Can you see the light?

Amazing findings can be made using fluorescence microscopy, but how do you choose the right equipment? **Iain Hagan**, **Agnes Grallert** and **Steve Bagley** give some practical help.

Simple and affordable steps to exploit recent advances in fluorescence imaging

While recent advances in imaging technologies have had a dramatic impact upon biology, the technical challenges and baffling array of options have tended to restrict these technologies to a limited number of *cogniscenti*. This is particularly true for imaging microbes where low intensity signals have conspired with the need for considerable magnification to present a formidable challenge. However, some straightforward changes to standard fluorescence microscopes can now satisfy this obsessive desire to maximize sensitivity and resolution.

Imaging platform: spinning, scanning – how convoluted can it be?

Laser scanning confocal microscopes (LSCMs) have a reputation as benchmark instruments for high-resolution imaging. However, they require very bright samples and most microbiologists have no need to take advantage of their ability to illuminate a constrained slice in thick sample. Although LSCMs are not ideal for imaging bugs, a second type of confocal microscope, the spinning disk microscope (SDM), is. Instead of scanning consecutive points in a series of lines across a field to build up an image line by line that is the hallmark of LSCM, SDMs simultaneously monitor 356 points which reduces off-site bleaching and combines with further technical advantages to give arguably the best temporal and spatial resolution available. However, SDMs are very expensive and rely upon bright lasers for illumination. Imaging multiple wavelengths invariably means using multiple lasers, further raising prices. Traditional, 'widefield' fluorescence microscopy, on the other hand is versatile, affordable and now sensitive enough to deliver top quality imaging. It works by bathing samples in light of a specific wavelength that is selected by the use of dedicated filters from a universal white light source. The secret is to ensure that as much of the light that leaves the specimen gets to the camera as possible.

▲ Microscope lenses. *David Parker / Science Photo Library*

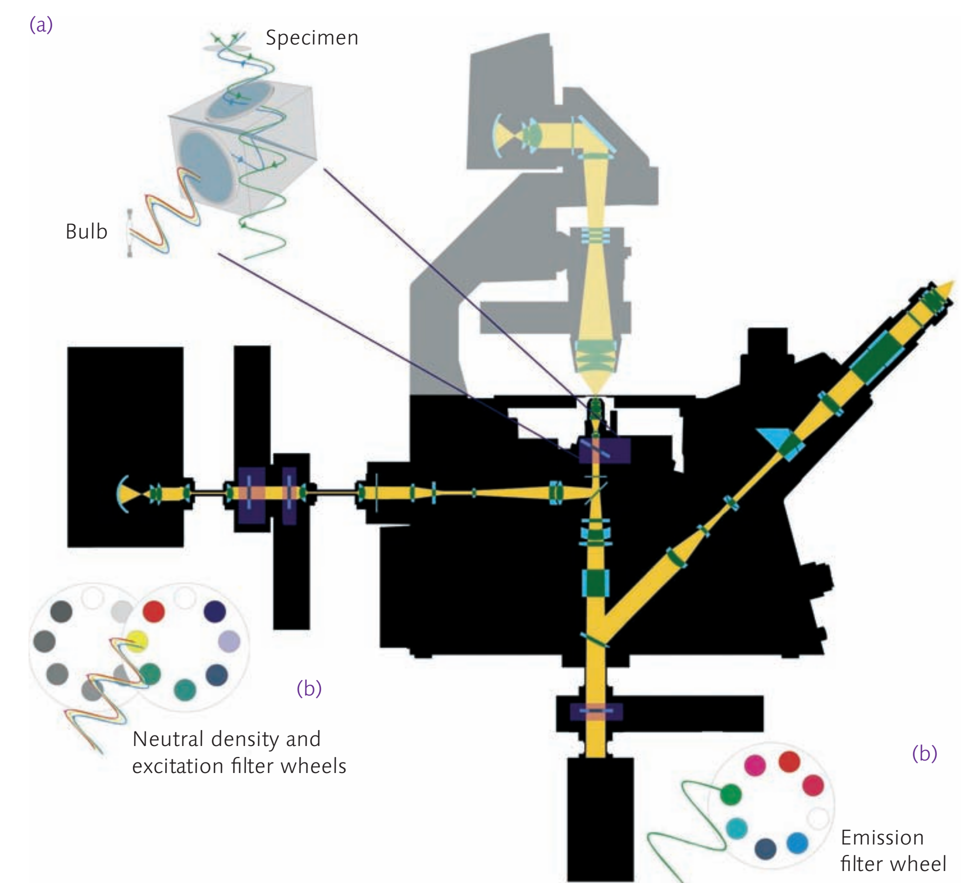
Enhancing an existing widefield system

1. Light path: provision

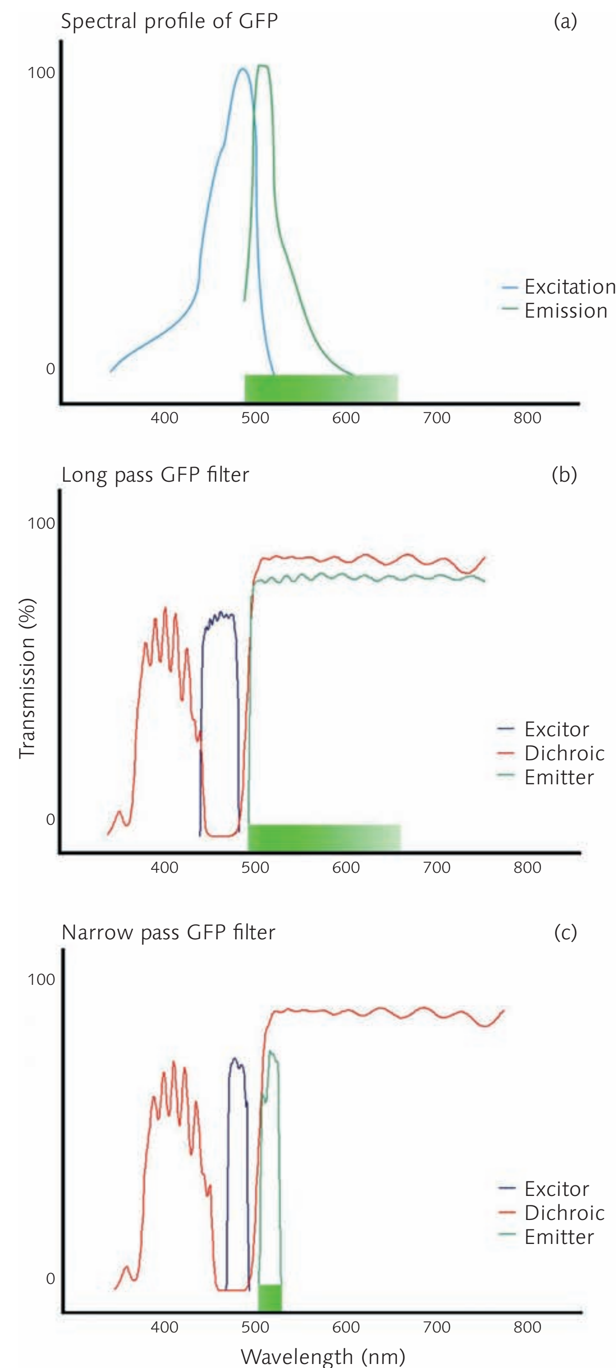
The most easily justified modification to an existing widefield system is to upgrade the light source from the traditional mercury bulbs to a tungsten/xenon system. As well as being brighter than mercury bulbs, tungsten/xenon systems are much cheaper to run (£0.35 per hour vs. £1.20 per hour). Thus, they not only boost sensitivity, but pay for themselves over a 2- to 3-year period, and then save money after that. A final perk is that they overcome the common pitfall of uneven illumination that has plagued the use of mercury sources. The xenon/tungsten systems use a fibre optic cable that scrambles the image of the bulb to deliver an even illumination that is invaluable for quantification.

2. Light path: transmission

When passing through, or bouncing off, an optical element some light is lost, eroding both the intensity and quality of the image. While the optical elements in microscope bodies have been reduced to a minimum, the demands of certain disciplines mean that objective lenses can contain a considerable array of additional elements. Pathologists and neurobiologists often want to see high-power magnification images of entire fields that are free from distortion and in sharp focus from one side to the other. This technically challenging demand is met by the inclusion of correcting elements to counteract distortions at the edge of a field of view. However, these extra elements reduce transmission. This cost is too great for microbiologists who would happily tolerate distortion at the periphery of a field that is often tens of cells wide, as the benefit of neglecting this correction is enhanced transmission. In other words because commercial suppliers offer a range of lens types for a range of



▶ Fig. 1. A cartoon showing the two options for filtration of light in a widefield microscope system. (a) Most systems use filter cubes that sit between the objective and the eyepiece; however, the use of filter wheels (b) enables rapid switching from one channel to another. *Steve Bagley*



▲ Fig. 2. Filtration of GFP emission by long and narrow band pass filters. (a) The excitation (blue) and emission (green) spectra of GFP. The green bar indicates the breadth of fluorescence emission that is fully sampled by the long pass (b) but not the narrow pass filter (c). *Steve Bagley*

► Fig. 3. Labelling living *S. pombe* cells with two fluorescent lectins facilitates the ultimate live cell imaging control – three distinct cell types imaged alongside each other. Red, cell type 1; green, cell type 2; black, cell type 3; blue, chromatin (Hoescht 33342). *Agnes Grallert*

applications it is vital to know what type of lens you are using. Does it have a lot of correction or maximal transmission?

The key term is the Numerical Aperture (NA) of an objective. The higher the NA, the higher the transmission. Quite fortuitously for microbiologists, a form of microscopy called total internal reflection microscopy (TIRF) that demands the highest NA possible has recently gained in popularity, inducing every commercial supplier to produce objectives with extremely high NAs. The catch is an extremely shallow depth of field – however, the size of microbes comes to our rescue again as depth of field is rarely a problem for a microbiologist. A further catch is that you are likely to be told by the sales representative that these lenses are only useful for TIRF and you should not try them! However, persist! Demand a loan for a trial run! A switch from a 100× 1.3 to a 100× 1.45 doubled the intensity of our yeast images.

Many microscopes have optional internal magnification lenses on sliders or wheels that can provide further magnification between the objective and the eyepiece/camera. Beware, these lenses can severely erode image quality and should be used with utmost caution.

3. Light path: wavelength selection

Fluorochromes are excited at one wavelength and emit light at a second, lower energy (longer) wavelength. Thus paired excitation and emission filters that select the appropriate wavelengths for a particular fluorochrome lie at the heart of any widefield microscope. Most fluorescence microscopes have filters paired up, either side of an appropriate (dichroic) mirror to reflect and transmit the appropriate wavelengths in specific blocks between the light source objective and eyepiece/camera (Fig. 1a). Alternatively, and more expensively, filters can be mounted on independent filter wheels to enable more rapid changes in wavelengths (50 ms vs 900–2000 ms for changes of internal filters) and to isolate the moving parts from the body of the microscope to minimize vibration (Fig. 1b). Whichever system is available it is important to understand the range of filters at your disposal.

Fluorophore emission spectra generally have a strong peak that trails off towards the red end of the spectrum (GFP in Fig. 2a). To distinguish between the signals from two different fluorochromes in a single specimen, two combinations of excitation and emission filters are required to select the appropriate portion of the spectrum to excite and observe one fluorochrome while excluding those that would excite/observe the signal from the other. Two issues arise here. The more restrictive the filtering, the more sophisticated is the coating on the glass needed to generate the filter. The more complex the coating, the less light is transmitted. Second, the selection of the narrow bands of the spectrum that are required to see multiple wavelengths as separate signals in one sample excludes the long tail in the emission spectrum of the fluorochrome and so effectively throws away up to 20 % of the signal from the sample. Therefore, if only one fluorochrome is to be imaged, 'long pass' filter sets (Fig. 2b), that need less coating and lack an upper cut off, enabling the full tail of the spectrum to be captured, give much better signals than the restricted and heavily coated 'narrow band pass' filters that permit selective imaging of different fluorochromes in a single sample (Fig. 2c). If you are just interested in GFP in an experiment, a long pass filter set will boost intensity by up by 20 %. Furthermore, the technology used to generate filters has been revolutionized in recent years so that simply replacing old filter sets with newer alternatives (splutter-coated filters) can increase the brightness by 20–30 %.

One aspect of light sampling that is often overlooked is the type of dichroic mirror used. Just like filters, the greater the demands placed upon the specificity of the mirror, the more sophisticated are the coatings, so less light is transmitted. Thus, the selection of a dedicated dichroic for a particular wavelength can also considerably boost transmission.

4. Detection: cameras

Cameras take us away from this article's theme of 'easily affordable' adaptation. However, changes in CCD technology that are transforming the quality of cameras in high-street stores are also transforming microscopy. For microbiologists, the key issue is the quantum efficiency of a CCD chip – literally, how much light arriving at the surface will be converted into an electrical read out. A technology in which light signals are converted into a stream of electrons has pushed quantum efficiencies of chips to greater heights. Switching to an 'electron multiplying CCD' camera has revealed signals that we could not detect with the previous generation of chips.

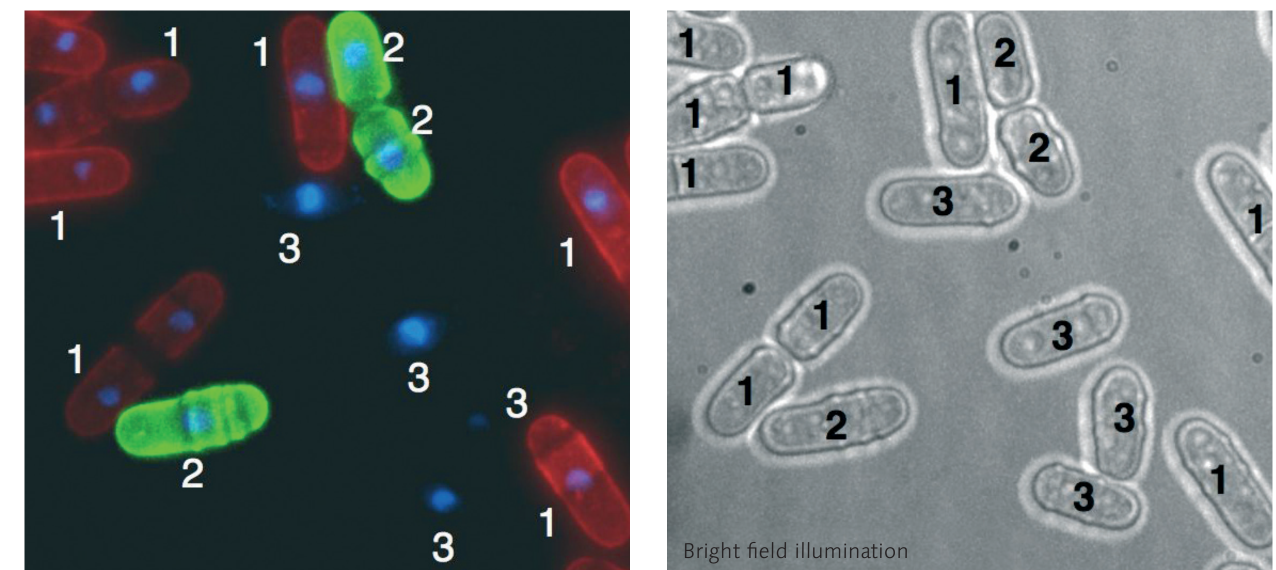
Some straightforward changes to standard fluorescence microscopes can now satisfy the obsessive desire to maximize sensitivity and resolution

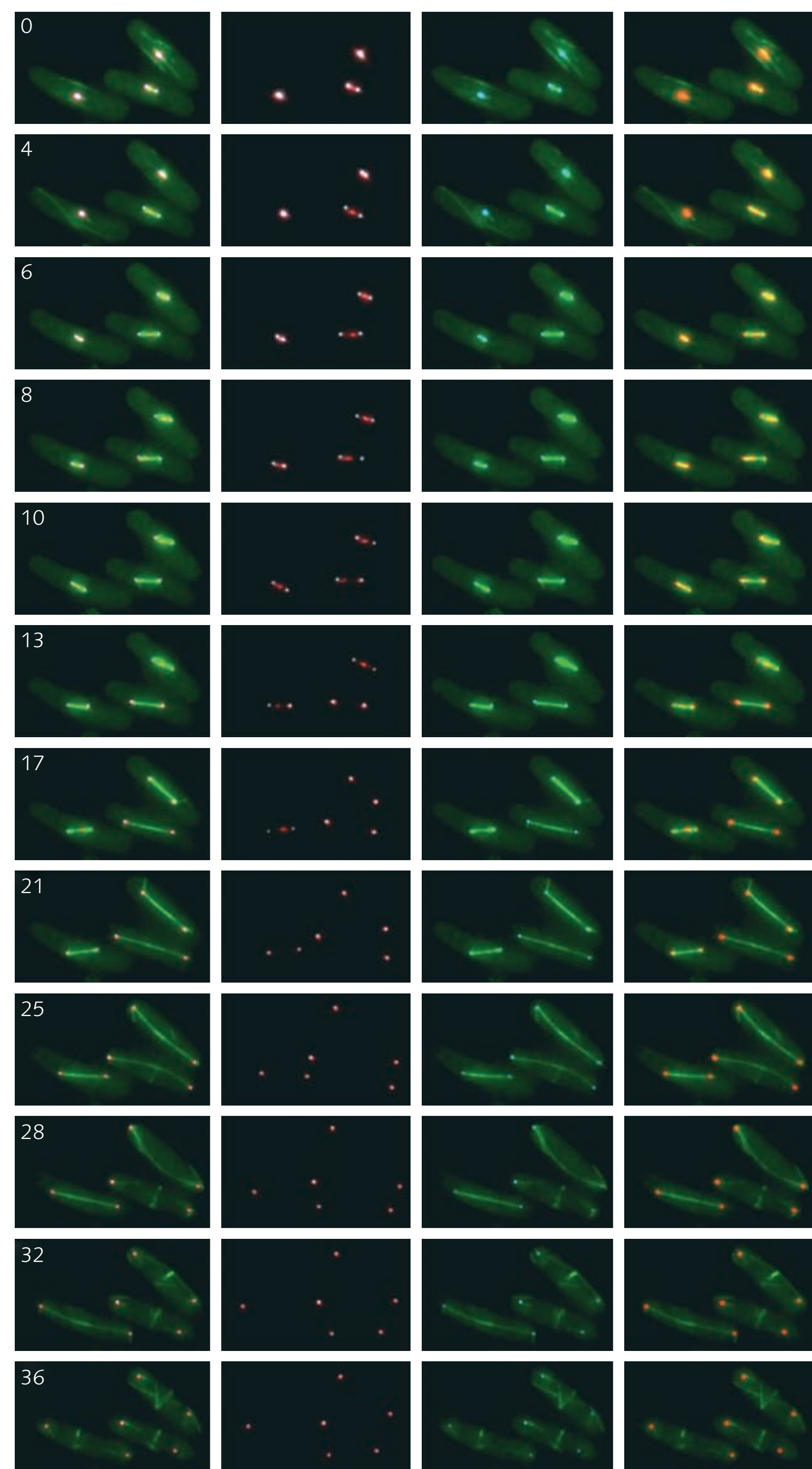
5. Optimal sample control: staying alive

Environmental control can be as critical as the optimization of the light path. For example, encasing the stage in a heated Perspex box might not be sufficient to give accurate temperature control. The metal of the objective lens is connected to the microscope body that lies outside the box. It can therefore act as a 'heat sink' that reduces the temperature in the one place where you do not want it changed – the field of view. This problem can be overcome with accurate and highly versatile commercial systems that control the temperature of the coverslip surface (for example, by heating a transparent coating of metal) and the temperature of the objective with a heating collar. A number of micro-perfusion chambers can be used to maintain nutrient supplies or deliver drugs or toxins on cue. Cells can be mounted on a range of transparent gas-permeable matrices to ensure the correct aerobic/gaseous conditions are maintained over extended periods.

6. Wall to wall imaging

Microbial cell walls can be exploited in a variety of ways to assist imaging. Coating coverslips in ligands that bind





▲ Fig. 4. Three colour images of dividing *S. pombe* cells in which the centromeres are marked by *cnp1.cherry*, the spindle poles with *sid4.tomato* and microtubules with *atb2.GFP*. A movie of this file is linked to this article on the *Microbiology Today* website (www.sgm.ac.uk). Green, tubulin; red, centromere; blue, spindle pole body. *Agnes Grallert*

cell-wall components, such as poly-L-lysine or lectins, is a simple way of ensuring a sample stays put during image capture. This is particularly important if medium has to be changed via perfusion. Another simple trick is the selective labelling of one cell type in a single field of view with a fluorochrome before they are mounted side by side in the same field. Thus, a mutant strain can be observed in exactly the same conditions as the wild-type control (Fig. 3).

Is it worth it?

A modest equipment grant of around £12,500 can transform an existing microscope system for imaging GFP to deliver some top notch images (light source, £3,000–4,000; specialized objective, £5,000–7,000; dedicated filter/dichroic set, £800). A quick trip to another lab that has the technology in place can determine just how great a difference can be made and whether it will be of any use. Invariably the gains obtained with these modest adjustments eventually provide justification to granting bodies for a new camera and more sophisticated image processing, leading to the ability to visualize three or more proteins for extended periods (Fig. 4). There has never been a better time to dispel the folklore surrounding imaging and have a go.

Iain Hagan[1], Agnes Grallert[1] & Steve Bagley[2]
[1]CRUK Cell Division Group and [2]Advanced Imaging Facility, Paterson Institute for Cancer Research, University of Manchester, Wilmslow Road, Manchester M20 4BX, UK (e ihagan@picr.man.ac.uk, agrallert@picr.man.ac.uk, sbagley@picr.man.ac.uk)

A supplement with further tips to negotiate imaging are linked to this article on the Microbiology Today *website at* ***www.sgm.ac.uk***