

Chapter 1

Principles of fluorescence microscopy

This chapter will introduce the concept of the light microscope leading into contemporary fluorescence microscopy. Basic geometrical optics and properties of light waves will be presented to understand the construction and functioning of light microscopes. The concepts presented here will be elementary and recalled in later chapters.

1.1 Light microscopy

1.1.1 Construction of light microscopes

The fundamental concept of a light microscope requires a set of lenses to relay and magnify the image of a remote point using light as a measurand. The sample is located in the front focal plane of the *objective lens* and the resultant image is focused onto the *primary imaging plane*, see Fig. 1.1. Light emitted by a point o_1 at the sample plane is transformed into a parallel ray bundle by the objective lens, which travel parallel to the optical axis. The tube lens then refracts the ray bundle back down onto its focus. The ray bundle for an a point away o_2 from the optical axis can be determined by the *chief ray* (r_c) which passes through the optical centre of the objective unperturbed, and the *marginal ray* (r_m) travelling parallel to the optical axis which crosses the back-focal point of the objective lens. Both of these rays propagate in parallel within the *infinity space* between the objective and tube lens, as do all sets of rays at any point o_n at the focal plane of the objective lens.

Magnification

Due to the parallel propagation of rays in the infinity space the distance between the two lenses may be spaced arbitrarily, though typically the back focal points are matched to create a *4f system*, see Fig. 1.1.

The marginal (r'_m) and chief (r'_c) rays incident on the tube lens then govern where a real image of the sample lies at the *primary image plane*. o'_1 The image size (I) in the primary image plane is set by the distance between the intersection of the primary image plane to the intersect of the the margin (r'_m) and chief rays (r'_c) at the tube lens.

$$\tan \alpha = \frac{O}{f_{\text{objective}}} = \frac{I}{f_{\text{tube lens}}} \quad (1.1)$$

It follows that the magnification of the sample is:

$$\implies M = \frac{I}{O} = \frac{f_{\text{objective}}}{f_{\text{tube lens}}} \quad (1.2)$$

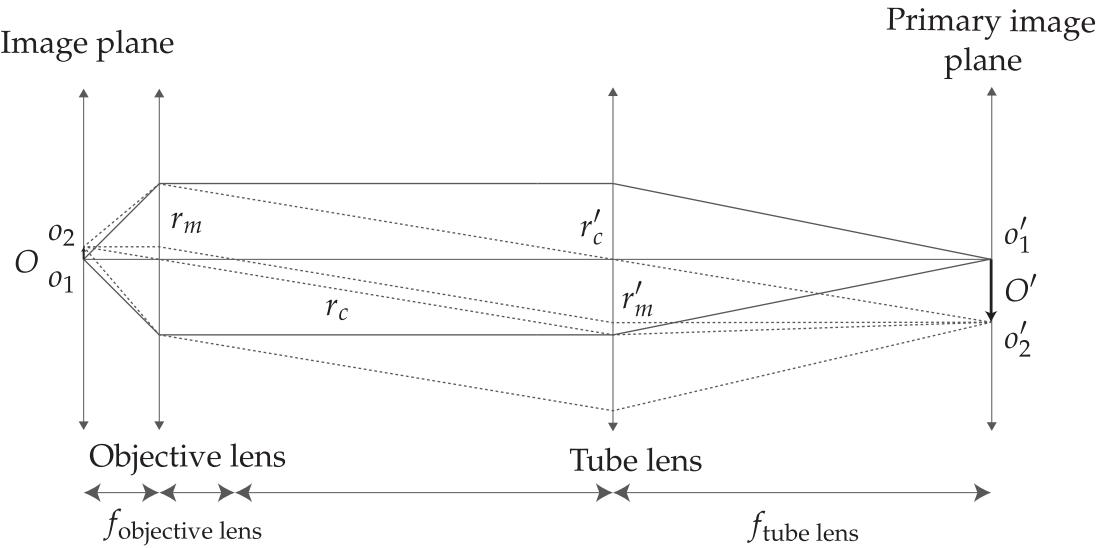


Fig. 1.1 Ray diagram of a two lens magnification system.

Field of view

The observable objective field (field of view) is limited by the aperture stop of the system typified as the field number (F_n) and the objective lens magnification M_{object} :

$$FOV = \frac{F_n}{M_{\text{object}}} \quad (1.3)$$

The field number of objective lens design is limited by the image degradation caused through optical aberrations, with modern objective technology reaching up to 28 mm from the previous standard of 20 mm.

Illumination

The illumination system defines the contrast mode, the resolution of the instrument, and the overall brightness. Two principally different optical setups are in use in optical microscopes. The optically simpler of the two is the source focus or critical illumination and the other, which is by far more prevalent, is called Köhler illumination.

Critical illumination uses a single *condenser* lens whereas Kohler illumination uses an additional *collector* lens. The use of two illumination lenses allows for a conjugate 4f system of illumination to the detection optics, this ensures that all ray bundles passing through the sample are parallel and the illumination brightness is

homogenous. Having a conjugated illumination also allows for alternative contrast methods to be implemented.

In the illumination beam paths discussed earlier, the specimen is placed between the light source and the objective lens. In many cases, however, it is advantageous to illuminate the specimen from the side of observation (*epi-illumination*). For instance, when looking at the reflection of opaque or fluorescent samples. Optically the illumination is the same or similar with the condenser lens then being the imaging objective lens as well.

1.1.2 Resolution

Resolution refers to the level of detail that can be recognised in an image, such as small and intricate structures or the distance between closely placed small objects. The latter distance is used to define and quantify the optical resolution.

Angular and numerical aperture

The maximum half acceptance angle (α) of an objective lens limits the amount of light that can be collected from the sample. An objective lens may be approximated to applying a Fourier transform of the imaging space, through an aperture with radius a with an imaging plane at the far field. This leads to high frequency information being omitted during the imaging process and the lens behaving as a low-pass filter. The electric field ($E(r)$) and resultant intensity ($I(r)$) distribution of a single point (delta function) at the image plane then becomes:

$$E(r) \propto E_0 \frac{J_1(2\pi r \sin \frac{\alpha}{\lambda})}{2\pi r \sin \frac{\alpha}{\lambda}} \quad (1.4)$$

$$\implies I(r) = I_0 \left[\frac{J_1(2\pi r \sin \frac{\alpha}{\lambda})}{2\pi r \sin \frac{\alpha}{\lambda}} \right]^2 \quad (1.5)$$

Where J_1 is a Bessel function of the first kind and α is the half opening angle of the tube lens. This function is more commonly known as an Airy disk 1.2.

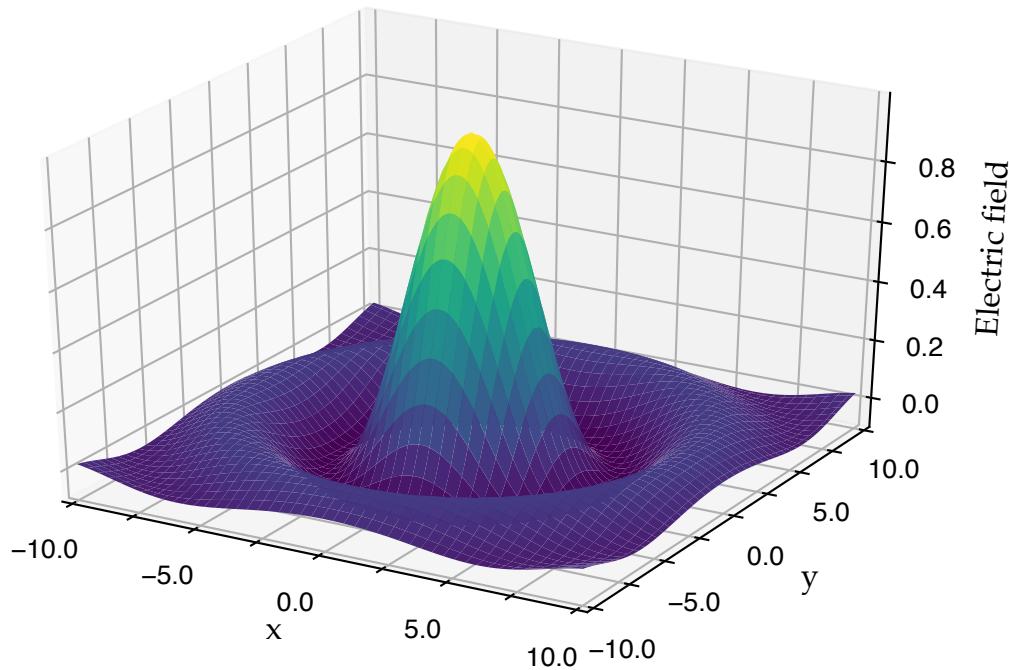
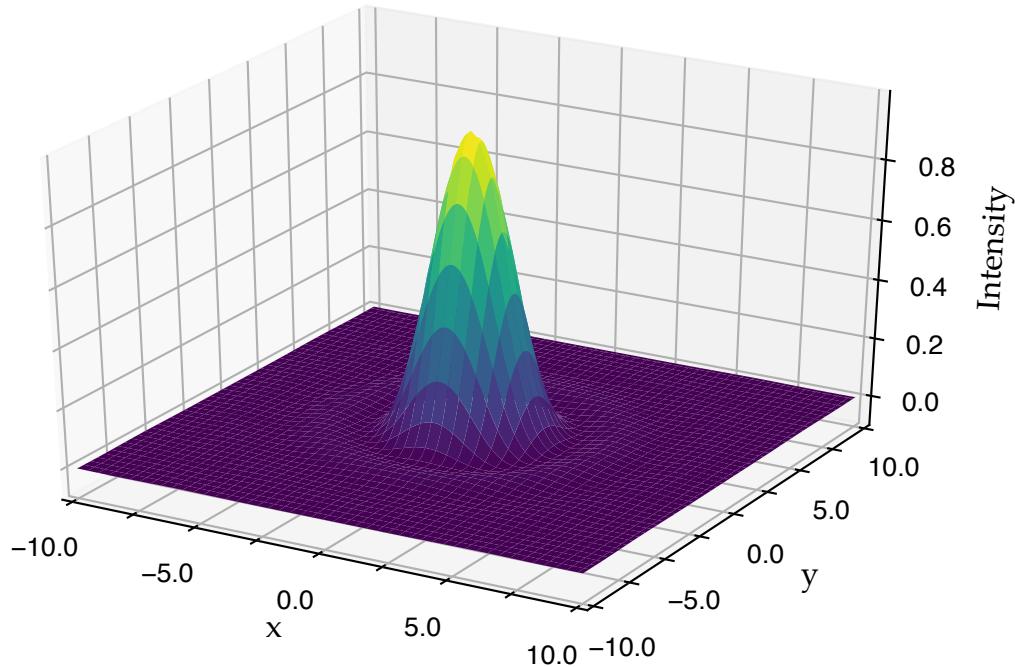
(a) $E(r)$, equation (1.4)(b) $I(r)$, equation (1.5)

Fig. 1.2 Electric and intensity amplitudes of a theoretical Airy disk in radial units of $2\pi r \sin \frac{\alpha}{\lambda}$

Lateral resolution

Point emitters are therefore imaged more faithfully with wider collecting angles and with higher frequency light. By placing two point emitters close such that the first zero crossing of the Bessel junction J_1 coincides with the centre of the second point emitter gives a distance of:

$$r_{0,\text{objective}} \approx \frac{0.61\lambda}{n \sin \alpha} = d_r \quad (1.6)$$

$$\implies d_r = \frac{1.22\lambda}{NA_{\text{objective}}} \quad (1.7)$$

The resulting distance is Rayleigh's criterion for resolution which provides a limit to the resolution of a system based on a dip in intensity maxima, between two neighbouring emitters, of $\sim 75\%$.

Axial resolution

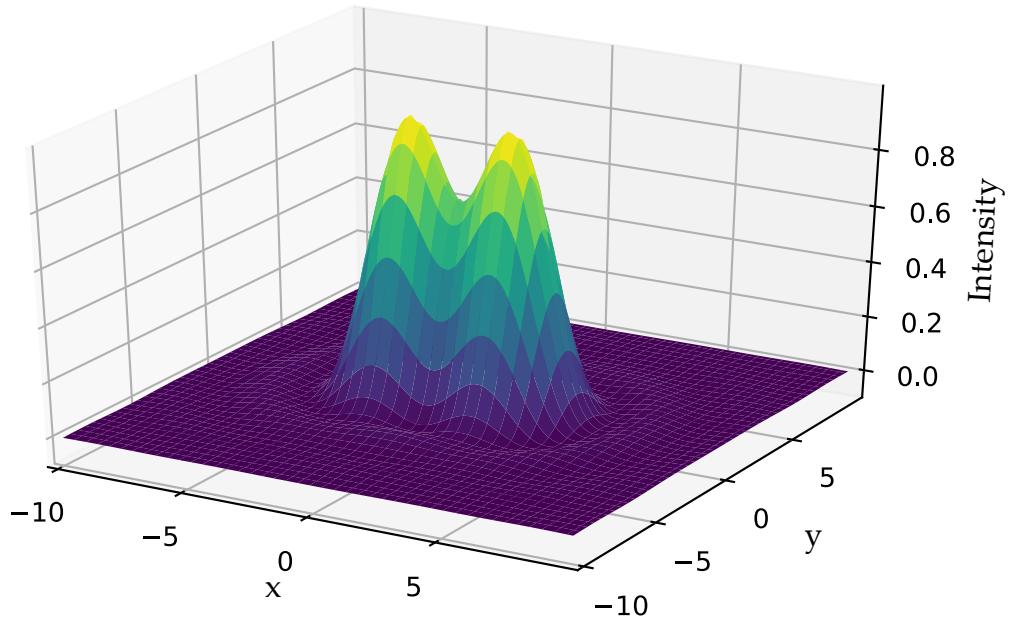
The Airy disk describes the in-plane lateral intensity distribution, with an analogous analytical function propagating axially. Once again, by comparing the the distance to the first zero in intensity along z an analytical definition can be formed:

$$z_{0,\text{objective}} = \frac{2n\lambda}{NA^2} = \frac{1}{2}D_{\text{objective}} \quad (1.8)$$

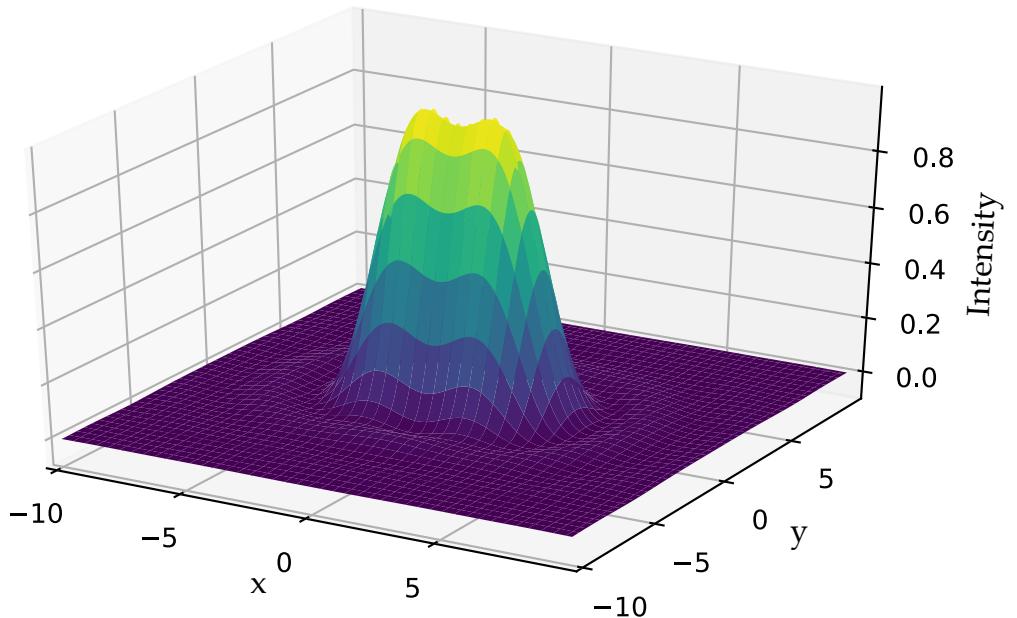
The achievable axial resolution is governed by axial extension of the PSF as is $2z_{0,\text{objective}}$ and commonly called the *depth of field* ($D_{\text{objective}}$). The depth of field physically refers to the distance a focussed object may be moved axially before losing image fidelity to defocus.

Sampling

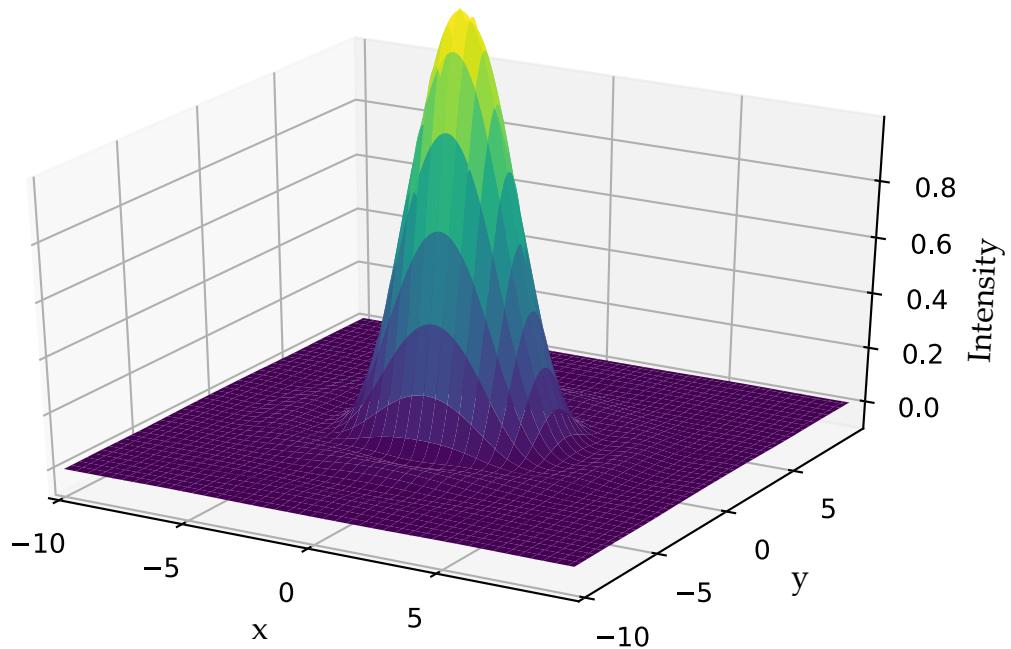
Once transmitted, the formed image information is typically recorded digitally using using a Charge-coupled device (CCD) array camera or similar device. According to Nyquist sampling theory the resolution of the detector required to resample the image information faithfully is $d_{\text{detector}} = \frac{d_r}{2}$. To then choose the correct system magnification (M_{system}) it follows that:



(a) The Rayleigh criteon, whereby the centre of the second emitter sits at the first zero of the first emitter

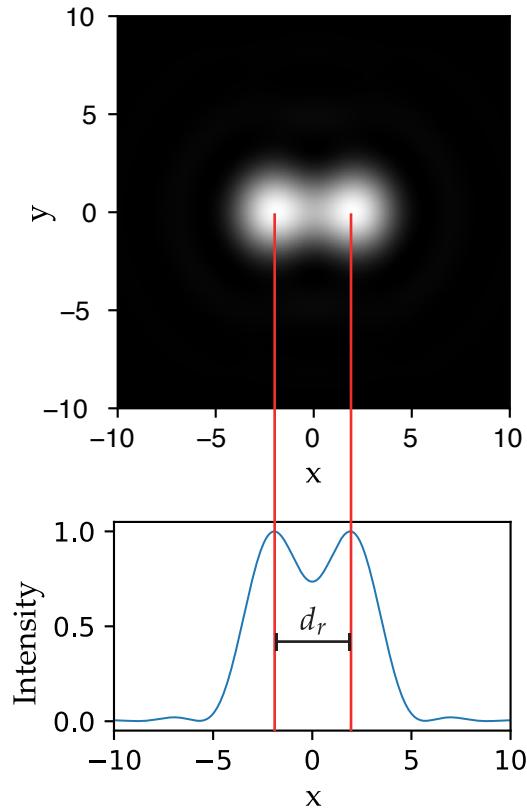


(b) The Sparrow criteon, whereby the centre of the second emitter is one FWHM of the function distant from the first emitter



(c) Unresolved, depicted here as being half the Sparrow limit

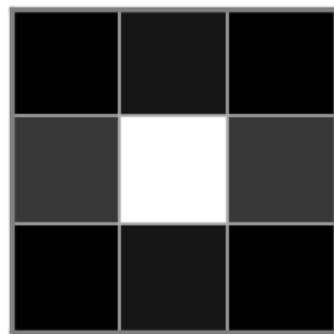
Fig. 1.3 The resolution of a system is governed by the resolving capability of two nearby point emitters. (a) and (b) show the Rayleigh and Sparrow criterions respectively with (c) showing point emitters too near to be resolved due to the lack of any intensity contrast between them



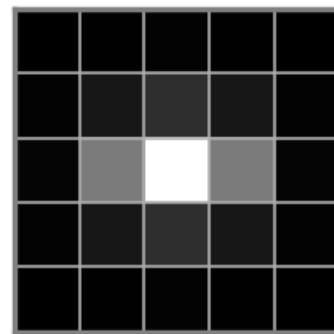
(a) Intensity image of a pair of resolved point emitters separated by the Rayleigh distance d_r

$$M_{\text{system}} = \frac{2d_{\text{detector}}}{d_r} \quad (1.9)$$

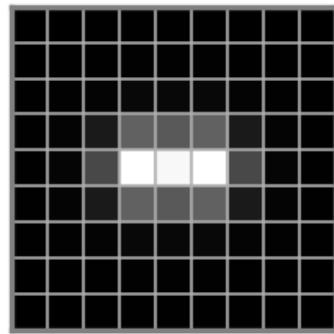
For a detector with $6 \mu\text{m}$ pixels, a magnification on the order of $50\times$ is sufficient for diffraction limited imaging using visible light. Magnifications in excess of this limit are deemed *empty magnification* and decrease the overall Signal to Noise ratio (SNR) of the recorded image. Over-sampling plays an important role in super-resolved systems where the additional pixel information, though diffraction limited, may be used to increase resolution computationally.



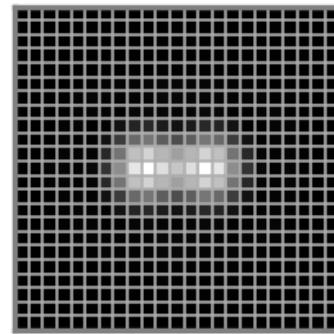
(b) Sampled using 3 by 3 pixels
 $d_{\text{detector}} = 1.5 \times d_r M_{\text{system}}$



(c) Sampled using 5 by 5 pixels
 $d_{\text{detector}} = 0.9 \times d_r M_{\text{system}}$



(d) Sampled using 9 by 9 pixels
 $d_{\text{detector}} = 0.5 \times d_r M_{\text{system}}$



(e) Sampled using 23 by 23 pixels
 $d_{\text{detector}} = 0.19 \times d_r M_{\text{system}}$

Fig. 1.4 Point emitters are sampled by a detector varying pixel sizes, analogous to varying magnification. (d), shows Nyquist sampling; (e) is over sampled, giving super detector resolution. (b) and (c) do not preserve resolution as they are below Nyquist sampling and hence detect a single emitter.

1.2 Fluorescence microscopy

1.2.1 Contrast in optical microscopy

1.2.2 Fluorescence

Fluorescent molecules absorb photons of a particular energy (wavelength) and a short time later re-emit a photon with lower energy, this energy difference gives rise to a red-shift of the emitted photon. This shift in colour allows for suitably coated glass to chromatically discriminate between the desired fluorescent signal and the undesired scattered incident light. Upon being exciting by incident light, an electron within a fluorescent molecule may excite to a higher energy level, provided it has sufficient potential energy to traverse the energy barrier. Once in the higher excited state (S_1), the electron will exist there for an average lifetime (τ), slowly losing energy to the surroundings through vibrations and molecular collisions. Once the electron has *trickled* down the energy levels, it will return to the ground state (S_0) emitting a photon with an energy less the amount of energy lost when in the excited state, the *Stoke shift*. Electrons may also return to the ground state through molecular collisions or an *intersystem crossing* whereby the electron finds a path via transient states with lower energy requirements, the *triplet state*. See Fig. 1.5

Multiple (split) energy levels slightly above the ground and excited states then give a distribution of multiple different emission wavelengths. Fluorescent molecules therefore have excitation and emission spectra rather than discrete excitation and emission lines, see Fig. 1.6 for typical fluorescent molecules used in microscopy. An important advantage of fluorescence microscopy is labelling *specificity*, which refers to the ability to accurately label markers, features or molecules within a biological sample. Multiple labels in different spectral windows can further elucidate how labelled entities interact within the sample.

Labelling

Fluorescent labels may be attached to a site on molecule directly, using primary antibodies or secondary antibodies. Primary antibody labelling involves attaching an antibody to the target molecule directly and the dye molecule attaching to another site on the antibody. Secondary antibody labelling attaches a secondary antibody to the primary antibody and a fluorescent dye to the secondary. Secondary antibody labelling has the advantage of the ease of labelling most target molecules with most

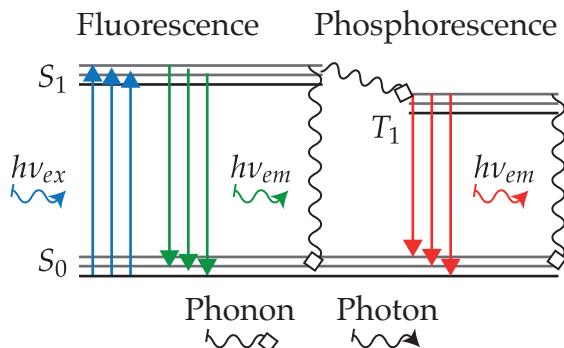


Fig. 1.5 Jablonski diagram representing a green fluorescent molecule. Transitions are represented by waving vectors. Electrons in state S_0 receive energy from blue incident light, this causes the electrons to transition to state S_1 . From S_1 electrons may lose energy no radiatively and re-emit a greener photon when relaxing back to S_0 . Once in S_1 electrons may transition to a triplet state, re-emitting a photon through phosphorescence. A molecule in a triplet state will be for a longer time to S_1 and with an increased chance of oxidation which causes photobleaching.

dye molecules. However, imaging resolution is lost the longer the ligand attaching to the target, but for convenience of dyes with the suitable anti-group being available. The staining process is further inhibited by cellular mechanics; a cell may not endocytose the stain or be it may degraded through autophagy in the lysosome. For non-membrane permeable dyes transfection techniques, though invasive, do exist [1]. Staining can also lead to non-specific binding of fluorescent molecules reducing confidence in specificity and increasing the overall background fluorescent signal in-turn decreasing the image contrast.

For live organism imaging, staining is impractical. Genetic manipulation can allow for fluorescent proteins to be expressed with high specificity as the cells themselves are producing the desired fluorescence; with good spatial homogeneity when compared to soaking samples in dye; and low sample toxicity.

1.2.3 Fluorescence microscopy

Illumination

The mercury arc lamp became the ubiquitous excitation source through it emitting in visible emission spectrum and more intensely than a standard halogen lamp. However, advances in light Emitting Diode (LED) and laser technology have caused a technological shift towards these alternative sources. Modern LEDs and lasers are

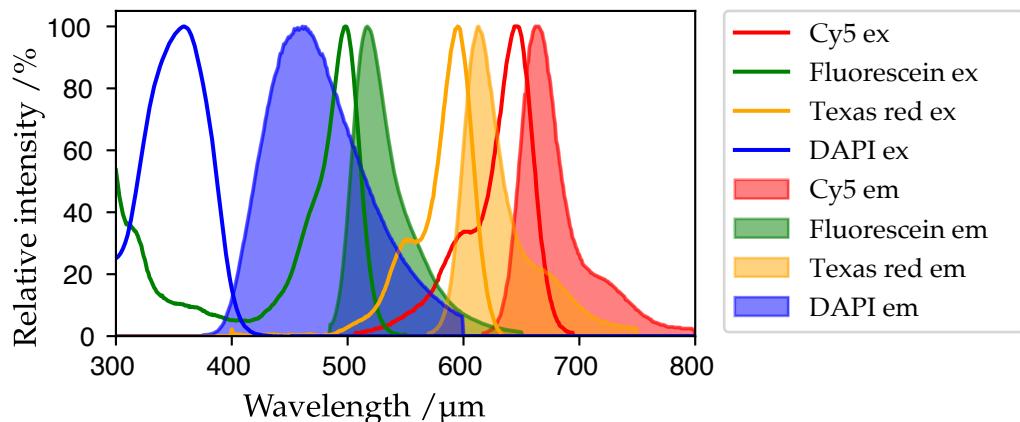


Fig. 1.6 Fluorescence excitation (lines) and emission (filled curves) spectra of the compounds Fluorescein, Texas red, Cy5 (all homocyclic) and DAPI.

now at the stability and intensity to compete with lamp based sources, with more available intensity and homogeneity.

LED sources are currently behind Lasers in terms of the intensity required for certain imaging applications due to the *etendue* of the emission. As etendue is preserved through any system of optics an LED source, having large etendue, will be very photon inefficient, to the point of being infeasible for applications such as point-scanning.

Lasers are limited to very specific excitation lines due to the materials used, particular in diode lasers. These sources are also optically coherent which causes self-interference in the illumination profile (speckle) and the sample. A super-continuum laser source uses non-linear effects, typically induced within a long photonic crystal fibre, to produce broad spectrum visible laser light. From this spectrum, wavelengths may then be selected using emission filters, as with lamp-based sources and white LED sources. This is in contrast to systems with monochromatic lasers as adding more laser lines requires the physical addition of a new laser, making super-continuum lasers versatile. However, the intensity of the pump laser source is spread into the entire spectrum causing narrow selected emission bands to have relatively low intensities.

1.2.4 Signal collection

Once created, the sample image is recorded for analysis and dissemination. For contemporary digital pixel (photosite) arrays are used in wide-field fluoresce microscopes and photo-multiplier tubes (PMT) in *most* laser scanning microscopy.

Detectors

Wide-field detectors come in many flavours, all of which exploit semi-conductor physics to convert incident photons into electrons. CCD based detectors collect electrons during an exposure and transfer in a serial manner through conversion electronics to create digital images. Intensified Charge-coupled device (ICCD) detectors follow the same protocol but use on-photo-site electron cascading to increase the signal and *intensify* the read image. Electron-Multiplying Charge-Coupled Device (EMCCD) detectors transfer their entire frame to a separate conjugate chip which digitises the image for computation. As the frame is being transferred the signal is amplified to multiply electrons collected in the conjugate digitisation site and intensify the image as in ICCD. Transferring the entire frame reduces the digitalisation time and increase the imaging frame-rate.

Complementary Metal-Oxide-Semiconductor (CMOS) chips directly output a digital value on a per pixel. The additional circuitry found off-chip in CCD detectors is embedded in each pixel. Though this reduces the overall fill factor feasible in each chip, this is recovered using a micro-lens to focus directly onto the active read area of the photosite. The per-photo-site architecture allows for a region-of-interest area to be addressed. CMOS detectors made specifically to address quantitative scientific (scientific Complementary Metal-Oxide-Semiconductor (sCMOS)) usage boast having: small pixel sizes; low read noise; large detection arrays; large dynamic range and no multiplicative noise.

Noise

Adverse noise arises in optical microscopes from several compounding effects. The larger the noise level the lower the SNR and the more degraded the recorded image will be. Increasing the integration (exposure) time of the detector will bring the desired signal out of random background noise. A detector with a large *quantum efficiency*, will be able to overcome noise more quickly. The *dynamic range* of the detector is the intensity range at which the weak fluorescent signal can be recorded

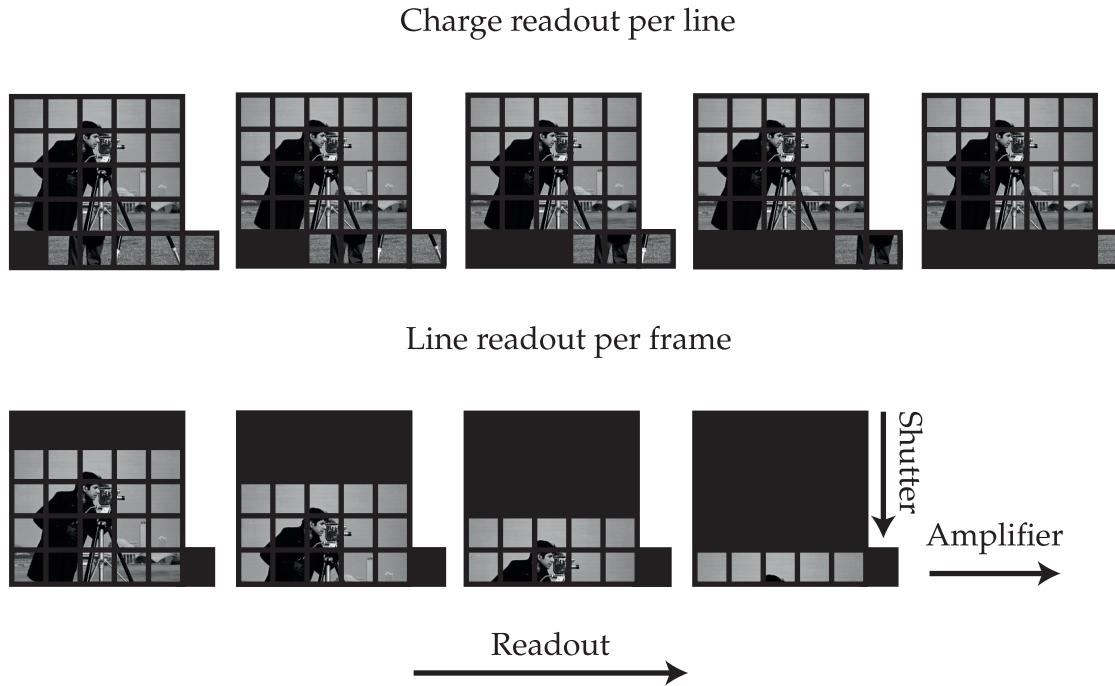


Fig. 1.7 Schematic of how electronic charge is transferred from a CCD array through an amplifier. **Charge readout per line:** charge is serially transferred along the line being read into a single amplifier. **Line readout per frame:** once the line is read, the entire frame is shifted down to be read, acting as a shutter. Serial reading through a single amplifier is slow.

above background noise. The *bit depth* (8 or 16 bit typically) of the detector defines the detectable intensity resolution, which is of particular use for very weak signals.

The *dark current* (noise) contribution occurs in the detectors themselves as the semiconductor material produces erroneous electrons from converting thermal phonons. Dark current increases linearly with integration time and so chip cooling is recommended. *Photon noise* (shot) originates from corpuscular photons arriving at the detector following a temporal Poissonian distribution. An image containing n photons will suffer a variation of \sqrt{n} in photons being emitted, this can appear as valid structure once recorded. *Read noise* is rooted in the conversion process of the analogue voltage of electrons, at each pixel, to digital values. The intensity response across a detector may also be inhomogeneous, this may be flat-field corrected using a calibration from a uniform intensity source.¹

¹manufacturers for sCMOS cameras apply this correction as standard

1.2.5 Limits of fluorescence microscopy

Photobleaching

Fluorescently stained samples will slowly fade in intensity over time as the dye molecules are photochemically destroyed through photobleaching. The process occurs typically through photo-oxidation, once the fluorophore is in an excited state it may then energetically fall into a triplet state where it is more likely to permanently bond with oxygen radicals. Dye medium can be buffered with scavengers of Oxygen radicals to mitigate the process of bleaching. Genetically modified organisms suffer less from photobleaching as molecules that have bleached are continually being replaced by newly expressed fluorophores.

Photobleaching can be exploited using Fluorescence Recovery After Photobleaching (FRAP) wherein an imaging region is purposefully bleached so that unbleached dye molecules may diffuse into the FOV. The rate of return of intensity in the imaging region then gives a measure of diffusion.

Phototoxicity

Fluorescent dyes can act as photosensitizers during live imaging, causing damage to functionality of the cell. Chromophores of fluorescent proteins are shielded by direct contact from molecular oxygen through protein moiety, making fluorescent proteins less phototoxic.

1.3 Three dimensional fluorescence microscopy

1.3.1 Confocal Microscopy

Marvin Minsky proposed the first confocal microscope in the late 1950s to image deep into brain tissue. A pinhole is placed in a conjugate image plane in the detection path which precludes out-of-focus light from being detected. The narrower the pinhole the better the optical sectioning, though sacrificing the received signal.

In Minsky's microscope the sample was mechanically scanned to build a volumetric image. By mechanically scanning the imaging speed is greatly reduced but to the speed of responses of the stage, the maximum speed of travel of the stage and the relaxation time of the stage. Specimens are prone to spatial shifts during scanning as well which causes distortions in the final image.

Modern confocal microscopes use galvanometric scanning mirrors to sweep a laser beam through the sample to build an image. Though faster than mechanical scanning, video-rate scanning confocal microscopy is only viable using resonant scanning mirrors and high power lasers.

1.3.2 Two photon (2P) microscopy

The photon rejecting pin-hole of confocal microscopes can be entirely avoided by using infra-red laser sources. Exciting a fluorophore to an excited state requires a quantised amount of energy, which is typically supplied by a single photon. Two photons, each with double the desired wavelength, will contain the requisite amount of energy to cause the same excitation. For this event to occur there need to be a high photon density surrounding the fluorophore. In a laser scanning system, this means that the beam focus of the objective lens is the most likely place for fluorescence to occur, with a sharp decline in axial intensity axially, providing optical sectioning.

By using 2-Photon (2P) excitation greater depth imaging (~6 fold deeper) can be achieved with reduced photo-toxicity, making the technique very useful for non-invasive live imaging.

Drawbacks

Water, which is abundant in biological specimens, has a large absorbance in the infrared spectrum; combined with the high energy needed to create the 2P effect at the focal point, this can cause localised heating which can in-turn be damaging to specimens and cause optical aberrations. Localised heating can be mitigated by moving the beam sufficiently quickly such that significant heating does not occur. Using infrared excitation also means that the 2P imaging has a much reduced theoretical lateral resolution when compared to visible confocal microscopy. Finally, infrared-red laser sources are prohibitively expensive and, until recently, reliable turn-key solutions were not viable.

1.3.3 Structured illumination microscopy

In Structured Illumination Microscopy (SIM), the sample is illuminated with a periodic sinusoidal pattern using a fast wide-field microscope. The mixing of

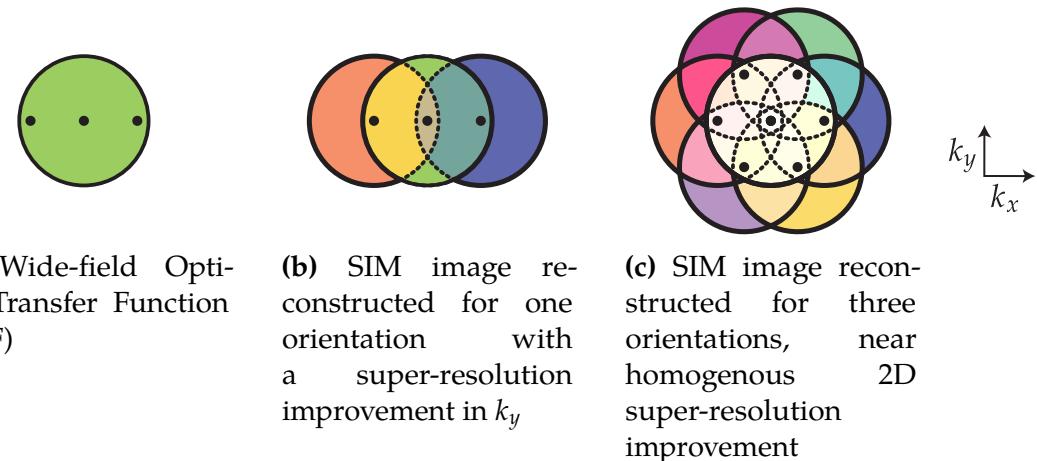


Fig. 1.8 In (a), the circular area corresponds to the passband of the objective lens, the edge being the frequency cut-off. The raw data in (a) consists of superposed original image information positioned at three different origins. Once delineated, the high-resolution information can be relocated to the correct position, resulting in a wider pass-band, (b). The process is repeated three times to isotropically fill the available frequency space by rotating the illumination patterns, (c).

this illumination frequency with the spatial frequency of the sample means that additional resolution can be extracted both in xy and z .

As discussed, an objective lens acts as a band pass filter for low-frequency information. From the *Convolution theorem* (see Appendix ??), the multiplication of two signals in real-space is the convolution of the two Fourier transformed signals in frequency space and, importantly, visa versa - the convolution of two signals in real space is the multiplication of the Fourier transformed signals in frequency space. Meaning that projecting a sinusoidal pattern in real space will convolve the Fourier transform of the sample signal in frequency space with the Fourier transform of the sinusoid signal. The Fourier transform of a sinusoid is three delta functions at $-1, 0$ and 1 with a separation governed by the pattern frequency: $k_1 = 2\pi\lambda$. And so, before the imaging system can band-pass the signal, the illumination pattern has forced three copies of the original image into the pass band, shifted by the vectors k_1 and k_{-1} such that high frequency information outside of the pass band has been cumulated. The overlaid information is then computational unmixed by imaging with three different sinusoid phases to delineate the $-1, 0$ and 1 orders for reconstruction, see Fig. 1.8.

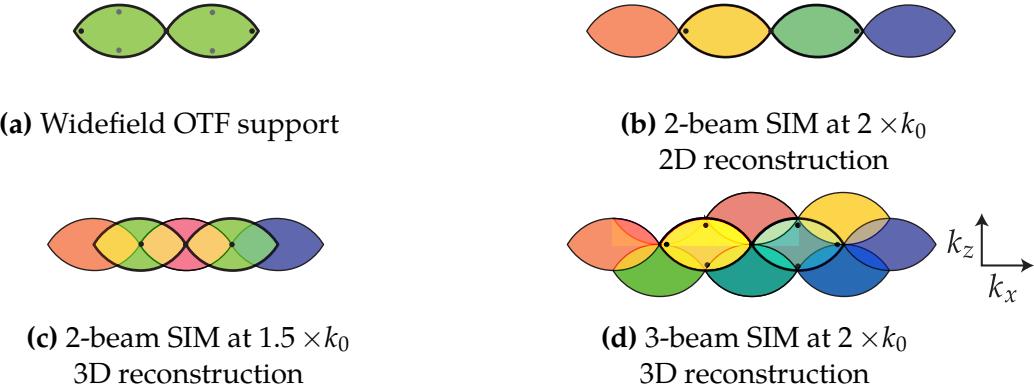


Fig. 1.9 Axial view of 2 and 3 beam illumination in SIM. Black points in (a) represent lateral frequency mixing from 2-beam illumination, the grey points represent axial frequency mixing from 3-beam illumination. (a) shows the lateral extent of 2-beam SIM at maximum ($2 \times$) excitation frequency, with no added axial resolution. (c) shows the lateral extent of 2-beam SIM at $(1.5 \times k_0)$, giving reduced resolution but providing increased axial resolution. (c) shows the lateral and axial extent of 3-beam SIM, doubling in resolution axially and laterally [gustasson]. The thick black outlines show the original OTF support of the widefield image.

Optical sectioning SIM

Three dimensional information can be extracted by manipulating frequency space even further. For instance, the *missing cone* (see Fig. 1.9) can be filled in by using an illumination pattern with k -vectors half the maximum available. In frequency space this pushes the region of the missing cone into axial resolution maxima of the OTF support (toroidal), giving three dimensional structure. By adding a third beam along the optical axis to interfere with the sinusoidal pattern, a further axial sinusoidal pattern can be created.

1.3.4 Selective plane illumination microscopy

The techniques as introduced above all provide volumetric imaging through reconstruction. Structured illumination techniques require computation reconstruction which is prone to artefacts. Confocal scanning is slow and generally lossy with signal. Light-sheet microscopy offers reconstruction free (SIM), fast, low photo-toxic volumetric imaging. The application of light-sheet microscopy is the focus of this thesis and will be covered in detail in the next following chapter.

