the white fibrous, protective, outer layer of the eye; the cornea is fused as an extension to this coat. A small part of the sclera is seen from close distance around the iris; hence the term 'the white of the eye'. The choroid is a pigmented black membrane that has two functions: it prevents unfocused light from shining through the sides of the eye and prevents light that enters the pupil from reflecting inside the eye. The third coat, the retina, lies directly on top of the choroid. The three transparent structures within the eye are the aqueous humour, the lens and the vitreous humour.

Any optical instrument – and the eye is no exception – must have its image-forming surfaces (e.g. the cornea and lenses) kept in a steady position with respect to each other and the detector (screen, CCD/sCMOS face-plate, PMT tube, film plane or retina) upon which the image is formed. The flexible tissues of the eyeball and the two surfaces of different radii, or 'figures', of the lens are kept firm by the pressure of the internal fluids of the eye. The aqueous humour lies between the cornea and front, or anterior, surface of the lens. It is continually drained and replaced by fresh fluid. The more gelatinous vitreous humour fills the larger space of the eyeball between the rear, or posterior, surface of the lens and the retina. The vitreous humour is not replaced like the aqueous humour and tends to liquefy very slightly as we age.

When the vitreous humour becomes less viscous, cellular debris or clumped strands from the vitreous humour can float freely. Being less transparent, these *floaters* cast fleeting shadows on the retina, following the motion of the eye whilst drifting through the vitreous humour. They are more prevalent in short-sighted eyes, and become increasingly annoying as we age (see also Chapter 9, Section 9.13). Floaters are most likely to be seen when using objectives and eyepieces of high magnification, especially if the objective is of relatively low aperture and the microscope is set up with the adjustment of the illumination set too low such that it does not fill the objective with light. The proper method of adjusting the microscope is explained in Chapter 9, Section 9.11. If a floater appears, moving the eye from side to side or up and down can create an internal current that will help move the floater away from the line of sight.

The lens of the eye is composed of transparent cells held in place by suspensory ligaments connected to the ciliary body, which focuses the lens. The majority of the focusing (approximately 60%) to form the image is done by the fixed cornea[5], while the bi-convex lens adjusts to allow fine focusing to maintain a clear image. This focusing is called accommodation and is discussed further below. The focal length of the relaxed lens is 17.1 mm and 14.2 mm when fully accommodated. The iris, which determines the pigmentation

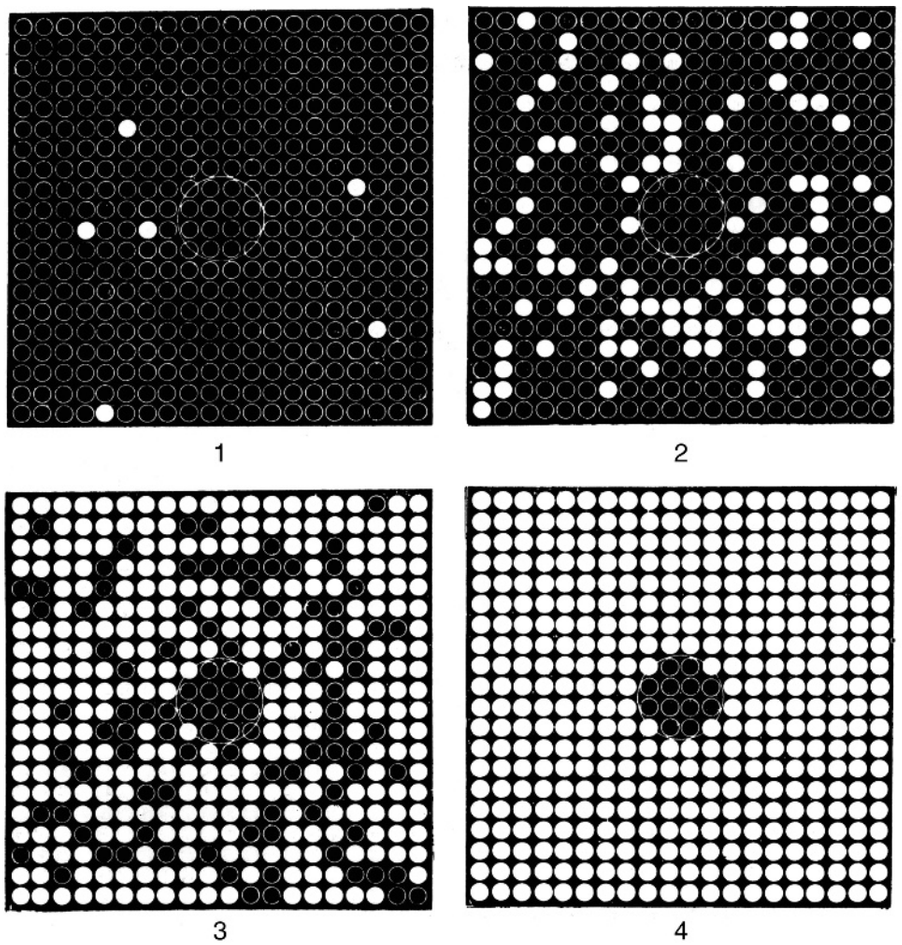

Figure 1.6 ***Sensitivity of scotopic vision*** *All four panels of the figure show the image on the retina of the same (bright) field containing a central dark patch. In panel 1 the illuminance is so low that only 6 of the 100 receptors receive a photon – at or just below the absolute threshold of vision. In panel 2 the illuminance is ten times higher, but the central dark patch is hidden within the noise of the random background photons. In panel 3, at 100 times threshold, the central dark patch is only just discernible but not convincingly so, and it is only in panel 4, at 1000 times threshold, that the central dark area is readily visible against the background with sufficient contrast. Source: Land and Nilsson 2001. Reproduced with permission from Oxford University Press.*

of our eye, dilates automatically in response to differing light intensities (irradiance), to regulate the diameter of the central opening or aperture – the *pupil* – of the eye[6]. In very bright light, the iris contracts to give a pupil of approximately 2 mm or *f*/8.3. This diameter increases in young adults to about 8 mm (or *f*/2.1) or in the elderly to about 5 mm (or *f*/3.3) at low-light intensities.

Within the retina, the sizes of the rods and cones differ slightly. Cones are typically 40–50 μm long. A rod photoreceptor is longer (about 60 μm) and narrower than a cone: about 2 μm in diameter, whilst a cone is 2–8 μm in diameter, depending upon its location in the retina. With rods we see in black and white, or monochrome. Overall, there are roughly 1.5×10^6 ganglion nerve cells per eye, with as many as 150 rods connected to a single nerve cell via bipolar cells. This summation, or convergence, increases sensitivity, which is useful when the light intensity is low. However, summation reduces definition – things look less sharp. A single rod is only slightly more sensitive than a single cone. However, cones are innervated as few as 2–6 per nerve cell, making them approximately 30 times less sensitive to light. Cones are essential for resolving fine detail sharply and for colour vision.

The area of the retina directly behind the lens is called the macula. It is densely innervated and contains the *fovea centralis*, which has the highest concentration of singly-innervated cones, giving excellent visual acuity in our central vision. The entire fovea is about 1.5 mm in diameter, comprising approximately 5° of the visual field. The rod-free area of the fovea is 0.5 mm in diameter, subtending 1.7°. Whilst the majority of cones (about 94%) are located in the peripheral retina, the 60 000 cones of the fovea are much slimmer. Here they measure 1–2.5 μm in diameter and are packed together in a hexagonal pattern. At the fovea, the layers overlying the photoreceptor layer are much thinner, giving the appearance of a pit (Figure 1.8) to the cross section of the whole retina at this point. Within the central

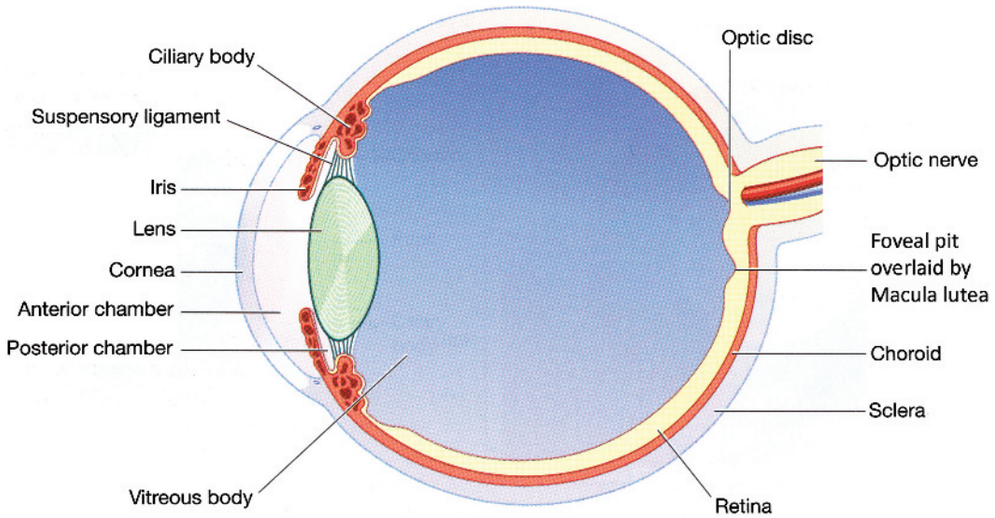

Figure 1.7 ***Anatomy of the eye*** *The blind spot occurs because no photoreceptors can exist where the optic nerve and retinal blood supply join the eyeball. Because the eye tiles images in the brain, this area is 'filled in' unconsciously, despite the fact that the blind spot extends to about 6° in diameter and is therefore quite large. The yellowish macula lutea overlies the fovea, the area of highest visual acuity, which lies directly on the visual axis of the eye. Source: Waugh and Grant 2001. Reproduced with permission from Elsevier.*

0.35 mm (1°) of the fovea is the very high-resolution area, the *foveola*, located at the bottom of the foveal pit. Here in the foveola there are about 12 000 cones; only a tiny fraction of the total number of photoreceptors are responsible for our high acuity vision.

The size of the cones in the foveal region, and their packing within the retina, has a direct bearing on visual acuity. The average cone-cone centre distance is 3.8 µm. In Chapter 3, Section 3.11, Box 3.4 is an explanation of how the packing of the light-sensitive cones, which make up the fovea, matches the maximum possible resolving power of the eye. At very best we cannot discriminate, or resolve, detail that is closer together than 72 arcseconds, (i.e. 36 cycles per degree, CPD; visual acuity is often measured in CPD), which is equivalent to resolving 0.09 mm (90 µm) at the standard reference viewing distance (which is also sometimes called the nearest, or least, distance of distinct vision) of 250 mm[7]. This holds true only during conditions of photopic vision at normal levels of light intensity; when we are dependent upon our rods alone, in scotopic conditions, our visual acuity falls off.

Outside of the fovea, there is summation of both rod and cone cells. Thus the bandwidth of visual processing is highest for visual fields directly in front of us. The fovea comprises less than 1% of the area of the retina, but its importance to our vision is demonstrated

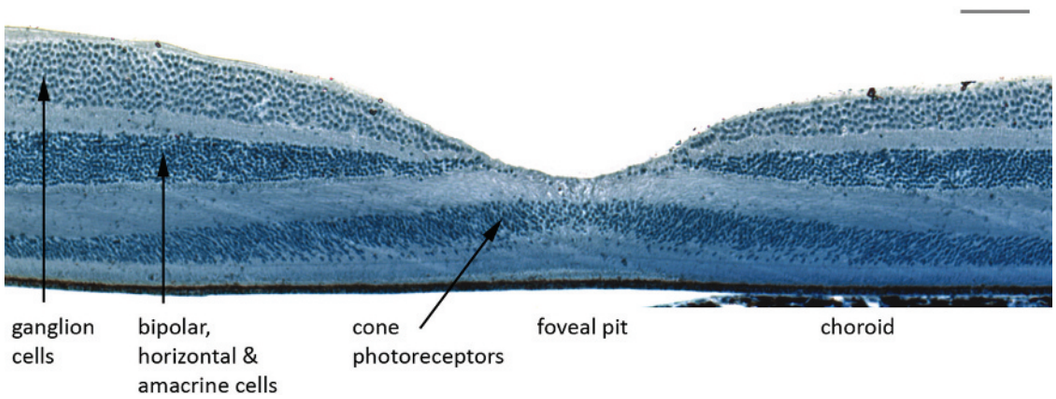

Figure 1.8 ***The fovea and foveal pit*** *This area of highest visual acuity contains slimmer cones and no rod photoreceptors. The organisation of each layer within the retina is also shown in Figure 1.3. Section: monkey, toluidine blue; scale bar = 100 µm. For further details, refer to Kolb, H. (2007); https://www.ncbi.nlm.nih.gov/books/NBK11556/.*

by the fact that over 50% of the visual cortex is devoted to processing the information derived from this small region. Since the fovea contains no rods, it is insensitive to low intensity light signals. Astronomers know this: in order to observe a dim star, they use averted vision.

Broadly speaking, the density of receptors and the degree of summation determine the degree of visual acuity: the greater the number of singly-innervated receptors, the greater the ability of the eye to distinguish individual objects at a distance. We have a mean value of about 200 000 cones per mm^2, which is good for a mammal but is quite low compared to a large raptor, which has about 400 000 cones per mm^2 as well as more ganglion cell connections between the receptors and the brain, since it requires a high visual acuity for hunting[8]. We may have limited visual acuity compared with a falcon, but it is much better than that of a honeybee, which has only 1% of our visual acuity and would not be able to see if there were any books on the shelf across the room, let alone read the titles. However, a honeybee does not need to read but does need to navigate. It can respond to the polarisation[9] vector of light and uses this to navigate to food sources.

Approximately two thirds of the population is right-eye dominant and one third, left-eye dominant, preferring visual input from one eye over the other. This has consequences for adjusting and using the microscope, which is covered in Chapter 9, Section 9.11. The diameter of the *exit pupil* of the beam of light leaving the eyepiece is determined by the diameter of the pupil of the iris, so that no information about the image is lost. This is explained further in Chapter 8, Section 8.6.

1.4 Aberrations of the Eye

Because our eyes have a single lens, they suffer to some extent from both spherical and chromatic aberration. Our eyes may also suffer from aberrations such as astigmatism, for which we require corrective spectacle lenses. The subject of optical aberrations and how they affect the performance of the microscope in forming an image is discussed later in Chapter 6. In the microscope these lens aberrations can be corrected by cementing single lenses together into doublet or triplet combinations. These lens groups require combining convex (positive) and concave (negative) lenses; animal eyes are only bulging, or biconvex – they have no negative configuration. Doublet or triplet elements have a longer focal length than is feasible in a small eye. The eye must therefore rely on natural methods to correct optical aberrations.

Spherical aberration manifests itself as unsharpness in the image (Figure 1.9). Spherical aberration in the eye is minimised by the different curvature (known as the 'figure' of the lens by opticians) of each of the two surfaces of the elliptical biconvex lens. Also, under daylight illumination, our pupils are smaller; this restricts light paraxially, close to the optical axis, and thus minimises the effects of both spherical and chromatic aberration. Additionally, a smaller pupil also ensures that light falls only on the central fovea for sharp vision. Whilst a larger pupil lets in more light to the eye and too small a pupil suffers from image blurring due to the effects of diffraction, there are no advantages in the eye having a pupil too large (Figure 1.10); otherwise, aberrations limit image quality. The direct effect of chromatic aberration in the eye manifests itself as different colours appearing slightly out of focus with respect to one another. Figure 1.11 demonstrates this.

The entire macula is covered with a yellow carotenoid pigment, very similar in hue to the yolk of an egg, called the macula lutea. Together the cornea, lens and macula filter out much of the harmful UV light. The pigmented macula absorbs blue light also. Since blue light refracts more than red light, absorption of these shorter wavelengths helps minimise the effects of chromatic aberration and glare from scattered light. We have approximately 64% of red (L) cones and 32% of green (M) cones but only 4% of blue (S) cones (Figure 1.12), and these are largely absent from the fovea, where visual acuity is highest. Additionally, our brains respond to light falling on-axis upon the fovea preferentially to other parts of the retina (the Stiles-Crawford effect). This reduces perception of any scattered blue wavelengths, helping further to minimise chromatic aberration.

Eyes (and lenses) suffering from astigmatism[10] have an unequal curvature of the cornea, the lens or both. This inequality causes the light to be refracted, or bent, to a different degree at different meridians of the cornea or lens. As Figure 1.13 shows, rays that propagate along two perpendicular planes, for example, will be brought to a focus by an astigmatic lens at different distances along the optical axis from each other. Figure 1.14 shows a test for diagnosing astigmatism. When using the microscope, it is necessary to determine whether your spectacles are corrected for astigmatism (see Box 9.5, Chapter 9). This dictates whether you must retain your spectacles for observation down the microscope, or whether you can remove them, if desired. Many people have a mild degree of astigmatism, often without realising it. The condition is severe enough to necessitate ophthalmic correction by spectacles or contact lenses in one third of the population.

Our eyes are sometimes likened to a photographic camera, but unlike the exposure of film in an analogue camera or the capture of photons and conversion to an electronic signal in a digital camera, our vision is not instantaneous but rather a continuous process. We build up a panoramic view by extremely rapid scanning microsaccadic movements, which our brains then tile into a sharp image. We do this for three reasons: scanning allows the fovea – the region of highest cone density and the greatest sensitivity – continually to image a scene. The fovea sees only the central 1.7° of the visual field, roughly equivalent to the width, at arm's length, of both thumbnails placed side by side. Without microsaccades, our vision would be severely limited: only our central vision would be at all sharp, with the rest an indistinct blur (Figure 1.15). Secondly, scanning allows the brain to tile images and thus fill in over the blind spot, the area without photoreceptors where the optic nerve connects to the brain. Thirdly, staring fixedly at a scene without microsaccadic scanning would also cause the rods and cones temporarily to cease operating, since they must constantly regenerate their pigment, which is bleached by light.

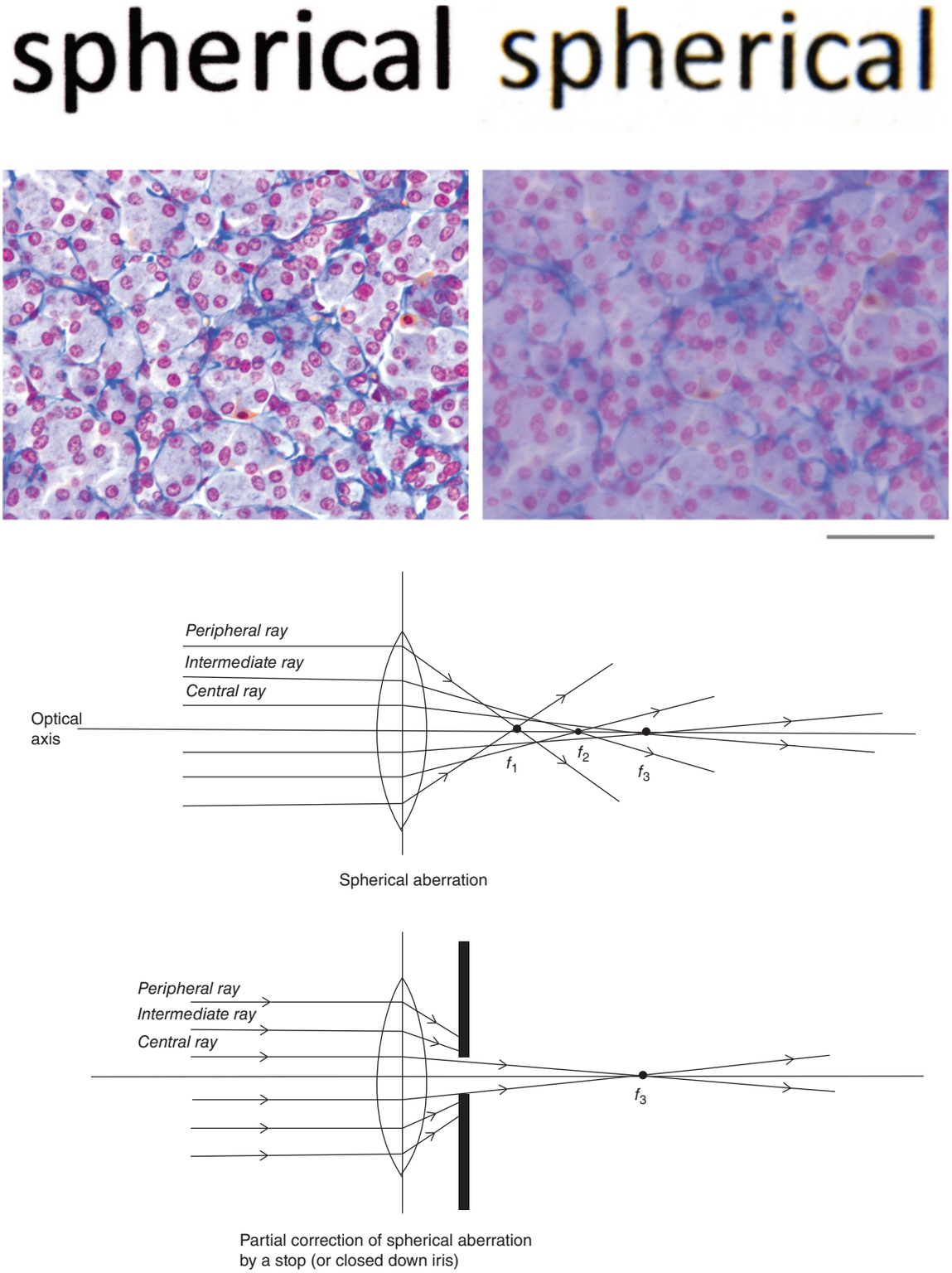

Figure 1.9 ***Spherical aberration*** *The top two illustrations were taken with a highly corrected camera lens (left) and a single lens that had failed in the quality control process (right), of the word 'spherical'. Spherical aberration is most noticeable at the edges of the image; notice how the letter R is smeared and unsharp. A smaller pupil ensures that only paraxial light rays fall on the central fovea for sharp vision.*

The bottom two illustrations show spherical aberration in the microscopical image. The image on the left is taken with a corrected lens. The right-hand image of the same field of view shows spherical aberration. The image is blurred and unsharp; image contrast is also severely degraded. Section: monkey pancreas, acid fuchsin & azan; scale bar = 50 μm. Source: (top) Courtesy of Mr K. Glover. (bottom) Bradbury 1967. Reproduced with permission from Elsevier.

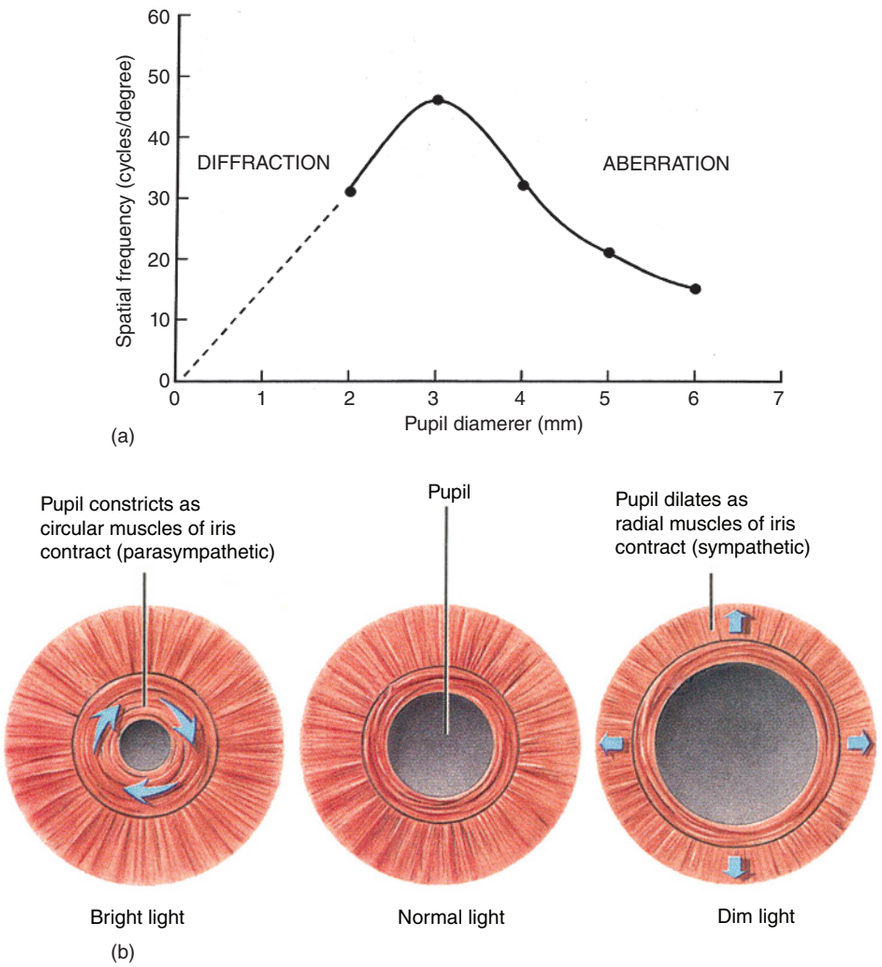

Figure 1.10 ***Optimum pupil diameter*** *In bright light, under photopic conditions, a pupil of 3 mm is optimal between the effects of diffraction and spherical aberration and (to a lesser extent) chromatic aberration. As illuminance falls, visual acuity is limited by photon noise rather than optical quality. Source: (a) Land and Nilsson 2001. Reproduced with permission from Oxford University Press. (b) Tortora and Grabowski 2003. Reproduced with permission from John Wiley & Sons.*

1.5 Binocular and Stereoscopic Vision

Despite the limitations of a single lens and consequent aberrations, our eyes are suitably adapted for their function. We can see into the distance and also close up to a near distance of distinct vision of about 250 mm. We can detect peripheral movement with ease, although with eyes at the sides of their heads, many animals are able to see very large visual fields, in some cases behind themselves, without moving their heads. This is clearly useful for detecting moving predators; the trade-off is having very limited 3D vision, which only extends in a narrow sector to their front for these animals. Predators, on the other hand, tend to have their eyes positioned on the front of their heads. We have forward-facing eyes on the front of our faces separated by the inter-pupilliary, or interocular, distance of between 50 and 75 mm. With each eye offset by about 5° from the optical axis through the centre of our head, this disparity gives us binocular vision. Each eye presents different views simultaneously to the brain. Complex neural circuitry matches each set of points from one view with the equivalent set from the other eye. We exploit binocular disparity to fuse in the brain the image from each eye to provide visual depth and three-dimensionality, allowing us successfully to navigate the world around us.

Parallax is the apparent displacement of an object due to a change in the position of the observer. For example, the needle on an analogue speedometer, because it is raised away from the face of the instrument dial, will indicate a different speed to a passenger in the car than the driver. The passenger sees the needle from an angle and observes an apparent speed; the driver observes the needle face on and notes the true speed of the car.

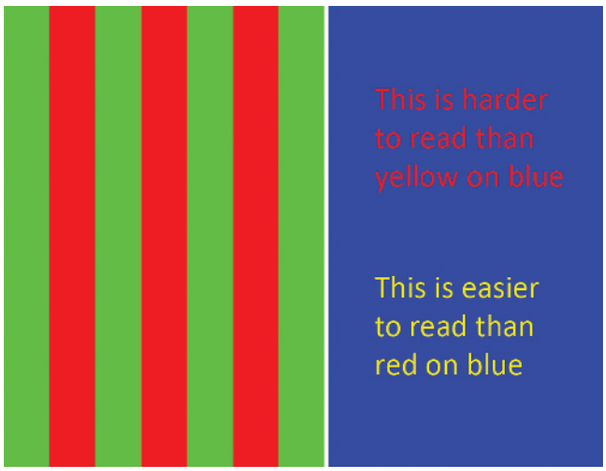

Figure 1.11 ***Chromatic aberration*** *Try to focus sharply on the red stripes, and the green stripes will appear slightly unsharp and out of focus. The brain will interpret each colour as being at a slightly different depth, with the red stripes appearing closer. Likewise, the red text on a blue background is harder to read (despite these colours being saturated) than the unsaturated yellow text on blue. This is because red and blue occur at opposite ends of the visible spectrum, and so the focusing mismatch arising from chromatic aberration is greater. The eye tires more easily trying to accommodate and focus on each colour separately. Source: Franklin et al. 2010. Reproduced with permission from John Wiley & Sons.*

The process by which the brain exploits parallax to compute depth perception and to estimate distances is known as stereopsis. The etymology of the word is from the Greek *stereo* meaning 'solid', and *opsis* meaning 'power of sight'. Birds, such as chickens and pigeons, who have eyes on the sides of their heads, are unable to view a single object stereoscopically with both eyes. They cannot use parallax to judge distances, as we do, and must judge distance by moving their heads – viewing an object with each eye independently. This is referred to as motion parallax[11] and is why pigeons can be seen continually bobbing their heads up and down. People who are stereo-blind, who lack vision in one eye, or have both long- and short-sighted eyes, often have poor depth perception, but it is not entirely absent. We learn strong monocular clues to judge both distance and depth. These include a change in the size of the retinal image and linear perspective: as a car drives away it apparently becomes smaller. With aerial perspective a change in colour occurs, that is, distant mountains appear blue. Experience and expectation of light and shade also help, as does interposition: an object which is overlapped appears further away. A lack of binocular vision affects about 12% of the population. Stereo-blindness will not prevent you from adjusting and using a microscope normally.

Béla Julesz, a Hungarian neuroscientist, developed the computer-generated random-dot stereogram that, with a stereoscope, allowed the brain to see 3D shapes from a two 2D flat images. Within the random dots a pattern is horizontally displaced in each image. Observers with binocular vision can process this disparate information to see a 3D image in depth above the random background. This proved that depth perception is a neurological phenomenon, rather than a purely visual process. Christopher Tyler, a student of Julesz, together with Maureen Clarke further developed the autostereogram (employing a repeating pattern) so that the 3D effect could be seen without the aid of special glasses or a stereoscope. These were later to give rise to the popular *Magic Eye* books.

1.6 Why We Need Optical Aids

When we want to see something in more detail, we bring it closer to our eyes. It is difficult or impossible, for example, to read a newspaper at a distance of several metres. As the newspaper is brought closer to the eye, the image is spread further over the retina (Figure 1.16), and the print becomes easier to read until a certain distance is reached, when it is no longer possible to see it clearly in focus. Alternatively, the object we are viewing may be so distant that it is not possible to get sufficiently close to bring it closer to the eye and see the detail that we would like.

In both cases we can use an optical aid to help us distinguish, or resolve, further detail. If we cannot get sufficiently close, we might use a telescope or a pair of binoculars. Where we can get close up to the object, we use a magnifying glass or microscope.

These examples highlight two of the limitations of the eye. When the newsprint is far from the eye, only a small image of it is formed on the retina. Several details may fall on any one sensitive photoreceptor so that individual fine details are not discriminated, or resolved. As the paper is moved closer, the size of the image increases and falls on more sensitive cells in the retina, enabling more detail to be

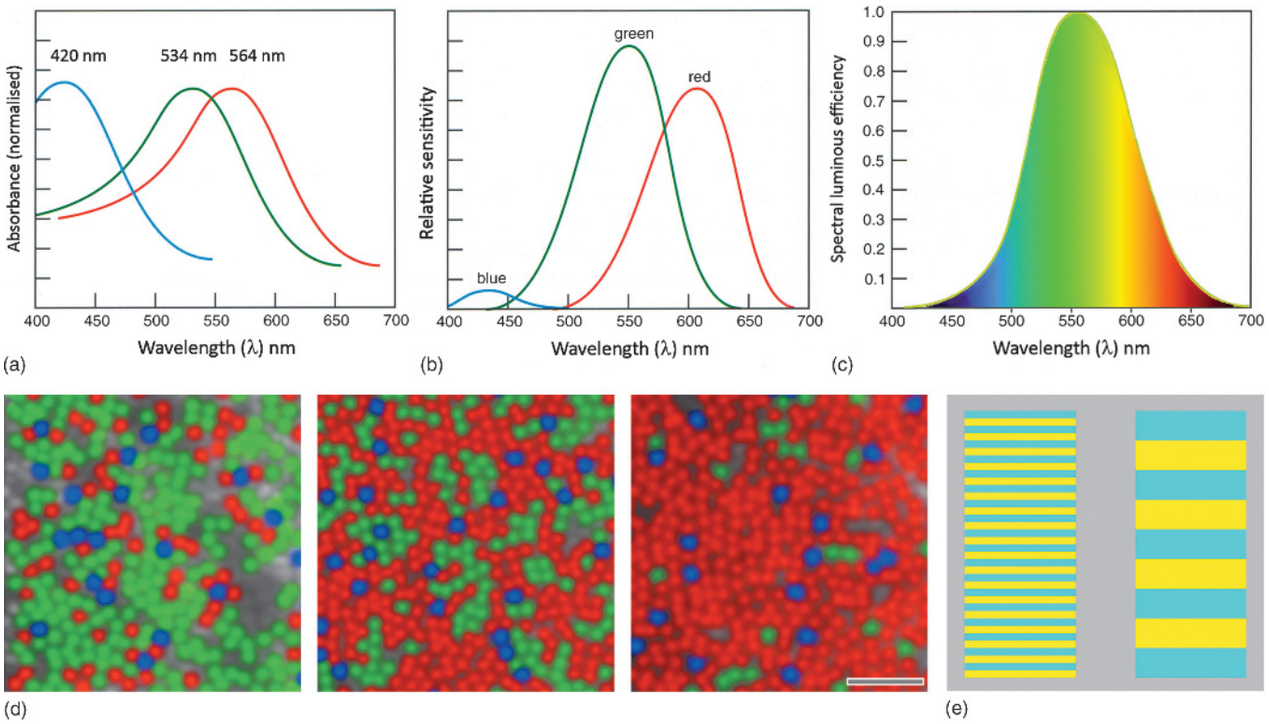

Figure 1.12 ***Distribution of cones in the retina*** *(a) shows the sensitivity of each of the three cones to the visible spectrum as a function of wavelength. (b) shows the relative sensitivity of red, green and blue cones to one another, and (c) shows the overall photopic sensitivity of the eye.*

We have very approximately 60% of red (L; long) cones and 30% of green (M; medium) cones but only 5% of blue (S; short) cones. These three types have peak wavelengths near 564 nm, 534 nm and 420 nm, respectively. The images are false coloured so that red, green and blue are used to represent the L, M and S cones respectively. (The true colours of these cones are bluish-purple, purple and yellow). The proportion of S cones is relatively constant across eyes, ranging from 3.9 to 6.6% of the total population of cone photoreceptors. However, the proportions of M and L vary widely. The mosaics in (d) illustrate the enormous variability in the L:M cone ratio – the left-hand panel has an L:M ratio of 1:3; the middle panel, 2:1 and the right-hand panel, an extreme 16:1. The scale bar represents 5 arcminutes, which is approximately equivalent to 25 μm.

It is because there are only a few blue (S) cones is that blue and yellow is seen with slightly poorer spatial resolution than other colours. This is demonstrated in (e). When held extended, at a large distance from the eye, the thicker blue and yellow stripes appear more saturated, and the thinner set of stripes appear less saturated. This is because the thin stripes are closer to the spatial resolution limit of the S cone mosaic. The consequence of this for microscopical fluorescence images (e.g. for CFP or YFP) is to pseudo-colour the label of interest white (for high contrast) or green (for high sensitivity) rather than cyan or yellow when discriminating fine detail in the image.

Source: (a,b) Tilley 2011. Reproduced with permission from John Wiley & Sons. (c) Skatebiker at English Wikipedia, via Wikimedia Commons. (d) Professor H. Hofer. Courtesy of Professor Hofer. (e) Adapted from Professor D. Heeger. http://www.cns.nyu.edu/~david/courses/perception/lecturenotes/color/color.html

discerned. The important factor here is the *viewing angle*, subtended at the eye by a feature in the object, and which increases as the object is moved closer to the eye.

The other limitation is exhibited when the lens of the eye reaches the end of its close-focusing adjustment (Figure 1.17, its *accommodation*), usually with the object at a distance of about 250 mm for a normal adult eye. Moving the object closer will now no longer help because, although the viewing angle is increased, the lens cannot bend sufficiently to maintain the image in focus. This *near point* defines the nearest distance of distinct vision, sometimes called the least distance of distinct vision. It is the point at which the lens of the eye is maximally accommodated.

Young people generally have a near point closer than 250 mm, but as we age, our power of accommodation decreases. The lens and ciliary muscle both become less flexible, and we gradually lose the power to accommodate. Our near point recedes, and we must perforce hold a newspaper (or this book!) further from our eyes[12] in order to read the print. This condition is called presbyopia (Figure 1.18) and is the reason why older people need spectacles for close vision.

Magnification can be expressed as a ratio between the tangent of the viewing angle when an object is observed with a magnifying system (such as a magnifying glass) and the tangent of the viewing angle subtended by the same object when observed by the naked eye at an agreed distance. A viewing distance of 250 mm, between the object and the vertex of the cornea, is taken as the International

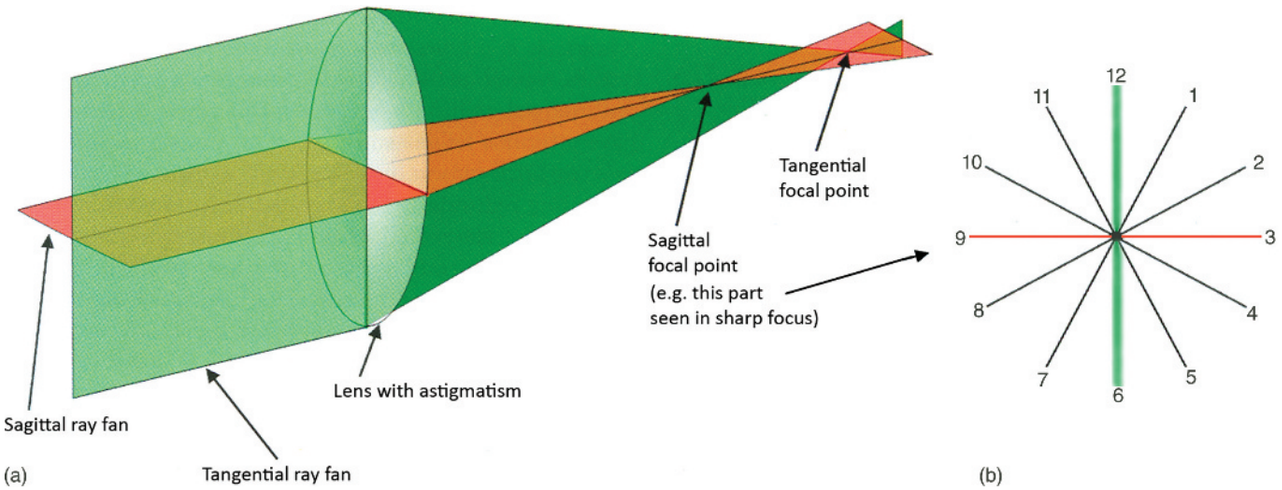

Figure 1.13 ***Astigmatism*** *The vertical and horizontal planes are identified as* tangential *and* sagittal *meridians, respectively. When imaged with an astigmatic lens, part of an object (e.g. a vertical bar) in the tangential plane (shown here in green) will be focused at a different point to a separate part of the same object (e.g. a horizontal bar) in the sagittal plane (shown here in red). This means that the entire object can never be wholly seen in sharp focus without the aid of corrective lenses. Source: Allen and Triantaphillidou 2011. Reproduced with permission from Taylor & Francis Group.*

Standard *Reference Viewing Distance* for expressing magnification. How the size of the image formed with a magnifying glass can be calculated is shown in Chapter 3.

With a magnifying glass or eyepiece, our eyes form a real image from a lens, which would otherwise form a virtual image. A magnifying lens in front of the eye enables the eye to focus an image from an object lying within the nearest distance of distinct vision. The magnifying glass produces an upright virtual image. This image is the same side of the lens as the object, apparently at infinity. The lens of the eye can thus relax to view the object instead of accommodating. The image is perceived by the eye as if it were at a viewing distance of 250 mm, the reference viewing distance. The eye can form a real image (Figure 1.19), yet the object lies only an inch or so away from the observer. A microscope eyepiece can conveniently be used as a simple magnifier: for example, for inspecting whether an objective requires cleaning. Invert the eyepiece and look through it in the 'reverse' direction. Since it has a short focal length (25 mm), the front of the eyepiece will have to be held very close to the surface of the objective, as explained in Chapter 3, Section 3.8.

In ophthalmology, and when using single lenses as simple magnifiers, the overall magnification is calculated as an *angular* measure. The angle θ subtended by the object at the nodal point of the eye determines the retinal image size. Sometimes it is advantageous not to have to calculate linear magnification from the ratio of image size to object size. Either it may not be possible to approach the object to measure its size or the image may be a virtual one so that its size cannot be directly measured.

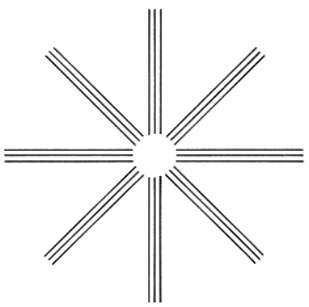

Figure 1.14 ***Test for astigmatism*** *Close one eye, and view this figure without glasses or contact lenses. Hold the figure sufficiently close so that all lines look blurred. Steadily move the figure away until all or only one set of lines is in sharp focus. If all lines are in sharp focus, you don't have astigmatism. If one set is in focus, rotate the figure to see if the lines become blurred (another set may become sharper). Re-orientate the figure as before; continue to move it until lines perpendicular to the first set are sharply focussed. Repeat the procedure with your glasses or contact lenses to see if your astigmatism is corrected. Source: Falk et al. 1986. Reproduced with permission from John Wiley & Sons.*

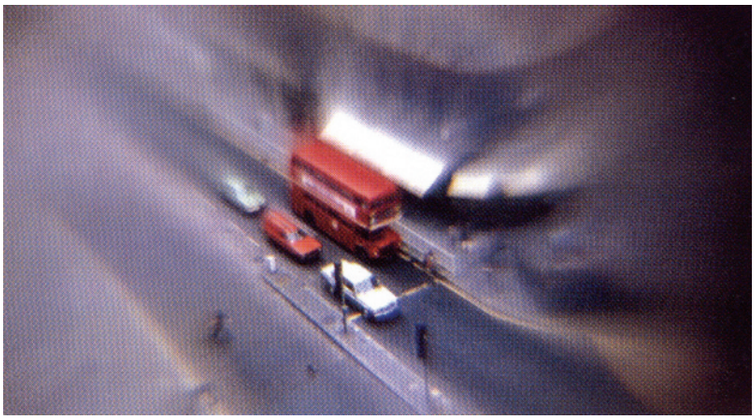

Figure 1.15 ***The visual field*** *If our eyes were to operate in a fixed fashion without employing microsaccadic movements to allow our brains to tile together a wider and sharper field of view, this is what we would see – assuming that the photopsins of the cones are not bleached. Our vision would suffer from spherical aberration, being particularly noticeable at the edges of our field of view.*

1.7 Using Lenses to Correct Eye Defects

The far point is the furthest point at which an object can be placed so that a clear image is formed on the retina. For people with normal vision, the far point lies at infinity (Figure 1.17). Emmetropia (from Greek *emmetros*, 'well-proportioned') describes the state of the normal eye when an object placed at infinity is in focus with the lens relaxed. For all practical purposes, infinity is considered to be greater than 30–40 times the focal length of the lens. For our eyes, this is greater than 6 metres or 20 feet away (6 metres is roughly 350 times 17 mm, the approximate focal length of the normal eye). The wavefront of light rays coming from an object at infinity are essentially parallel, and the rays are focused on the retina without any effort of accommodation.

Myopia is a condition, often called near-sightedness, whereby images that are far away from the eye appear blurred. This is either due to axial myopia or refractive myopia (Figure 1.20). In the case of axial myopia, the globe of the eyeball is too long, so the surface of the retina does not lie at the rear focal plane of the lens. Consequently a clear image cannot be formed. If the eyeball is of the correct dimensions but excessive curvature exists either in the cornea or the lens, then refractive myopia occurs.

Hyperopia (or hypermetropia; far-sightedness) exists when an unsharp retinal image is again formed (Figure 1.21), but in this case behind the retina, rather than in front as for myopia. Either the globe is squashed and too short, which is axial hyperopia, or the cornea

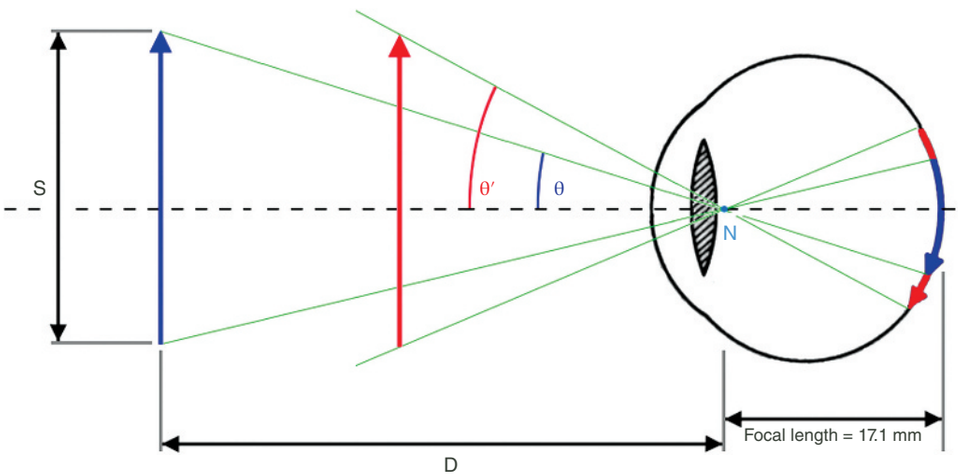

Figure 1.16 ***Ray diagram of an image being formed on the retina*** *The diagram shows the ray construction of a distant arrow in blue with the central ray passing through the nodal point of the lens of the eye. The nodal point, N, lies between the front and back focal points of the relaxed eye, which is 17.1 mm; it is effectively the centre of the lens. The visual angle θ is the angle subtended by the blue arrow at the eye, usually stated in degrees of arc. As the arrow is brought closer to the eye, the image is spread further over the retina, is detected by more and more cones and becomes easier to see. The visual angle, θ, in degrees, is given by $\theta = 2 \times \arctan(S/2D)$. Source: Adapted from Abbott 1984 with permission from Pearson.*

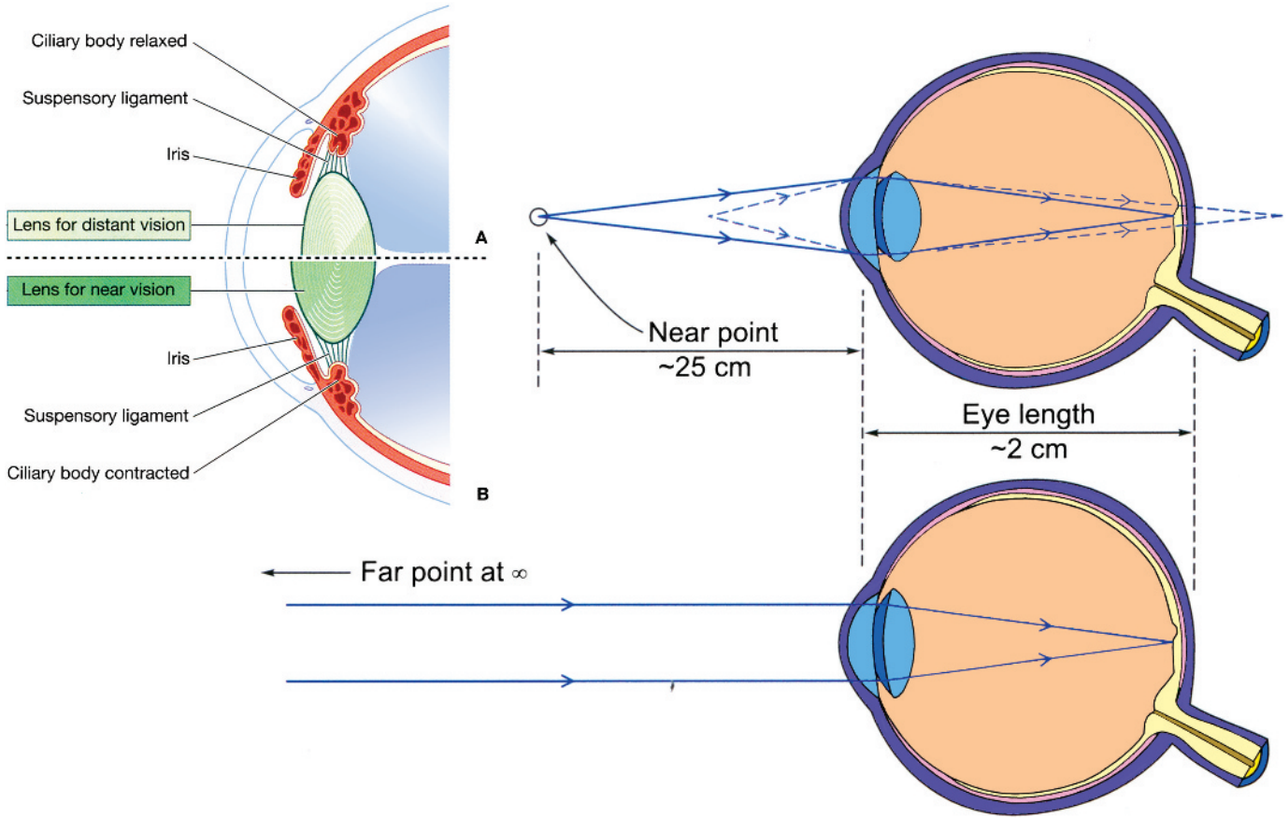

Figure 1.17 ***Shape of the lens during accommodation*** *In the relaxed eye the lens is about 3.6 mm thick at the centre; in the accommodated eye, it thickens to about 4.5 mm. The focal length changes from about 17 mm to about 14 mm, respectively. Source: (left) Waugh and Grant 2001. Reproduced with permission from Elsevier. (right) Adapted from Franklin et al. 2010 with permission from John Wiley & Sons.*

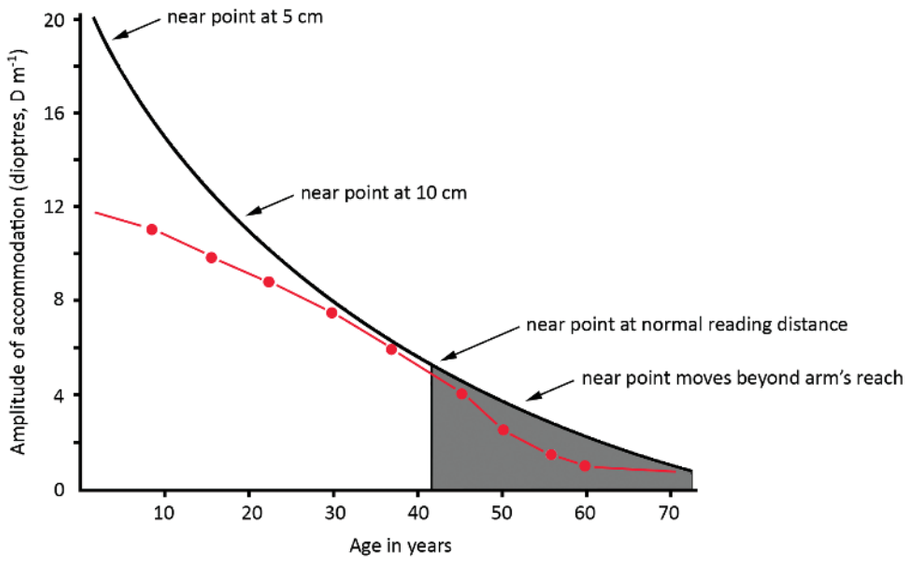

Figure 1.18 ***Presbyopia*** *As we age, our ability to accommodate is gradually lost as the lens and ciliary muscle become less flexible. The inverse hyperbolic curve shows how the near point recedes with age. The red line shows how the dioptric power of our eye decreases with age, from around 10 dioptres in mid-teens to 1 dioptre at 60-plus years. The dioptre measures the refracting power of a lens and is the reciprocal of the equivalent focal length in metres. The greater the dioptre value, the shorter the lens focal length. Source: Dr P. A. Howarth. Adapted with permission from Dr Howarth.*

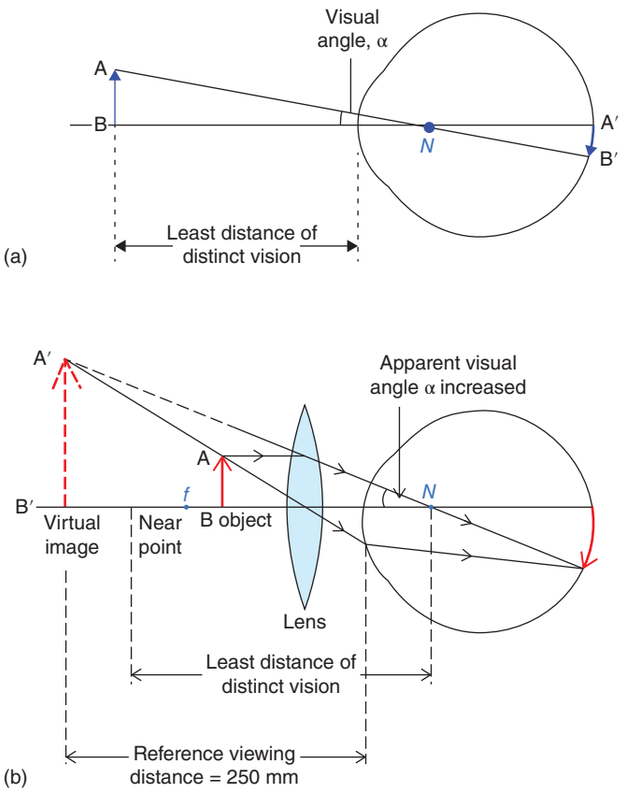

Figure 1.19 ***Action of a single lens to increase visual acuity*** *A positive (biconvex) lens acting as a magnifying glass or simple microscope. The object is located within the near point of the eye and within the focal length of the lens. The virtual image so formed is erect, laterally correct and magnified, and the apparent visual angle subtended at the eye is increased. Source: Bradbury 1967. Reproduced with permission from Elsevier.*

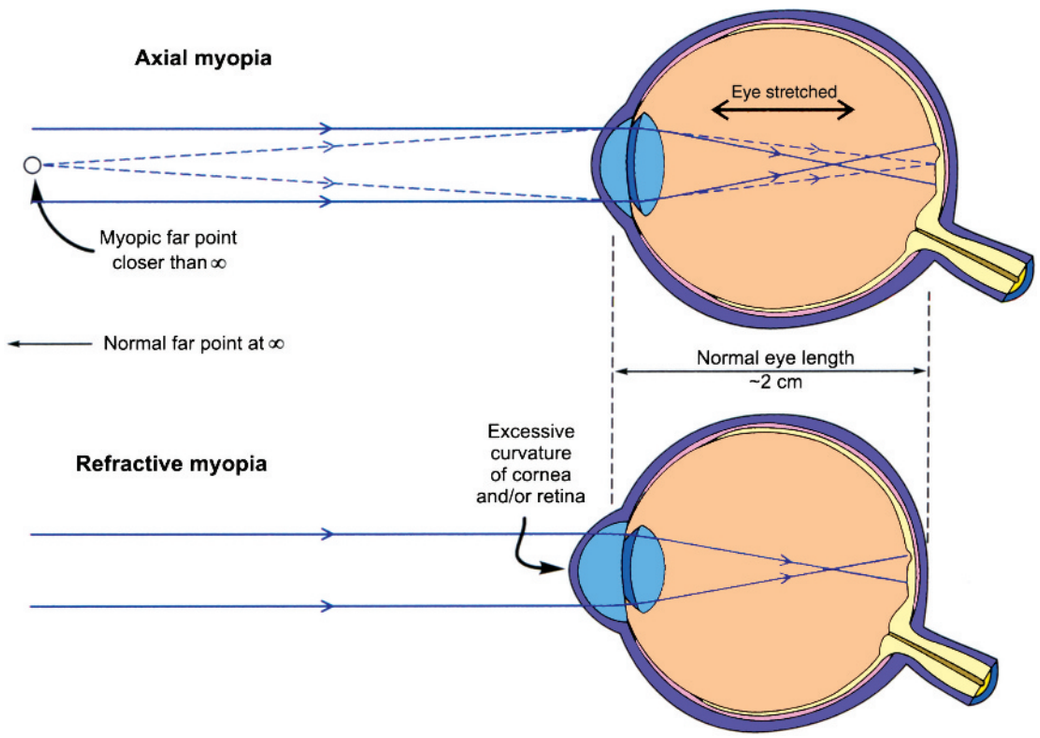

Figure 1.20 ***Myopia*** *In myopia the image is focused in front of the retina. Source: Franklin et al. 2010. Reproduced with permission from John Wiley & Sons. Compare this figure to Figure 1.17.*

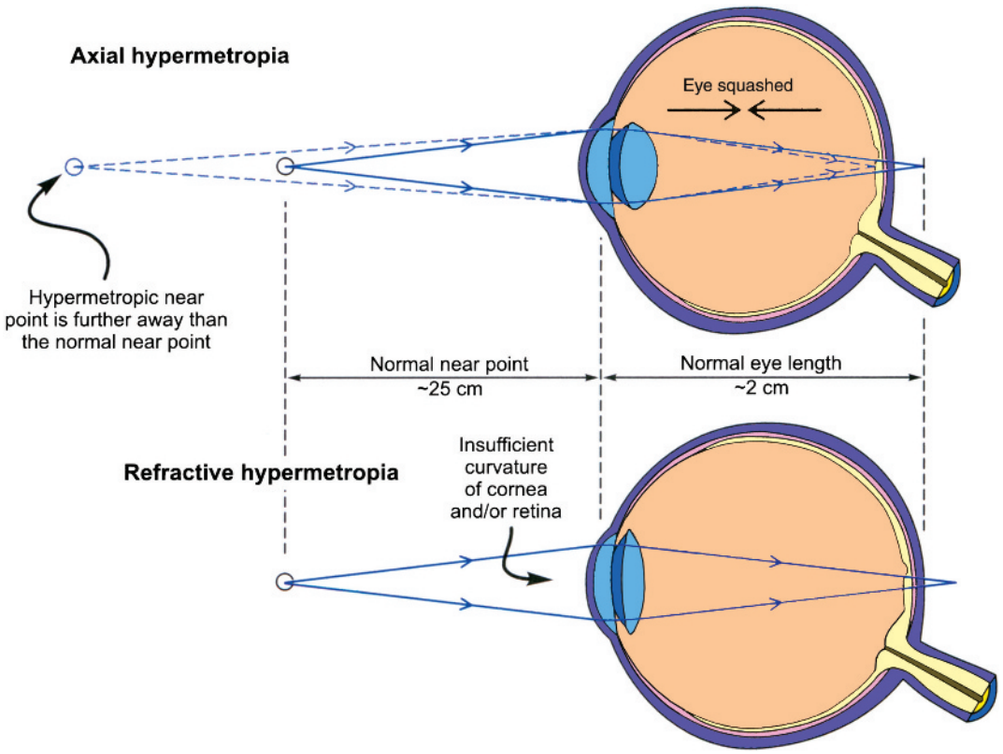

Figure 1.21 ***Hyperopia*** *In hyperopia the image is focused behind the retina. Source: Franklin et al. 2010. Reproduced with permission from John Wiley & Sons.*

and/or lens cannot bend or refract the rays of light sufficiently to form an image focused onto the retina. This is refractive hyperopia. The near point of a hyperopic eye lies further than the conventional 250 mm. The near point also recedes as presbyopia gradually occurs (Figure 1.18). Opticians normally prescribe biconvex, plano-convex or positive convex-concave (i.e. convergent) meniscus lenses (Figure 1.22) to correct these defects.

1.8 Seeing the Scientific Image

When viewing an object in our immediate environment, we generally have a rough idea of its size by comparison with other objects or people around us or from our own knowledge. We form this knowledge through experience of our three-dimensional world and

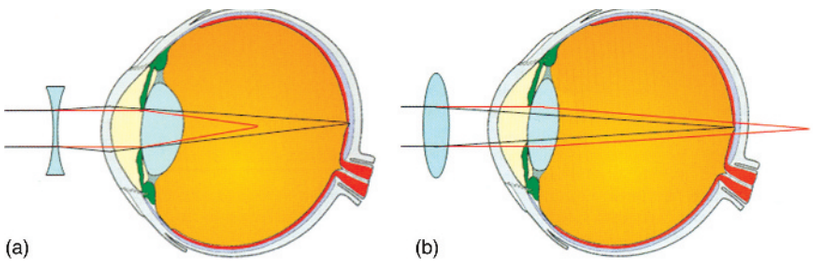

Figure 1.22 ***Correction of myopia, hyperopia and presbyopia*** *A myopic eye (a) will have excessive positive dioptric power (see Chapter 3, Section 3.4, for an explanation of dioptres) and is corrected with a negative (diverging) lens to refocus the image upon the retina. A hyperopic eye (b) has insufficient positive dioptric power, requiring correction with a positive (converging) lens to refocus the image. The same is true for a presbyopic eye, which has lost the power to accommodate sufficiently to form a sharply focused image upon the retina. Source: Allen and Triantaphillidou 2011. Reproduced with permission from Taylor & Francis Group.*

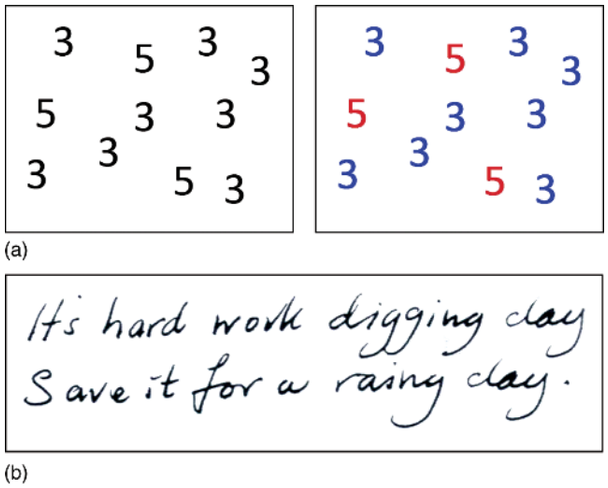

Figure 1.23 ***Using colour to discriminate patterns and detail*** *The eye is good at recognising patterns, colours and shapes: colour is useful for perceiving related groups quickly; pattern recognition helps to understand an otherwise ambiguous message, arising from use of the identically written words 'clay' and 'day'. Source: Russ 2004. Reproduced with permission from John Wiley & Sons.*

its perspective. The eye is very good at colour and pattern recognition and separating shapes from each other. The primary function of human vision is recognition, whether searching for food, avoiding danger or seeking a mate. We do this by comparison, based on experience. This hardwired ability is excellent in enabling us to cross the road and meet a friend, for example, but it is not suited to analysing scientific images, for which we have no requirement in evolutionary terms.

Because we scan our visual field rapidly and our mind 'fills in' details from previous experience, we naturally invest only the briefest amount of time assessing our environment for food, danger or mates. We can recognise a familiar scene with the barest minimum of visual clues because of the human ability to discriminate patterns, colours and shapes. This is what cartoonists rely upon to convey their message. Figure 1.23 illustrates our ability to employ colour to lift specific details from amongst a pattern or to use both context and redundancy to correctly understand a message when the image is wrong or ambiguous. The drawback is that we may miss subtle changes in our images: alterations in contrast, grey levels, colour or dynamic changes over time, if these changes are not abrupt. We certainly cannot hold all the details of an image in our mind's eye; our mental images are transient, comprising scant detail at low resolution. We are also susceptible to change blindness, and may fail to notice subtle changes details in successive images.

Computers, however, are very good at processing large amounts of data, at analysing and measuring, which we cannot do effectively (e.g. particle tracking through a Z-stack data set: we track particles by toggling back and forth between consecutive frames to decide on correspondences). Many microscopical samples are sections of tissues or flat surfaces of opaque materials. In these cases the images are two-dimensional representations of part of a 3D object. Without specific experience of the object being studied, it is very difficult for most people to place images of plane sections into a proper context. We are more used to seeing the external surfaces of solid objects. For most people it is challenging to imagine the appearance and shape of a 2D section cut through a familiar object (Figure 1.24). Without prior experience, it is more difficult to perform the exercise in reverse and to reconstruct a 3D object from a set of plane images (Figure 1.25). Yet this is what microscopists have to do.

While computers excel at handling data sets in ways that we find challenging or impossible, it is, however, very difficult to write effective algorithms and programmes to enable computers to fully 'discriminate' and analyse patterns and shapes in the way that humans do. Facial recognition is a case in point: we perceive a face as very different if only a few key features are altered, but it is very difficult to programme a computer to do the same with equal sophistication. When we recognise someone or something, we unconsciously endow the familiar person or scene with a full set of characteristics, according to our expectation and preconceptions. In short we have 'seen and interpreted' not '*observed* and interpreted'. This makes us prone to bias. When publishing scientific images, we can easily be biased; journal editors are aware that our 'typical' cell may really be 'the nicest picture that we have'.

Images are not merely pretty pictures, they are scientific data: a microscopical image is essentially a sampled 'photon intensity map'. The context of the image is important for interpreting data correctly. This point is made in the introduction to this book, in advice given by Henry Baker from one of the earliest instruction manuals on microscopy, when instruments collected far less complex data sets than they do nowadays.

1.9 Sizes of Objects

The eye-brain combination is very poor at estimating – let alone quantifying – size, length, area or volume. While we have a very well-developed sense of recognising familiar objects and patterns, we find it harder to describe and may fail to detect those that are

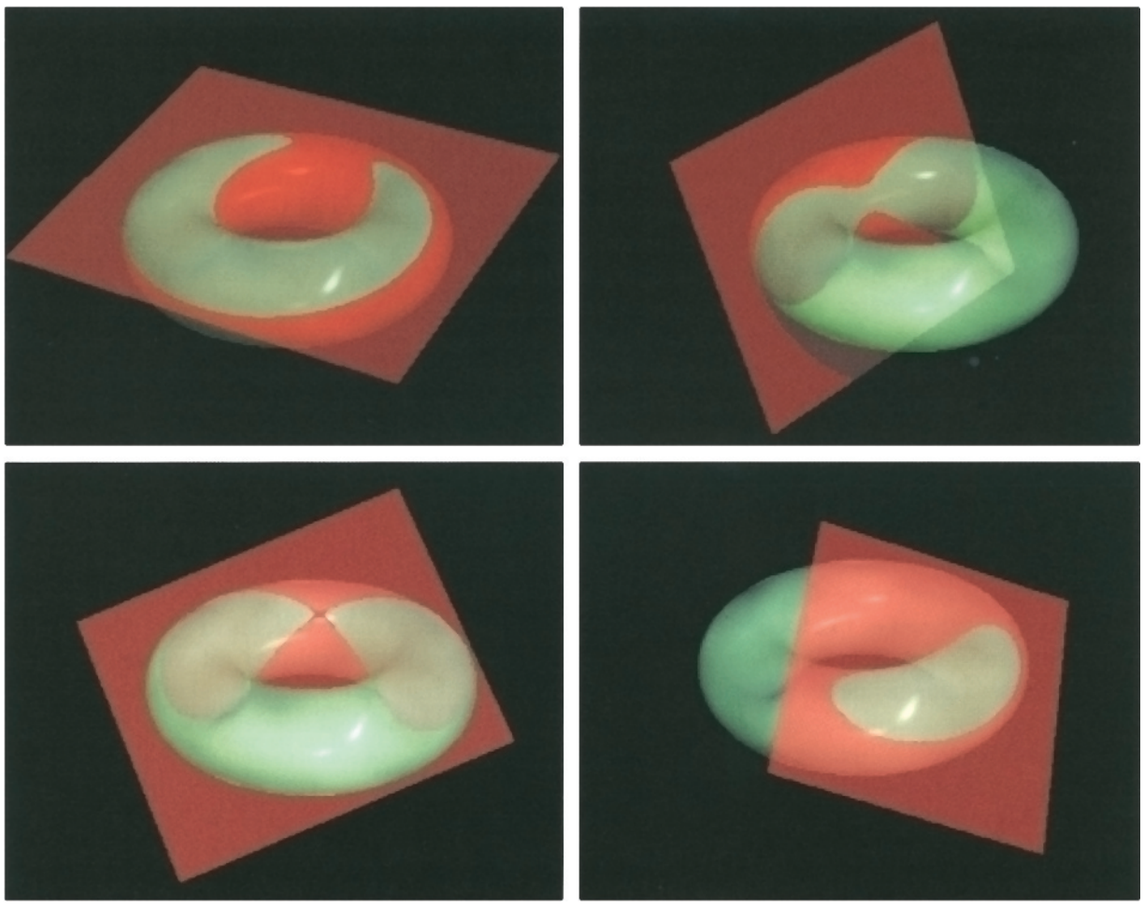

Figure 1.24 ***Calculating the appearance and shape of a 2D section*** *A bagel is a simple torus, which we recognise easily. It is more difficult to predict the shape of various surfaces as the bagel is cut through with a knife. Source: Russ 2004. Reproduced with permission from John Wiley & Sons.*

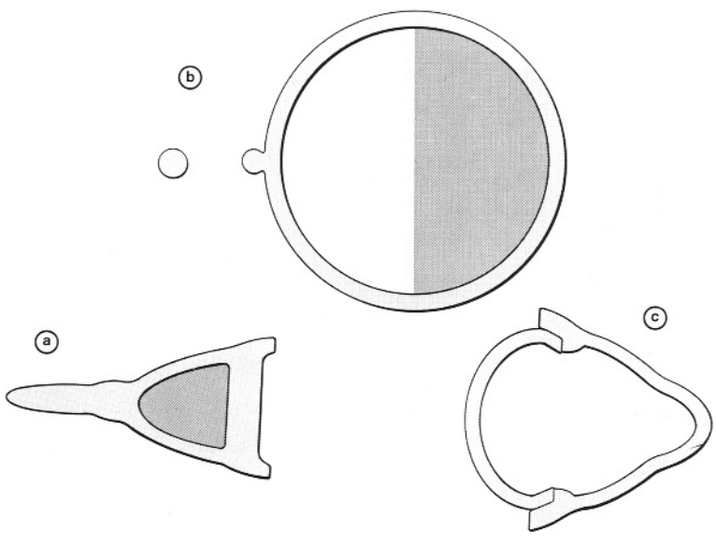

Figure 1.25 ***Reconstructing a 3D object from a set of plane images*** *Here the reverse problem to that presented in Figure 1.24 is illustrated. Three 2D sections derived from cutting through a familiar object are shown. It is difficult to visualise the original 3D object in our minds. Once presented with the answer (which may be found at the end of the chapter) the solution appears very simple! Source: Rogers 1983. Reproduced with permission from Elsevier.*

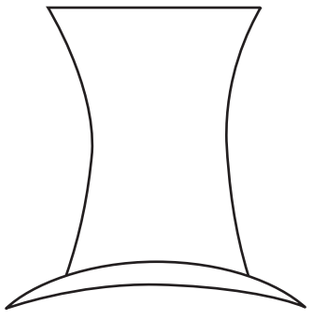

Figure 1.26 ***Top hat illusion*** *Our brains generally consider vertical dimensions to be more important than horizontal ones. Both dimensions are actually equal in length. Source: Russ 2004. Reproduced with permission from John Wiley & Sons.*

unfamiliar. Optical illusions depend upon these two weaknesses in order to work. It is also why eyewitness accounts of accidents or crime scenes vary so much and are often unreliable. Figure 1.26 shows a simple example: our brains generally consider vertical dimensions to be more important than horizontal ones. The top hat is, in fact, drawn within a square, and both dimensions are equal in length. Nevertheless, measurement is an essential part of the description and understanding of any object and/or its image – to put it into a proper perspective.

In microscopy, unless we are very familiar with the two-dimensional image presented to us, we have no idea of scale. The problem is compounded when studying a small field of view (at high magnification) with few or no features of reference that we can recognise. Is the image taken with a 10x or a 100x objective? Has it been further enlarged? Unless such features whose size we know are present in the image, we have no way of knowing the overall size of our image or the size of smaller components within it. We need to include an absolute linear scale with our images (for a description of how to do this, see Chapter 31, Section 31.8), or else we need to know the size limits of the objects we are studying. An appreciation of the scale and size of the images that we take with the microscope is important (although the importance of this is often overlooked) so that we can communicate this to our audience. Quite apart from being scientifically rigorous, giving our audience a scale with our image(s) imparts confidence and trust in what we have to say.

This book is only concerned with the light microscope, which will magnify from approximately 10 times[13] at the lower extreme to about 1500 times as an upper limit and resolve detail down to about 0.2 µm. The light microscope uses photons to illuminate the object and so form an image, but much greater detail may be resolved and seen if electrons are used. An electron microscope can resolve detail down to 0.2 nm under favourable circumstances.

A useful way to remember these values is 'the rule of twos'. The limit of resolution of the human eye is very approximately 0.2 mm at 250 mm distance; the limit of resolution of the light microscope 0.2 µm; the limit of the electron microscope, approximately 0.2 nm (depending on the sample).

From ants to atoms, there is a widely diverse range of both living and nonliving biological objects studied using the microscope. They range in size from metres to nanometres (Figure 1.27). Representative sizes of cells and organelles are given in Box 1.1. Until recently there was a dearth of published information on the sizes of cells and organelles, but the very useful BioNumbers online database (for the URL Internet link, refer to the end of the chapter) has redressed this issue. Entries have supporting references and a unique ID number; hence an audit trail underpins all data entered. Although the life we see around us appears very diverse, it is remarkably similar at the molecular level in the size range from 10 µm to 0.1 nm. The light microscope is used to study the upper part of this range: from 10 µm to 0.2 µm. The rapidly developing field of so-called super-resolution microscopy (see Chapter 26) – better called diffraction unlimited fluorescence microscopy – is driving the lower limit of resolution down towards 0.1 µm and beyond.

Box 1.1 The Size of Biological Structures

Many microscope users who come to use core facility microscopes often come to acquire images of labelled proteins and cell structures within tissues with little, if any, idea regarding the size of the structures they wish to study. So much thought and preparation has gone into the experimental design and study of the biological relevance of their proteins of interest that they have neglected to think sufficiently about the size, thickness and volumes of the samples they have prepared. A proper understanding of the sizes of the cells and tissues you look at under the microscope will allow you to decide whether to acquire an image at the limit of resolution or not, to choose the correct objective(s) and make any necessary adjustment to the pixel size of the PMT or camera detector for successful image acquisition. The associated database can be found at http://Bionumbers.org.

SnapShot: Key Numbers in Biology

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Cell

Cell size

Bacteria *(E. coli):* ≈0.7-1.4 μm diameter, ≈2-4 μm length, ≈0.5-5 μm^3 in volume; 10^8-10^9 cell/ml for culture with OD_{600}≈1

Yeast *(S. cerevisiae):* ≈3-6 μm diameter ≈20-160 μm^3 in volume

Mammalian cell volume: 100-10,000 μm^3; HeLa cell: 500-5000 μm^3 (adhering to slide ≈15-30 μm diameter)

Length scales inside cells

Nucleus volume: ≈10% of cell volume

Cell membrane thickness: ≈4-10 nm

"Average" protein diameter: ≈3-6 nm

Base pair: 2 nm (D) x 0.34 nm (H)

Water molecule diameter: ≈0.3 nm

Energetics

Membrane potential ≈70-200 mV → 2-6 k_BT per electron ($k_BT \equiv$ thermal energy)

Free energy (ΔG) of ATP hydrolysis under physiological conditions
≈40-60 kJ/mol → ≈20 k_BT/molecule ATP; ATP molecules required during an *E. coli* cell cycle ≈$10-50 \times 10^9$

ΔG^0 resulting in order of magnitude ratio between product and reactant concentrations:
≈6 kJ/mol ≈60 meV ≈2 k_BT

Concentration

Concentration of 1 nM:
in *E. coli* ≈1 molecule/cell;
in HeLa cells ≈1000 molecules/cell

Characteristic concentration for a signaling protein: ≈10 nM-1 μM

Water content: ≈70% by mass; general elemental composition (dry weight) of *E. coli:* ≈$C_4H_7O_2N_1$; Yeast: ≈$C_6H_{10}O_3N_1$

Composition of *E. coli* (dry weight): ≈55% protein, 20% RNA, 10% lipids, 15% others

Protein concentration: ≈100 mg/ml = 3 mM. 10^6-10^7 per *E. coli* (depending on growth rate); Total metabolites (MW < 1 kDa) ≈300 mM

Division, replication, transcription, translation, and degradation rates

at 37°C with a temperature dependence (Q10) of ≈2-3

Cell cycle time (exponential growth in rich media): *E. coli* ≈20-40 min; budding yeast 70-140 min; HeLa human cell line: 15-30 hr

Rate of replication by DNA polymerase: *E. coli* ≈200-1000 bases/s; human ≈40 bases/s. Transcription by RNA polymerase 10-100 bases/s

Translation rate by ribosome: 10-20 aa/s

Degradation rates (proliferating cells): mRNA half life < cell cycle time; protein half life ≳ cell cycle time

Diffusion and catalysis rate

Diffusion coefficient for an "average" protein: in cytoplasm D≈5-15 $\mu m^2/s$ → ≈10 ms to traverse an *E. coli* → ≈10 s to traverse a mammalian HeLa cell; small metabolite in water D≈500 $\mu m^2/s$

Diffusion-limited on-rate for a protein: ≈10^8-10^9 $s^{-1}M^{-1}$ → for a protein substrate of concentration ≈1 μM the diffusion-limited on-rate is ≈100-1000 s^{-1} thus limiting the catalytic rate k_{cat}

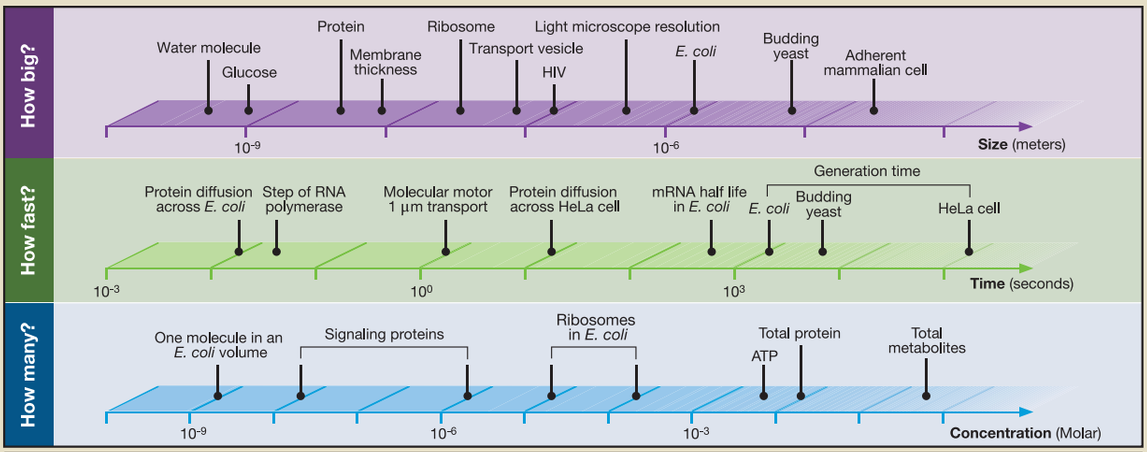
Genome sizes and error rates

Genome size:
E. coli ≈5 Mbp
S. cerevisiae (yeast) ≈12 Mbp
C. elegans (nematode) ≈100 Mbp
D. melanogaster (fruit fly) ≈120 Mbp
A. thaliana (plant) ≈120 Mbp
M. musculus (mouse) ≈2.6 Gbp
H. sapiens (human) ≈3.2 Gbp
T. aestivum (wheat) ≈16 Gbp

Number of protein-coding genes:
E. coli = 4000; *S. cerevisiae* = 6000; *C. elegans, A. thaliana, M. musculus, H. sapiens* = 20,000

Mutation rate in DNA replication:
≈10^{-8}-10^{-10} per bp

Misincorporation rate:
transcription ≈10^{-4}-10^{-5} per nucleotide
translation ≈10^{-3}-10^{-4} per amino acid



Useful biological numbers extracted from the literature. Numbers and ranges should only serve as "rule of thumb" values. References are in the online annotated version at www.BioNumbers.org. See the website and original references to learn about the details of the system under study including growth conditions, method of measurement, etc.

1 Cell *141*, June 25, 2010 ©2010 Elsevier Inc. DOI 10.1016/j.cell.2010.06.019

See online version for legend and references.

Source: Moran et al. 2010. Reproduced with permission from Elsevier.

These values are approximate. Prokaryotes can range between 1 and 10 μm; eukaryote animal cells can be from 10 to 30 μm and higher plant cells generally have a wider distribution of overall size from 10 to 100 μm.

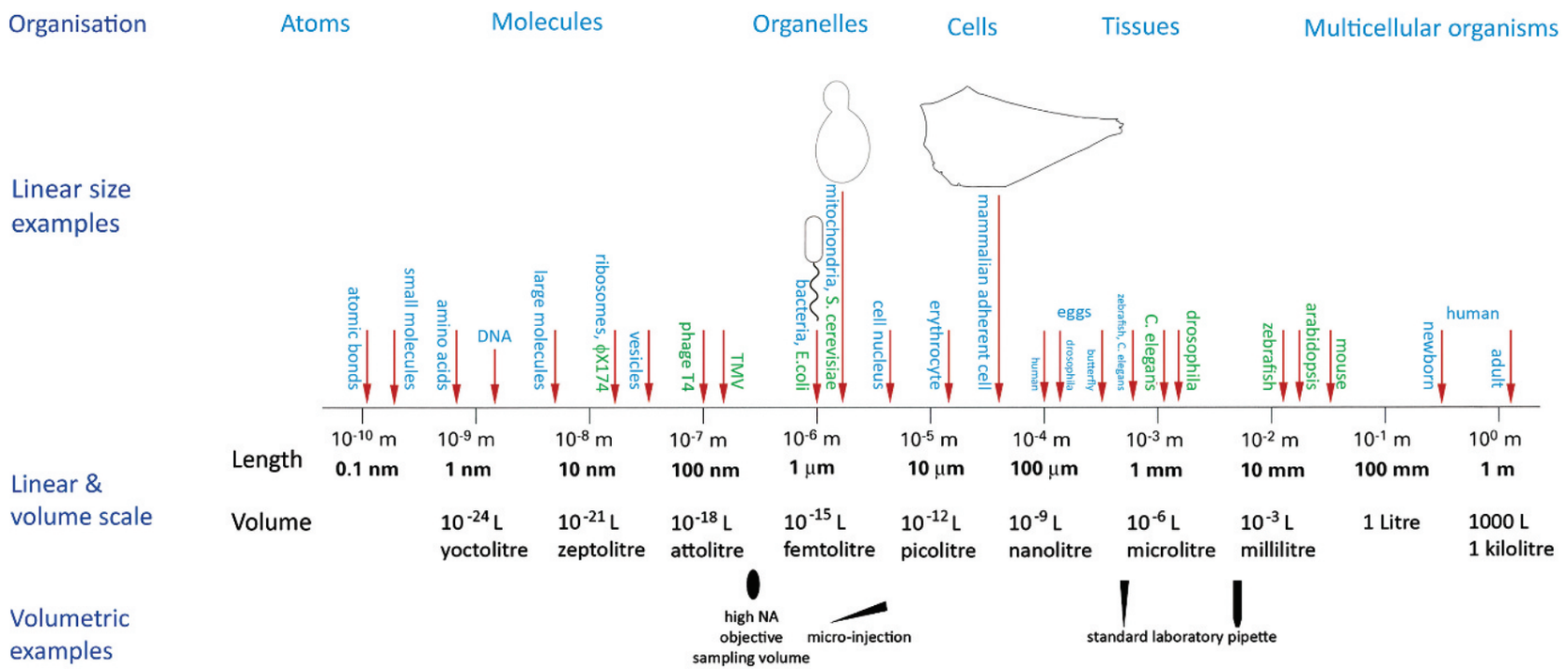


Figure 1.27 ***The size range of specimens studied with microscopes*** *The human eye will resolve details down to approximately 0.2 mm; the light microscope will resolve detail down to 0.2 μm; the electron microscope will resolve detail as fine as 0.2 nm. These are best-case values in each case, but it is possible to study fine detail throughout nine orders of magnitude. Source: Adapted from Davey and Lord 2003 with permission from Oxford University Press.*

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West, MJ (2012) Estimating volume in biological structures *Cold Spring Harb. Protoc.* 2012 *11*: 1129-1139. doi: 10.1101/pdb.top071787

The sizes of particles, crystals and fibres encountered in materials science are often larger than biological specimens. They do not usually approach the limit of resolving power of the light microscope, as biological cells, organelles and macromolecules do. In metallurgy, fine crystals are generally 0.1 mm in diameter or length; exceptions include silicon chip components and sintered metal products. The standard thickness of a petrological section is 30 µm, which places a limit on resolving sub-micrometre detail, and the structures observed are generally from 1 µm up to millimetre size.

1.10 Nomenclature and History

The term 'microscope' comes from academic medieval Latin, *microscopium*. It means literally 'an instrument for viewing what is small', derived in turn from the Greek prefix *micros* (small) and Greek *skopein* (to look at). Similarly, telescope comes from the Greek prefix *tele* (afar).

The term microscope should not be abbreviated merely to 'scope'. This is imprecise: there are over three dozen scientific or engineering instruments which use the suffix 'scope', and it could be shorthand for something as diverse as a polemoscope or 'jealousy glass' for use at the opera, or a hagioscope (a hole or squint in a church wall) enabling lepers to view the elevation of the host from outside a medieval church – a microscope is not designed for squinting through! Scope, used as a word in its own right, means opportunity, or else refers to the slack left in an anchor cable.

Knowing the evolution and historical development of the light microscope is not necessary to a practical understanding of how to operate it successfully. For those interested in a short account of the origin of the light microscope, refer to Appendix 6. Appendix 7 lists significant dates in the history of microscopy and optics within a wider scientific context.

1.11 Chapter Summary

At the end of this chapter you should understand how the eye works and its limitations. You should also know why we need a microscope to study small objects and have an idea of the size ranges of the samples and specimens that are examined with the microscope.

1. Any image, whether we observe the image directly or first record it, must ultimately be seen by the eye.
2. Our eyes respond to light from near UV at a wavelength of 380 nm to deep red at 710 nm.
3. We discriminate colour very well, although brightness less so. Consequently we must use look-up tables (LUTs) to set the limits of dynamic range for digital cameras and photomultiplier tubes.
4. Our colour vision and visual acuity are determined by the cone photoreceptors in our retina; the rods control the sensitivity of our dark-adapted sight. Humans possess good visual acuity, being able to see high contrast detail approximately 0.1 mm in size. We possess stereoscopic vision and have good contrast discrimination.
5. The single-lens eye suffers from both spherical and chromatic aberration. These defects are minimised by both physiological design and psychological interpretation of the image: by tiling a narrow paraxial visual field, by optimising pupil size and by reducing our response to blue light.
6. We require lenses to view small objects, when our eyes cannot accommodate sufficiently to see a sharp image at sufficient size in order to resolve fine detail. We also require lenses to correct for common eye defects, such as myopia, hyperopia, presbyopia and astigmatism.
7. The eye is very good at colour and pattern recognition and separating shapes from each other. The brain is poor at reconstructing a 3D object from a set of plane images, or quantifying sizes. We are prone to bias when analysing and interpreting scientific images.
8. The light microscope will magnify from approximately 10 times to about 1500 times as an upper limit and resolve detail down to 0.2 µm.

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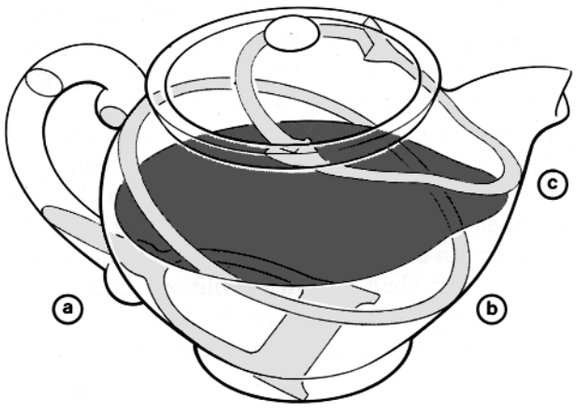
Answer to Figure 1.25



Source: Rogers 1983. Reproduced with permission from Elsevier.

Key Reading

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- A comprehensive chapter, with all the facts and figures about the eye well explained and easily accessible. At the time of writing, it is available as a free download from the Wiley website.

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Originally published as three articles in the *Proceedings of the Royal Microscopical Society*, the article is available on-line from John Russ' website: http://www.drjohnruss.com/downloads/seeing.pdf (accessed 1 June 2018)

Part 1: *Proceedings of the Royal Microscopical Society* June *39*/2: 97–114.
Part 2: *Proceedings of the Royal Microscopical Society* Sept *39*/3: 179–193.
Part 3: *Proceedings of the Royal Microscopical Society* Dec *39*/4: 267–281.

Internet Resources

BioNumbers database. URLs:
1. http://bionumbers.hms.harvard.edu/
2. http://en.wikipedia.org/wiki/Bionumbers

Optical radiation terminology and units (Newport). URL: http://assets.newport.com/webDocuments-EN/images/Optical_Radiation_Terms_And_Units.PDF

Notes

1. The familiar terms 'intensity' and 'brightness' are subjective attributes, having no measurable units. The term 'irradiance' or 'radiant flux density', which measures the radiative flux of electromagnetic radiation incident on a surface in watts per square metre (W/m^2) should be used instead of 'intensity'. However 'intensity' is widely understood as a popular term for the qualitative measure of the amount of light present, or incident, upon a surface. Two parallel systems are used to measure light. Radiometric quantities measure absolute electromagnetic power; photometric quantities characterise light as perceived by the human eye. The eye has different sensitivities to light of different wavelengths within the visible spectrum. The photometric unit equivalent to irradiance (E_e) is illuminance (E_v). Standards organizations recommend that photometric quantities be denoted with a suffix 'v' (for visual) to avoid confusion with radiometric quantities.

   Luminous flux (Φ_v) is the measure [in lumens; lm = cd·sr] of the perceived power of light, the rate of flow of energy – over the visible spectrum – emitted by a source in all directions. When adapted for bright conditions (photopic vision), the eye is most sensitive to green-yellow light at 555 nm. Luminous flux (also called luminous power) is the photometrically-weighted radiant flux, or power. Light with the same radiant flux (Φ_e; watts) at other wavelengths, say red, has a lower luminous flux. A 1000 watt heater gives off a large radiant flux, but as a light source, it radiates very few lumens. Most of the energy is in the infrared, leaving only a dim glow visible to the eye. Luminous flux differs from radiant flux, which is the measure of the *total* power of light emitted in all directions and measured in watts (W), because luminous flux is adjusted to reflect the varying sensitivity of the human eye to different wavelengths of light. For monochromatic light of 555 nm, 1 watt is equal to 683 lumens. The solid angle in every direction all around a point is equal to 4π sr (steradians). The total luminous flux of a one-candela point source is 4π lumens.

   Luminous intensity (I_v) is slightly different from luminous flux. Luminous intensity is the perceived power *per unit solid angle* projected in a given direction, a measure [in candelas; cd = lm/sr] i.e. of luminous flux per unit solid angle. Radiant intensity (I_e), the corresponding radiometric unit, is measured in watts per steradian (W/sr).

   Illuminance is a measure [lux = lm/m^2] of how much luminous intensity from a source is incident on a surface, spread over a given area; it is a measure of the *intensity* of the incident light. Illuminance is inversely proportional to area. Luminance (L_v) is illuminance ($lm/m^2/sr$) per unit solid angle, whilst the radiometric equivalent is radiance (L_e), which is irradiance ($W/m^2/sr$) per unit solid angle per unit projected area: the flux per unit area received on a surface, for example, by a detector.

   Luminance is a measure [cd/m^2] of the 'density' of the luminous intensity per unit of light travelling in a given direction, and of how much luminous flux will be detected by an eye looking at an illuminated surface. It describes the amount of light that passes through or is emitted from a particular area, and falls within a given solid angle. Luminance most resembles the perception of brightness. Luminance is often used to characterise emission or reflection from flat, diffuse surfaces. Lightness is the perceptual correlate of diffuse reflectance. For example, a well-lit room has a combined high luminous flux; the light is divergent, so it fills the room. A laser pointer has very low luminous flux (it cannot illuminate a room) but a dangerously high luminous intensity (and luminance) off a shiny surface. Luminance is sometimes incorrectly referred to by the term luminosity.

   Luminous efficacy (η) is a measure [lumen per watt = lm/W] of how well a light source produces visible light. It is expressed as the ratio of luminous flux to radiant flux or luminous flux to power consumption, depending on context.
2. For those interested, this discrimination of luminance is discussed further in Box 11.1. It is because of the logarithmic response of the eye to luminance that gamma correction is required in digital displays.
3. The fovea is a tiny depression in the retinal tissue (see Figure 1.8) within the centre of the macula lutea of the retina which contains only cones. In the light-adapted state, the fovea not only has the highest visual acuity, but also especially high sensitivity for luminance and colour discrimination. Approximately half of the nerve fibres in the optic nerve carry information from the fovea, whilst the remainder serve the rest of the retina.
4. A darkroom should not be painted black, but have white walls and controllable lighting. The white walls reflect rather than absorb the light, so allowing management of the intensity level and colour of the illumination. EM Nelson, a famous 19th century microscopist (Box 31.2) took great care to ensure his night vision was well adapted for all his high-resolution microscopy.
5. When we swim under water without a face mask, because the refractive index of the cornea (n = 1.376) is very close to that of water (n = 1.333) we are reminded of the role of the cornea in focusing the image onto the retina. Whilst swimming we can only see an image which is very blurred indeed, if at all. This is because hardly any focusing occurs at the submerged cornea; we lose 40 dioptres of focusing power. For an explanation of how dioptres are calculated, see Chapter 3.
6. See the definition and meaning of the term 'aperture' in Note 2, in the Notes section.
7. This minimum resolved distance is a rule of thumb, since the eye, like any device, has an MTF (modulation transfer function) response (see Chapter 10). Absolute visual acuity depends upon the nature of the object, but for adjacent high-contrast black-white bars, the calculation is as follows: If the eye can differentiate either a black bar or a white bar of a high-contrast pattern in 72 arcsec then, since one minute of arc (60

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arcsec) = 1/60 degree, a single line pair (1 cycle) must be twice the width of each line, so there are 30 cycles in a degree, and (30 x 1.2) = 36 cycles per degree in 72 arcsec. For individuals with 6/4.5 Snellen visual acuity (see Rostgaard and Qvortrup, 1999) the maximal resolving power is 0.8 arcminute (24 CPD) or 3.8 μm at the retina, which is the approximate diameter of cones within the fovea. See also Box 3.4, Chapter 3, Section 3.11.

8. The size of the retinal area, and hence visual acuity, is determined by the size of the eyeball. Provided that the centre-centre spacing between the photoreceptors remains constant, a larger posterior focal length improves visual acuity. A focusing lens allows for close vision and spreading a larger image over the retina. A large eye size also improves sensitivity, since a large pupil reduces diffraction and lets in more light. A larger pupil relative to eye length gives a smaller *f*-number and increased image brightness; for example, the giant squid in the deep ocean have the largest eyes of the animal kingdom: a 27 cm eyeball with a pupil of 90 mm and aperture approximately *f*/1. Visual acuity is also dependent upon the irradiance and contrast of the object. Humans have better contrast discrimination than most birds and better visual acuity.
9. Polarised light propagates as a wave within a certain field. That is, the vibrations are partially or completely supressed in certain directions, whereas natural light contains multiple wave trains of different amplitudes, wavelengths and polarisation states all travelling, or propagating, in the same direction. Plane-polarised light (see Chapter 13) has the electric field vector of its wave trains all oriented parallel to one another, such that there exists only one vibration direction. With circularly polarised light, the orientation of the field changes, and includes all planes of orientation.
10. On-axis astigmatism is described here; third-order, or off-axis, astigmatism is described in Chapter 6. It is a misconception to think that laser beams are naturally highly collimated: laser diodes have highly astigmatic beams that require optical correction in order to be useful.
11. Our eyes lock onto the fixation point in order to keep a specific 3D location at the centre of our vision. The locus of fixation points that produce a single image at corresponding retinal points of the two eyes, when the eyes are fixating binocularly, is called the horopter. With motion parallax, objects nearer than the point of fixation will move in a direction opposite to the direction of head movement, and those objects farther away will move in the same direction. Employing motion parallax in this way can be useful when focusing onto the film plane or CCD faceplate with a focusing graticule on a photo-microscope.
12. As the near point recedes with age, this causes a corresponding decrease in visual acuity and resolving power of the eye, which occurs without the quality of the retina being compromised, or involved.
13. This is only true for 'high power microscopes' and not stereomicroscopes, which can reduce the size of the image in relation to the object. Super-resolution microscopes can circumvent the classical resolving power limit.