Epifluorescence Microscopy

3

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Contents

What You Will Learn in This Chapter

In previous chapters, we have seen that the interaction of light with matter produces one or a combination of the following phenomena: transmission, absorption, reflection, scattering and diffraction, refraction and polarization, phase change and fluorescence emission [1]. Each one of these effects can be used to generate contrast and hence create an image. In this chapter, we will discuss the light-matter interaction that leads to the absorption of a photon and the subsequent emission of a photon with lower energy: Fluorescence. We will explore its principles, advantages over classic bright field techniques, limitations and some of its main applications in life and material sciences.

By providing technical analysis as well as a step-by-step protocol, the reader will be able to understand the concept of fluorescence microscopy, get an introduction to

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labelling techniques, understand the components of a fluorescence microscope and learn how to design and set up experiments with the optimal compromise between Acquisition Speed, Signal-to-Noise Ratio and Resolution.

The chapter will be divided into four sections: theoretical aspects of fluorescence microscopy, microscope setup, sample preparation and key applications of widefield fluorescence microscopy.

3.1 Fluorescence

Fluorescence is a natural property of individual molecules. These molecules absorb photons of specific wavelengths and subsequently emit photons of red-shifted wavelengths. Considering red light has lower energy than blue light, the term red-shifted signifies that some of the energy absorbed is lost due to vibrational relaxation, dissipation or other intramolecular processes. It is worth noting that, since fluorescence covers the visible range, the term 'colour' and wavelength are used interchangeably.

3.1.1 Jablonski Diagram

In order to understand the mechanism behind fluorescence, we use a Jablonski diagram (Fig. 3.1). This diagram shows the different energy states of a particular

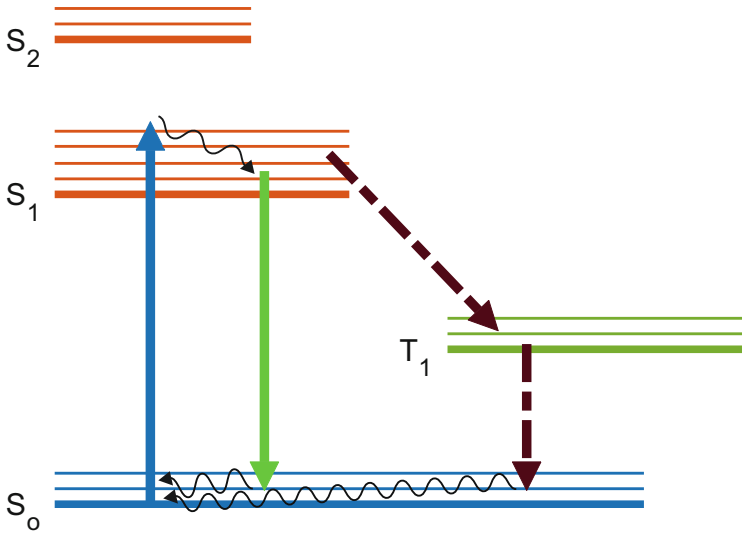

Fig. 3.1 A Jablonski diagram displaying the molecular singlet and triplet states and possible transitions for a fluorophore. Continuous arrows show radiative transitions (excitation and emission), dashed arrows show nonradiative transitions

molecule that govern the spectroscopic transitions of its electrons. Quantum mechanics dictates that the energy levels are distinct and that a transition between states occurs when so-called quanta with energies matching the energy gaps are absorbed or emitted. These transitions are either radiative or nonradiative. Radiative transitions indicate that a photon is absorbed or emitted. Nonradiative transitions do not involve photon emission. Depending on the electron spin state, they can happen in the same singlet state and are called internal conversions or between a singlet and a triplet state, where they are called intersystem crossings.

In its natural ground state G_0, a molecule is described as being in a singlet state. When a fluorescent molecule, a fluorophore, is exposed to light with high energy, photons are absorbed, and electrons get transitioned to the excited state S1 (Fig. 3.1). This absorption typically happens in the time scale of femtoseconds. The excited molecule can relax radiatively by emitting a photon with lower energy. This fluorescent de-excitation takes place in the time scale of nanoseconds [2]. Alternatively, the molecule can transition into a triplet state T_1 via intersystem crossing by flipping its spin. The triplet state is more stable than the singlet state, and the relaxation to the ground states happens in a matter of seconds to hours and is called phosphorescence.

3.1.2 Fluorescent Markers

Fluorescent molecules, or fluorophores, can be divided into three families: fluorescent proteins that can be natural or engineered, organic fluorophores, which are small molecules extracted from natural compounds and nanoparticles that are generally made from molecules like Cadmium, Zinc or rare-earth elements.

All fluorophores have five main properties:

1. Stokes shift (SS): The Stokes shift is defined as the difference between the absorption and emission peaks (Fig. 3.2) and corresponds to the average energy loss during the fluorescence process. The Stokes shift is the most important property of any fluorophore because it allows the separation between the excitation light and the emission light, significantly increasing the contrast. Most fluorophores have a Stokes shift of 30–50 nm. The higher this shift is, the better and easier the separation becomes. However, a high stokes shift reduces the total number of dyes that can be imaged together.
2. Fluorescence Lifetime (FL): This is the average time it takes a fluorophore between the absorption of a photon and the emission of a Stokes-shifted photon. The fluorescence lifetime ranges from a few nanoseconds to multiple seconds and can also be used to increase contrast in Time-Correlated-Single-Photon-Counting applications.
3. Quantum Yield or Efficiency (QE): It is the ratio between the number of absorbed photons and the number of emitted photons per unit of time and is used as an indicator of the efficiency of a particular fluorophore. QE is an important

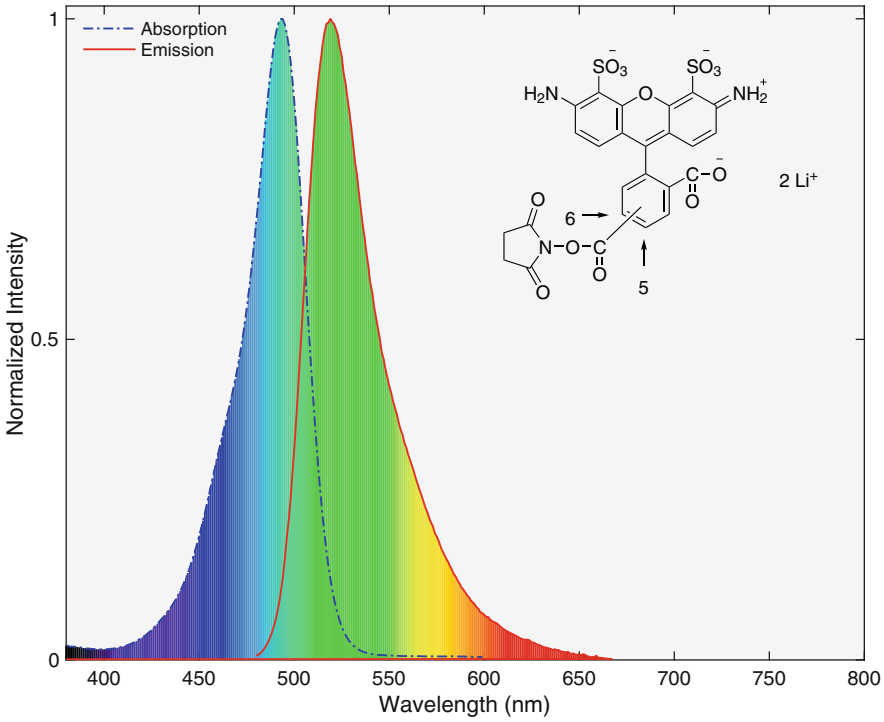

Fig. 3.2 Excitation, emission spectra and molecular structure of Alexa 488. Alexa 488 has an absorption maximum at 490 nm (blue) and an emission maximum at 525 nm (green)

parameter to be considered when choosing the right fluorophore since a high QE allows for low excitation powers.

4. Extinction Coefficient (EC): It is also known as the attenuation coefficient and describes the probability of absorption of a photon at a particular wavelength. It is usually measured at the absorption maximum.
5. Photon Yield (PY): It is the total number of photons a fluorophore emits before going extinct and varies between different fluorophore types. The Photon Yield is a critical parameter for single-molecule applications.

3.2 Fluorescence Microscope Setup

The fluorescence microscope consists of four parts: the excitation module, the emission module, the filter cube and the objective that focuses the excitation light on the sample and collects the emitted light (Fig. 3.3). The fluorescence microscope exists in two illumination forms: transmitted light illumination, also called *dia*-illumination and reflected light illumination better known as *epi*fluorescence

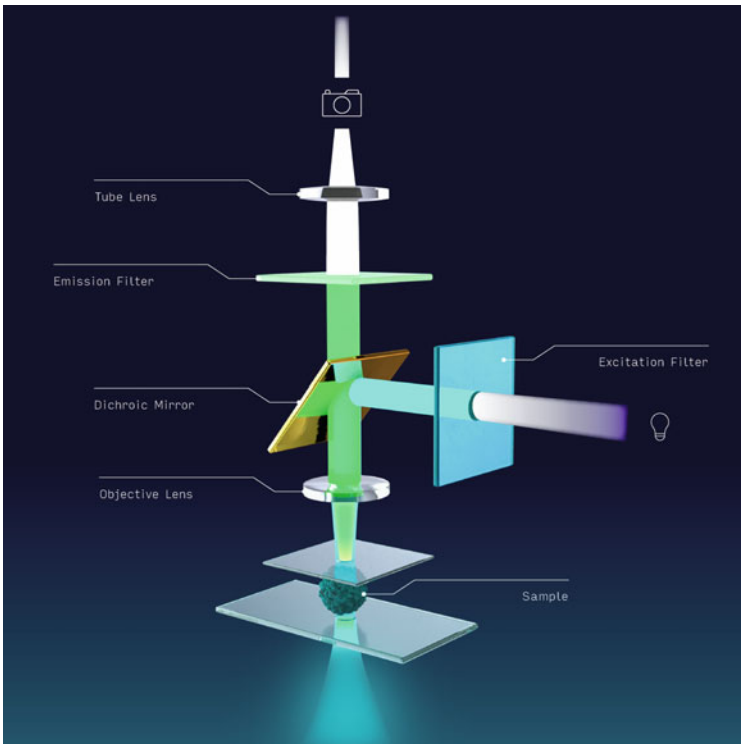

Fig. 3.3 Fluorescence microscopy setup consisting of four units: Excitation unit, filter unit (excitation filter, dichroic mirror and emission filter), magnifying unit (objective and tube lens) and detection unit (camera)

Table 3.1 Comparison of different excitation sources for fluorescence microscopy

	Mercury	Xenon	Solid State	Lasers
UV brightness	+	–	–	++
Blue-green brightness	+	+	+	++
Red brightness	–	++	–	++
Stability	–	+	++	++
Lifetime	–	+	++	++[b]
Safety	–[a]	+	++	–
Cost	++	+	–	–

[a]UV light, Heavy Metal and Ozone exposure
[b]valid for diode-based lasers

microscopy. In this chapter, we will focus on the epifluorescence setup as it is the most common type of microscope.

In epifluorescence, the excitation light (Table 3.1) is directed on a dichroic mirror that reflects it perpendicularly through the objective (Fig. 3.4). The objective focuses the light on the sample. Fluorescent light is emitted from the sample in all directions,

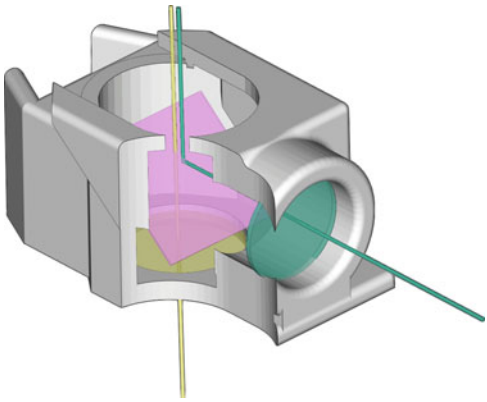

Fig. 3.4 Typical filter cube setup used in fluorescence microscopy. Green: Excitation Filter. Purple: Dichroic Mirror. Yellow: Emission Filter

and only part of it will be collected through the same objective where it passes through the dichroic mirror, the emission filter as well as the tube lens to reach the detector. Since this type of illumination covers the whole field of view of the objective, a camera is used to collect the fluorescence.

3.2.1 Excitation Module

Mercury Lamps Mercury lamps are the oldest light sources used in fluorescence microscopy. They use vaporized Mercury through which an electric arc is created to produce light. They are cost-effective and have the highest UV irradiation among other white light sources. However, Mercury lamps are hazardous as they contain mercury, can generate ozone and have a short lifetime of 200 h. They also need to be aligned for optimal illumination of the sample.

Metal Halide Lamps Similar to mercury lamps, metal halide lamps are gas discharge lamps that use a mixture of mercury and metal halides like bromine or iodine. They have a continuous spectrum with peaks at different ranges of the visible spectrum. They have sufficient intensity in the UV as well as the far-red range. Compared to Mercury lamps, metal halide lamps are more energy-efficient and have a longer lifetime (2000 h).

Solid State Light Sources or Light Emitting Diodes (LED) LEDs are the newest light sources available for fluorescence microscopy. Previously mainly available in the blue, green and red ranges, their UV emission had improved in recent years. LEDs have three main advantages. They can be switched on and off in milliseconds, have a lifetime of more than 10,000 h, and their intensity can be electronically controlled. An example is shown in Fig. 3.5.

Lasers Lasers are usually reserved for high-end widefield microscopy techniques like Total Internal Reflection Fluorescence Microscopy (TIRF) or Stochastic Optical

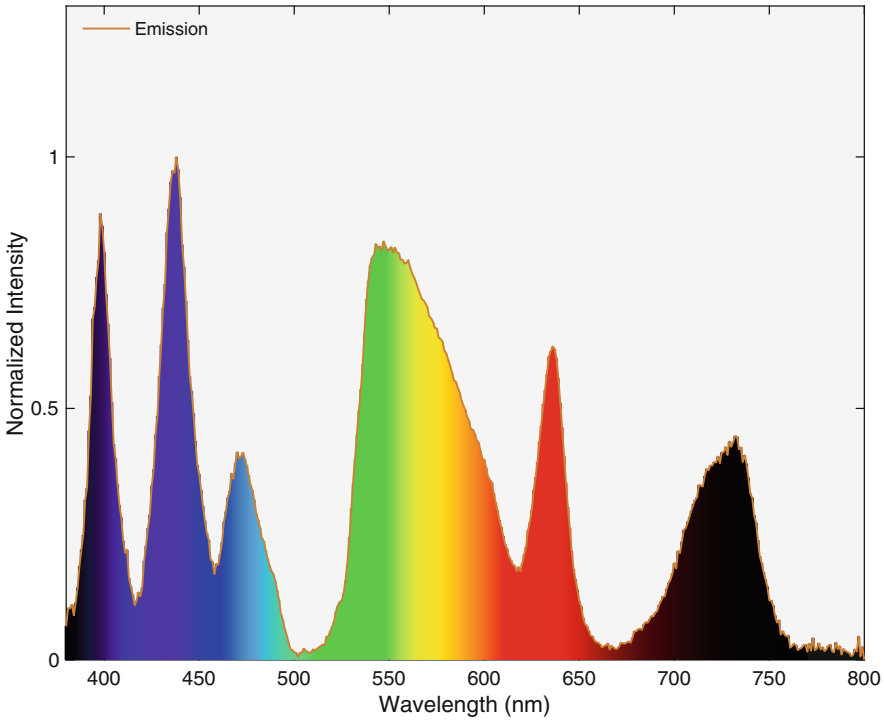

Fig. 3.5 Lumencor Spectra X emission spectrum. The light engine has several emission peaks that are matched to common fluorescence dyes like DAPI, FITC, TRITC or CY5

Reconstruction Microscopy (STORM) due to their higher cost and to laser safety regulations. They have the advantage of being: i) monochromatic, which removes the need for an excitation filter and ii) coherent, which gives the best sample illumination profile. However, this optimal sample illumination profile is highly dependent on the optical alignment requiring frequent servicing of the microscope.

3.2.2 Objective

In an epifluorescence setup, the objective has two functions: it is both the condenser that focuses the excitation light on the sample and the lens that collects the fluorescence signal, magnifies it and creates the image that will be projected on the detector. The objective is the heart of any microscope. Successful imaging requires careful consideration of the different specifications of any objective: magnification, numerical aperture, field number, immersion medium, working distance, contrast method (brightfield, phase contrast, differential interference contrast DIC). Many of these objective properties are interdependent and have been described in more detail in previous chapters. It is important to note that each part of the setup contributes to the

final resolution of any microscope. However, the objective is usually the limiting factor due to diffraction (Chap. 2) and residual aberrations.

Unlike brightfield microscopy, fluorescence microscopy relies on the generation of light from the sample. This process is extremely inefficient, and it is estimated that for every 10^6 photons emitted from the excitation source, only one photon reaches the detector. It becomes therefore essential to collect as many photons as possible that are emitted from the sample since each photon contributes to the brightness of the imaged structures. The brightness of the image is a function of the numerical aperture of the objective and the magnification. For a fluorescence microscope is given by:

$$\text{Image Brightness} = c \cdot \text{NA}^4 / M^2,$$

where c is a constant, NA is the numerical aperture and M the magnification of the objective. The numerical aperture defines the maximum angle of collection of light by the objective.

The above formula shows that in order to maximize the image brightness, the numerical aperture needs to be as high as possible and the overall magnification minimized. When choosing between two objectives with identical numerical aperture, the objective with the lower magnification will give the brightest image. When choosing between two objectives with identical magnification, the objective with the highest numerical aperture will give the best resolution and image brightness.

Another part of the fluorescence microscope is the tube lens. With an infinity-corrected objective, its role is to focus the light on the detector and to correct for some of the optical aberrations. Some microscopes have more intermediate lenses that can be used to magnify or demagnify the image on the camera in order to match the sensor's pixel size.

3.2.3 Fluorescence Filters

Fluorescence filters are a key component in fluorescence microscopy. They separate the light emitted from the sample from that used to excite the fluorophores, thereby allowing the image to be seen or detected with sufficient contrast. In conjunction with advances in camera technology, fluorescence filters have contributed significantly to the success of fluorescence microscopy in life science research. They are usually mounted in a cube containing the excitation filter, the dichroic mirror and the emission filter (Fig. 3.4). The excitation and emission filters are usually bandpass filters that have been designed to transmit only a specific part of the spectrum that can be matched to the excitation and emission of a specific fluorophore.

Dichroic mirrors, on the other hand, are filters with a cut-off wavelength at which they switch from transmission to reflection (Fig. 3.6). Dichroic mirrors are optimized for an incident angle of 45°, while excitation and emission filters work best with an incident angle of 90°. All three components are made of a glass substrate coated with several layers of metals or metal oxides with different thicknesses. Depending on its

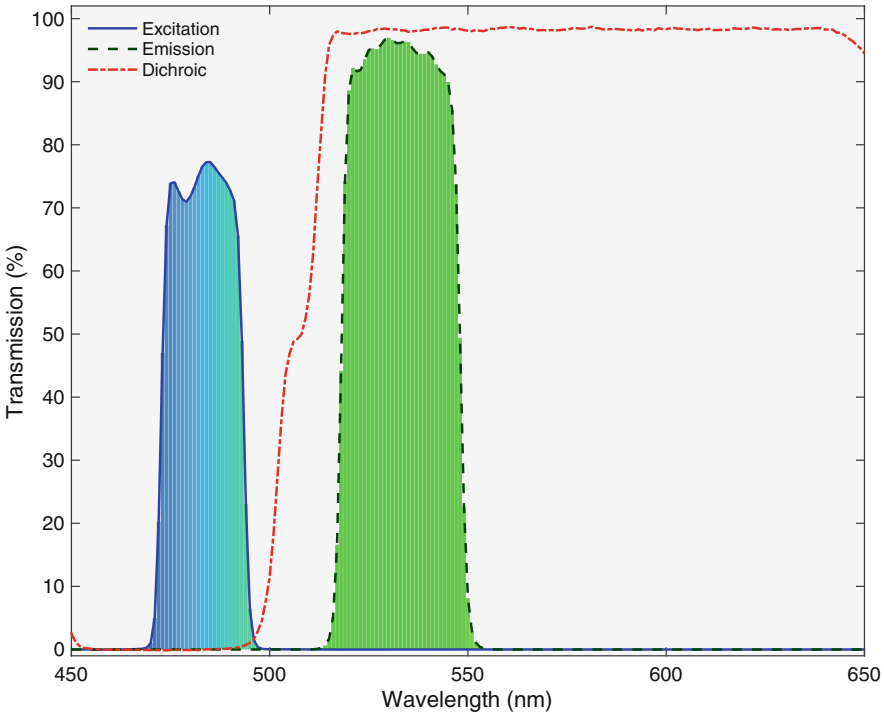

Fig. 3.6 Transmission curves for an Alexa 488 filter cube. Blue line: excitation filter. Green dashed line: emission filter. Red dash-dot line: dichroic mirror

incident angle, the light can either be reflected, transmitted or absorbed at the different layers giving the filters its optical properties. The visible spectrum has traditionally been divided into four primary colour ranges known as the blue, green, red and far-red ranges, with recent advances permitting a fifth colour in the near-infrared range.

3.2.4 Emission Module

Charge-Coupled Device CCD and Complementary Metal Oxide Semiconductors CMOS are the main two types of cameras used for widefield microscopy. CCDs and CMOS cameras are two-dimensional arrays of photosites (pixels) that convert the fluorescence light to a digital signal in a process involving:

1. Light to electron conversion. Here the photons hit the active surface of the chip and get converted to electrons through photo effect. The efficiency of this process is called Quantum Efficiency QE and varies across the spectral range reaching more than 95% in the green range for high-end cameras.

2. Charge accumulation: The electrons accumulate in each pixel for a specified duration, the so-called 'exposure time'. The number of electrons that a pixel can hold before becoming saturated and leaking charges to neighbouring pixels is called well capacity.
3. Charge transfer and amplification. The key difference between CCD and CMOS cameras is in the way the charge is converted and amplified. In CCD chips, the charge is transferred line by line and pixel by pixel to a single amplifier that amplifies the signal and converts it to a voltage making them slower than CMOS sensors. CMOS chips, on the other hand, have the amplifier built in each pixel, and the resulting voltage is transferred line by line and pixel by pixel to the analog-to-digital converter.
4. Analog-to-digital conversion: Here the amplified voltage is converted to an 8, 12, 14 or 16-bit digital signal that can be read by the computer and converted into brightness levels on the final image. It is important to note that there is a direct relationship between the output format of the camera and its well capacity. For example a CCD chip with a well capacity of 3000 electrons, will require a digitalization of 12 bit (4096). In comparison, an EMCCD camera with a well capacity of 60,000 electrons will require a digitalization of 16 bits (65,536) to be able to use the full capacity of the chip.

CCD or CMOS?

CCD and CMOS cameras have been around for decades. The traditional choice was CCD sensors for low light applications thanks to their higher signal-to-noise ratio and CMOS sensors for speed applications or in consumer electronics thanks to their smaller size and lower power consumption. However, recent developments of both technologies made this choice a little more complicated. For example EMCCDs have been developed in the early 2000s making video frame rates possible at full frame and reducing blooming effects. Meanwhile, CMOS cameras have seen an increase in their quantum efficiency as well as a decrease in noise levels, making them a valuable option for low light applications.

Due to the difference in sensor and pixel sizes, it is challenging to compare CCDs and CMOS camera performances. We have therefore summarized the main specifications of two similar high-end sCMOS and EMCCD cameras in Table 3.2.

Camera Noises

There are four primary noise sources: (i) The photon noise or shot noise is due to the Poissonian distribution of the fluorescence emission and is proportional to the square root of the number of emitted photons. (ii) The readout noise is the result of the charge/voltage transfer through the chip. This noise can be reduced by changing the readout speed at the cost of lower time resolution. (iii) The dark noise is an inherent noise to each chip and is associated with thermally generated charges on the photosites. The dark noise can be reduced by cooling the camera chip with forced air or liquid. (iv) The amplification noise is the noise created during the amplification process. This noise is the same across all pixels for CCDs as they have one amplifier

Table 3.2 Technical specifications of Andor Ixon 888 and Photometrics Prime 95B

	Photometrics Prime 95B	Andor Ixon888
Sensor type	sCMOS	EMCCD
Pixel size resolution	11 µm 1200 × 1200	13 µm 1024 × 1024
Sensor diagonale	18.7 mm	18.8 mm
Readout rate and noise	1.6 e RMS	<1e @ 30 MHz
Well capacity	80,000 e	80,000 e
Max frame rate	41 @ 16 bit 82 @ 12 bit	26 @ 16 bits
Quantum efficiency	Up to 95%	Up to 95%
Dark current		
Air Liquid	0.55 e 0.3 e	0.00025 e 0.005 e

but varies with each pixel on CMOS cameras. It is hence given by its Root Mean Square (RMS) or Median Value.

Tips

- Match the camera's sensor (Fig. 3.7) to the Field Number (FN) of the microscope and objective. Modern CMOS cameras are available with a 25 mm diagonal chip size. When combined with an FN 25 objective, they offer 70% more area for the same magnification compared to the standard FN of 19 mm.
- Minimize over- or under-sampling by matching the objective's theoretical resolving power to the pixel size of the camera (see step-by-step protocol).
- Binning should be used when: (i) resolution is not essential and (ii) the sample has low fluorescence emission. Binning increases acquisition speed, especially in CCD cameras, improves the signal-to-noise ratio and also reduces the file size, which improves processing speeds. For example binning is recommended when performing long-term live-cell imaging with sCMOS cameras as it allows the user to reduce the exposure of the cells to high intensities of light, ensuring cell viability.
- Cool the camera with forced air or liquid to reduce the dark noise in low light level conditions and lower the readout speed when time resolution is not limiting to reduce the readout noise.

3.3 Basics of Sample Preparation for Fluorescence Microscopy

Sample preparation can be divided into two groups: live sample and fixed sample preparation. In live sample imaging, the specimen needs to be kept in its natural environment or the closest possible to it. This usually means using an incubator with temperature and gas control for cells or tissues and a perfusion system for organs like the mouse brain [3]. In order to fluorescently label the specimen, the fluorophores need to be small enough to penetrate the membrane and bind to the desired

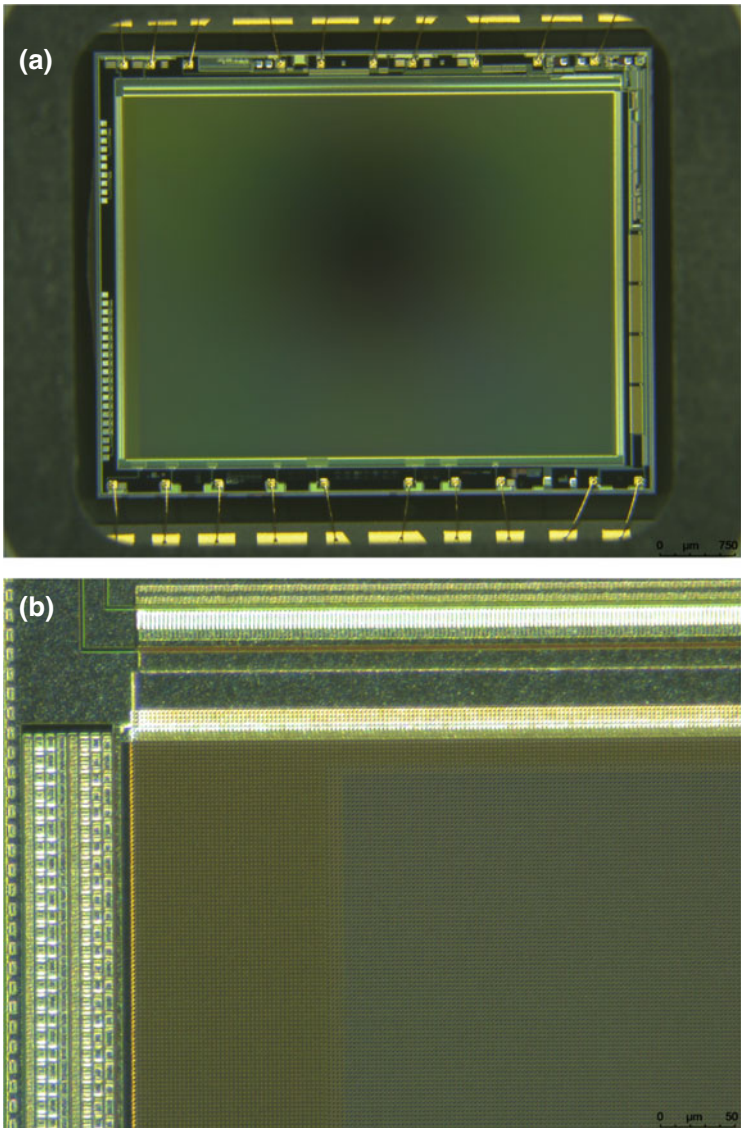

Fig. 3.7 (**a**) Image of a Sony ICX274 CCD chip with 1600*1200 pixels. (**b**) Zoom on the top left corner showing individual pixels

structures, or they have to be genetically engineered using fluorescent proteins vectors. For live sample imaging, the sample needs to be placed in a dish that is suitable for the type of microscope used. In case of upright microscopy, dipping objectives are generally used for high-resolution imaging and require dishes with

diameters of 35 mm. For inverted microscopy, glass-bottom single or multiwell dishes can be used.

In the case of fixed samples, the specimen needs to be sliced to 2–4 μm slices for widefield microscopy, fixed using fixatives like paraformaldehyde or glutaraldehyde, permeabilized with detergents like Triton and then labelled using immunohistochemistry protocols. This includes blocking non-specific epitopes with blocking reagents like Bovine Serum Albumin (BSA), incubation with primary antibody and then with a secondary antibody with several wash steps in between.

Sample mounting is the last important step before imaging and is as essential as the previous steps. Mounting has two objectives: (i) avoiding physical damage or deformation and (ii) matching the refractive indices of mounting medium and sample. Mounting media with different refractive indices can be made by mixing water and glycerol. By changing their relative concentrations, the refractive index can be tuned to match that of the specimen of interest (organelles, cytosol, mitochondria, tissue type) [4]. To reduce photobleaching, antifade agents should be added to the solution. Alternatively, a multitude of mounting media is commercially available (Vectashield, Prolong series, Moviol etc...).

Sample preparation and mounting is a tedious process. Although a plethora of protocols can be found in the literature, each user needs to optimize the predefined protocols to obtain the best results. For the sake of conciseness, detailed labelling protocols are referenced at the end of this chapter [5–9].

Several companies (incl. Sigma Aldrich, Thermofisher Scientific, Spirochrome, Ibidi) have specialized in developing and commercializing fluorophores for both live and fixed sample preparation. Their products include antibodies, plasmids for cell transfection as well as stains for the most common cell organelles. They also provide labelling kits with organic or silicon rhodamine dyes conjugated to maleimides, NHS ester, carboxylic acid groups that can be used to label a variety of biomolecules.

Tips

- Use 0.17 mm coverslips (thickness 1.5) for magnifications above 20×. Most objectives are corrected for this type of coverslips. Some objectives have correction collars that can be used to adjust for different thicknesses.
- Try different staining protocols and vary the concentration of the fluorophores (organic fluorophores or primary and secondary antibodies). Use multiwell dishes for initial testing.
- High concentrations of markers lead to non-specific binding.
- There are 6.022×10^{23} molecules in 1 mol! When changing concentrations, use factors 10 and 100 to notice a change.
- Avoid mounting media that contains DAPI.
- Ensure proper sample mounting and seal the coverslip with nail polish to avoid contaminating the microscope.

3.4 Main Applications of Fluorescence Microscopy

Fluorescence microscopy covers a wide range of applications in life sciences research and diagnostics. Classically used as a structural analysis tool, fluorescence is nowadays a key component in many research areas. Modern fluorescence microscopes offer not only high resolution and, in some cases, super-resolution capabilities, but also high-speed functional imaging capabilities. Moreover, other tools like flow cytometers and multimode readers have emerged with high-speed imaging capabilities. With the advances in camera technology, it is possible today to image with more than 100 frames per seconds with relatively high resolution.

Most modern high-end methods are based on standard fluorescence microscopes. Drawing a sharp line between widefield microscopy and other imaging modalities like TIRF, STORM or even spinning disk confocal microscopy is a difficult task. Although all these techniques provide unique features and advantages, they are essentially an improvement of the standard fluorescence microscopy setup. This setup is in contrast to another technique in microscopy, Laser Scanning Microscopy (LSM), that is based on sample scanning for imaging. The specimen is illuminated point-by-point with one or more lasers, and the fluorescence emitted from the sample is collected and associated with one pixel in the final image. A raster scan reconstructs the full image.

Several Laser scanning techniques like confocal microscopy or multi-photon microscopy will be discussed in the next chapters. Table 3.3 summarizes the most common techniques in microscopy.

3.4.1 Structural Imaging

Being an imaging technique, microscopy is fundamentally a structural analysis tool. The microscope not only magnifies an object but also resolves the spatial distribution of individual molecules of interest within that object. The key success of fluorescence microscopy comes from the fact that only labelled structures are visible. This

Table 3.3 Fluorescence microscopy techniques separated by image acquisition method

Widefield techniques	Laser scanning techniques
– Epifluorescence Microscopy – Total internal reflection fluorescence Microscopy TIRF – Photoactivated localization Microscopy PALM – Stochastic optical reconstruction Microscopy STORM – Light-sheet Microscopy – Spinning disk Microscopy – Structured illumination Microscopy – Fluorescence recovery after Photobleaching FRAP	– Confocal Microscopy – Multi-photon Microscopy – Fluorescence (cross) correlation spectroscopy FCS and FCCS – Raster image correlation spectroscopy RICS – Fluorescence recovery after Photobleaching FRAP – Stimulated emission depletion Microscopy STED – Minimal photon flux MINFLUX – Fluorescence lifetime imaging Microscopy FLIM

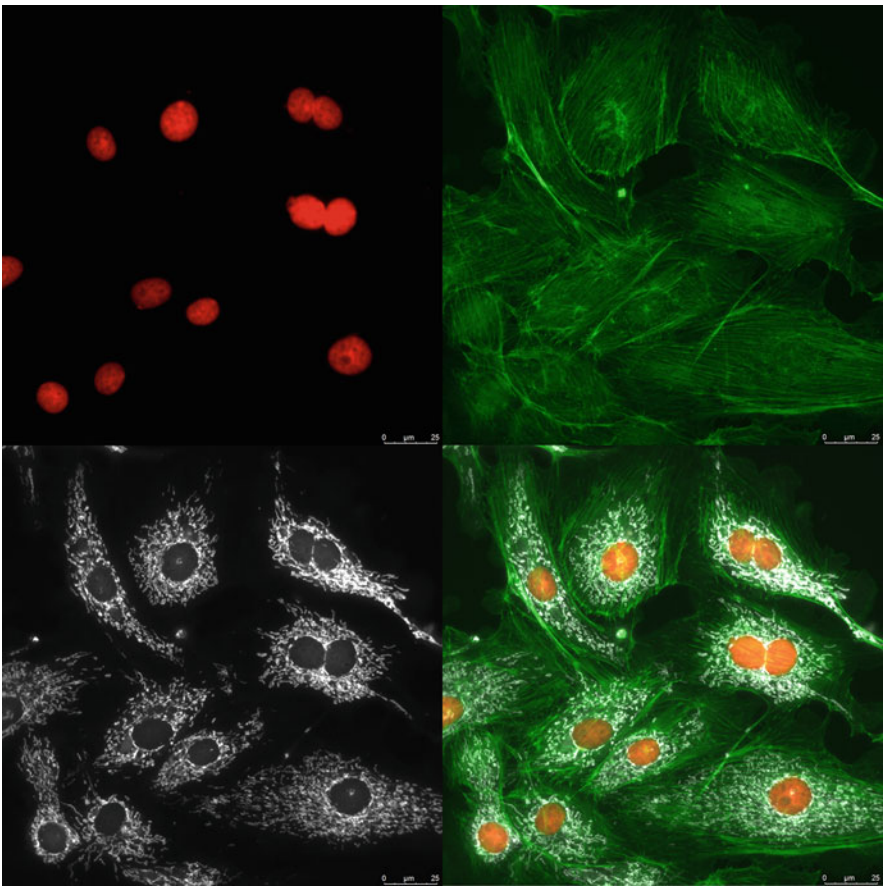

Fig. 3.8 Fluorescence image of bovine pulmonary artery endothelial (BPAE) cells stained with three colours. Nuclei are shown in Red, F-actin in green and Mitochondria in white

means that any molecule that can be labelled with a fluorophore can be made visible. By targeting specific lipids, peptides, amino acids or proteins with fluorophores, subcellular structures like the nucleus, the mitochondria, the actin filaments or any other molecule of interest can be imaged with high levels of detail.

Another advantage of fluorescence microscopy is its capability to use the full spectrum of visible light. A fluorophore's excitation and emission usually extend around 50 nm. This makes it possible to divide the visible spectrum into 4–5 channels (colours) which can be imaged simultaneously or sequentially (Fig. 3.8). This type of multiplexing has made colocalization studies possible: Instead of imaging only one element at a time, several molecules can be labelled and imaged during the same experiment. Here, the co-distribution of these biomolecules is analyzed, and their interaction is assessed.

3.4.2 Functional Imaging

Functional imaging essentially adds a time dimension to the imaging process. Here, structural information, in two or three dimensions, is combined with time information. Cellular dynamics can be resolved with nanometre and millisecond precision. These so-called time-lapse experiments can be performed to study molecular processes like replication, transcription or DNA repair as well as cellular processes like proliferation, immune reactions or migration. Other applications include Fluorescence Recovery After Photobleaching (FRAP) to investigate kinetics processes and Förster Resonance Energy Transfer (FRET) to study protein-protein interactions.

Protocol for Successful Imaging
Step-by-Step Protocol

In this protocol, a step-by-step guide is provided, and methods are presented on how to navigate iron triangle (Resolution–Speed–Signal to noise ratio). This protocol is optimized for 0.17 mm coverslips (thickness 1.5).

Before You Start:

1. Start by defining the purpose of the microscopy experiment: Is it structural analysis, colocalization studies, nuclei phenotyping, tracking of dynamic processes or others?
2. Estimate the number of images that will be needed in the study: for example, structural studies do not require multiple images when the resolution is sufficient, tracking dynamic processes needs a high number of images to achieve great statistical significance.
3. Identify the type of images to be acquired. XY (2D), XYZ (3D), XYT (time-lapse), XYZT(4D) and the number of colours that will be used. Modern microscopes can easily image four colours (three fluorescence channels and one brightfield channel). However, the more channels are required, the bigger the bleed-through between channels and the more control experiments will be required. It is advised to use a brightfield channel, especially for live-cell imaging, as it shows the overall condition of the sample.
4. Estimate the resolution required. This is a critical step as it can vastly decrease the duration of the experiment. A common pitfall is to go with the highest resolution simply because the images look beautiful. In other words, do not try to resolve details that will not be used in post-processing. For example if subcellular structures need to be resolved, then the feature size will be limited by diffraction, and the resolution will be equal to roughly 200 nm. Alternatively, if cell nuclei need to be imaged for counting or phenotyping, then a resolution of 1 μm will be sufficient.
5. Match the desired resolution to the microscope camera to find out which objective to use. You need to know the pixel size of your camera for this calculation. This can be easily found out online and is available on the software interface in modern commercial microscopes. It is usually between 5 and 15 μm. According to Nyquist's Sampling Criterion, the pixel size should be twice to three times

smaller than the size of the features that need to be resolved. Divide this pixel size by the desired resolution to get the magnification. Here it is assumed that the camera diagonal is matched to the microscope field number, and no intermediate magnification lens is used.
Example: a feature size of 600 nm corresponds to a pixel size of 200–300 nm in the image plane as per Nyquist's Sampling Criterion. With a physical pixel size of 10 μm, a 20× or 30× objective will be required. 30× objectives are uncommon, so a 40× is more appropriate. Note: magnification is in each dimension. A 20× objective will have a field of view that is four times bigger than a 40× objective. Considering that a plan-apochromat 20× objective has a NA of 1.0 and an equivalent 40× objective a NA of 1.4, the latter will bring 40% more resolution at the cost of losing 75% of the imaged area.

6. Choose the appropriate mounting medium/immersion medium combination [10]. For optimal resolution, the refractive index of the immersion medium should match the refractive index of the sample medium. Where possible, use water, glycerol or silicon oil immersion objectives for live-cell imaging and oil immersion for fixed samples with hardening mounting media like Prolong Gold or Prolong Glass.

On the Microscope:

1. Place the sample on the holder and fix it with clamps or tape.
2. Make sure the stage is levelled and that the inserts are properly placed. This is important to avoid unnecessary reflections from the coverglass. Some stages have a spirit level built in.
3. Use brightfield microscopy to focus on the sample. This greatly reduces photobleaching. In some cases, like thin sections, it is hard to find the sample. Here, use phase contrast or DIC contrast to locate them. Alternatively, look for the reflection of the covergalss or close the aperture diaphragm. Use fluorescence only when brightfield microscopy does not work.
4. Start with the objective that has the lowest magnification (5× or 10× objective). This objective will have the largest depth of focus and will make it easy to focus on the sample.
5. Use the binoculars to focus on the sample. Modern microscopes have an autofocus feature that can focus on the sample. This autofocus has improved over the years, but a trained eye is still better and faster.
6. Find the area of interest and centre it. Use the crosshairs on the binoculars, if available. Many commercial companies offer them as an option.
7. Set up the imaging channels in the software. Use multi-band filters only when acquisition speed is limiting. Otherwise, use single band filters that reduce cross excitation and bleed-through.
8. Set up the camera exposure and excitation power in a way that utilizes the whole dynamic range of the camera. Avoid saturation as it reduces resolution. We recommend starting with a few milliseconds of exposure for brightfield imaging and a few hundreds of milliseconds for fluorescence imaging and adjusting

either exposure or light attenuation until some of the pixels get saturated. Use a high-low colourmap/look-up table, if available, as it shows saturated pixels in a different colour (blue or red).
9. Set up the other dimensions (time, Z-stack) depending on the experiment.
10. Start your experiment.

Tips for multi-position long term live-cell imaging

- Turn the incubator on well before the start of the experiment (1–2 h) and make sure the temperature sensor is placed as close as possible to the sample.
- Make sure the stage is levelled, and the sample dish/slide is fixed on the stage. Use putty-like adhesive (Blu-tack) if necessary.
- Use hardware focus control systems, if available (Leica Adaptive Focus Control, Olympus Z-Drift Compensation, Nikon Perfect Focus System, Zeiss Definite Focus). Alternatively, make sure the microscope is in a location with minimal vibration (basement) or placed on an anti-vibration table.
- For suspension cells, wait 30 min or 1 h before starting your experiment. This will ensure that the cells reach the dish bottom.
- Use a slow stage speed (100–250 μm/s).
- Use the highest gain and lowest exposure possible.
- Add the individual positions in spiral-like shape to minimize stage movement.
- Use Z-stacks only when necessary. This will greatly increase the viability of the cells. Most cells are small, and a 2D image will deliver most of the required information.
- Make sure the shutter goes off after each image to avoid unnecessary exposure.

Take-Home Message

- Epifluorescence microscopy is a widefield modality of microscopy that uses fluorescence contrast to generate images.
- Epifluorescence microscopy is the most common type of imaging in life science research.
- The main advantages of epifluorescence microscopy are:
  - A dark background (high contrast) thanks to the separation of excitation and emission.
  - The labelling specificity: only the desired structures are visible on the image.
  - Its versatility: the possibility to combine multiple colours.
- The main disadvantages of epifluorescence microscopy are:
  - The indirect nature of the technique: The fluorescence is emitted from the fluorophore and not from the molecule of interest.
  - Photobleaching: the emission of fluorescence is finite in time. Photobleaching can be critical for long-term experiments.
  - Bleed-through from fluorophores with spectral overlap. Bleed-through is critical for colocalization studies.

- For optimal imaging results, excitation, emission and sample have to be matched and optimized for each other.
- Many high-end techniques are based on the epifluorescence setup.

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