Microscopy & Microtechniques Focus

THE BIGGER PICTURE – A MACRO VIEW OF FLUORESCENCE

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To see anything through a microscope requires a certain amount of contrast. For bright field this is achieved through the use of a number of different histological stains, which bind to specific proteins to differentiate cell types, organelles and inclusions for example. Most of these stains though require samples to be fixed and processed and therefore risk the introduction of possible misleading artefacts. Techniques such as fluorescence have revolutionised microscopy to enable the clear identification of many parameters by naturally producing contrast in the properties of light and refractive indices of the different components of a sample.

Fluorescence techniques place numerous benefits in the hands of researchers wishing to exploit the upper limits of sensitivity and resolution in microscopy. Beyond the scientific benefits, simply studying fluorescence images can sometimes offer a new insight into a reality which is usually hidden from view from the world.

"ACTING AS LIGHT SOURCES, FLUORESCENT MOLECULES INDICATE THEIR LOCATION IN A CERTAIN AREA OF A SPECIMEN WITH LIGHT OF A SPECIFIC COLOUR"

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WHY USE FLUORESCENCE?

Using fluorescence can be likened to a teacher asking pupils if they have completed their homework. The rapidly changing face colours of the 'guilty' students provide conclusive 'results'! When used to address specific questions regarding life or materials science specimens, fluorescence can also visualise the result in a certain colour. For example, immunohistochemistry can identify the distribution of a specific protein within a tissue, where a fluorochrome is used to mark the protein via an antibody.

Although histological staining procedures for transmission light microscopy have a long history in microscopy, fluorescence microscopy holds a key advantage due to the enhanced resolving power that it can deliver. Naturally occurring fluorescent molecules (eg GFP, Green Fluorescent Protein) enable this, as even if a structure is too small to be resolved by a light microscope, the emission light still remains visible. Further advantages of fluorescence over staining procedures include the reduction of potential artefacts and the facilitation of live cell observations.

Acting as light sources, fluorescent molecules indicate their location in a certain area of a specimen with light of a specific colour. To emit light, these indicators require energy supplied to the fluorochrome by excitation light provided by the microscope light source. A specific range of wavelengths is required to excite a specific fluorochrome, as every fluorochrome has its own excitation and emission spectra. For example, a range of blue wavelengths around 480 nm can excite the FITC fluorochrome. The microscope must, therefore, be perfectly equipped to visualise this fluorescence accordingly.

FLUORESCENT MOLECULES

There are two options for using fluorescent microscopy, depending on what is being investigated: either the specimen itself naturally contains fluorescent molecules (autofluorescence); or specific fluorochromes have to be added to the specimen. Fluorochromes themselves can be divided into at least three groups. The first group require other molecules, such as antibodies or lectines, to bind to specific targets. This rapidly growing group of fluorochromes includes longstanding ones such as FITC and TRITC. Most of these fluorochromes are sold together with the specific target-finding molecule (e.g. a goat anti-mouse IgG antibody Cy5 labelled). Quantum dots are also partial members of this group, but different in structure and theory. They are nanometer-sized crystals of purified semiconductors and exhibit long-term photo stability, as well as bright emission.

The second group have inherent binding capacities, such as the DAPI nucleic acid stain intercalating with DNA or the DiI anterograde neuron stain. This group also contains fluorochromes that change their fluorescent properties when bound to different amounts of molecules such as calcium (e.g. Fura-2). These fluorochromes are used directly and do not necessarily require a transportation system such as an antibody. The third group contains the fluorescent proteins produced by organisms themselves such as GFP. This makes it possible to set up experiments in an entirely different way and are frequently used for live cell imaging or developmental studies and molecular biology. All fluorochromes show distinct spectral properties and can often be combined for a multicolour specimen analysis.

FLUORESCENCE ADVANCES

With an explosion in available fluorochromes in recent decades, fluorescence detection techniques now play a major role in the functional analysis of organisms since they can be applied on a whole organism right down to tissue level. This not only requires a wider field of view, but also the addition of different illumination technologies to ensure fluorochrome excitation. Consequently, fluorescence systems that incorporate these requirements to offer a macro-view down to a micro-view can prove to be an extremely useful tool for any research laboratory.

Advances in the technology behind microscopes, such as the new Olympus SZX16 stereo-zoom (*Figure 1*) and the MVX10 mono-zoom microscopes (*Figure 2*), are certainly addressing these needs. Such technological developments have now made it possible to apply advanced techniques at low magnifications, such as fluorescence, when using 'zooming' microscopes and gain completely clear 3D views from whole organism right down to fine details.

When using microscopes to study whole organisms, the working distance is a key consideration. Therefore, achieving the necessary resolution for fluorescence techniques using a macro-zoom microscope can prove a challenge, since there is a fine balance between offering increased magnification combined with greater resolution, versus a greater working distance. Higher Numerical Apertures (NA) offer increased resolution and increased light collection efficiency to result in brighter fluorescence. Consequently, for a macro-zoom system to be capable of high quality fluorescence imaging, there is a need for specially designed optics to accommodate this need for ever-greater working distances coupled with higher numerical apertures and increased field size.

To increase NA and light efficiency, whilst still maintaining the working distance, both of Olympus's new macro-zoom microscopes for fluorescence applications, the SZX16 and MVX10, support larger lenses than conventional zooming microscopes. Further boosting light efficiency, the advanced Olympus objective line used for these microscopes has a significantly increased transmittance across the spectrum, but especially in the near IR regions. This is achieved by novel multi-layer coating which enhances sensitivity to allow the rapid collection of more light.

Specially developed lead-free materials used in the optics also drastically reduce the autofluorescence of the components, enabling detection of even faint fluorescent signals.

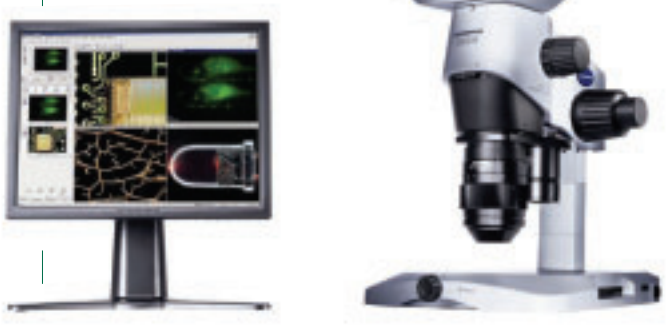

Figure 1: The Olympus SZX16 research stereo microscope

A NEW STEREO RESOLUTION

With the increasing range of microscopy techniques for research, the instruments required have become increasingly sophisticated to give clear views of ultra-fine details. Stereo microscopes are designed for low magnifications compared to compound systems. This is because as magnification and numerical aperture increase, the depth of field (amount of image in sharp focus) decreases (and vice versa). Therefore, there is a limit to how much an object can be effectively magnified on a stereo microscope. The Olympus SZX16 though, is a completely new stereo microscope designed with enhanced technological features to extend the stereo effect, providing enhanced 3D imaging for micromanipulation and meet ever evolving modern research needs.

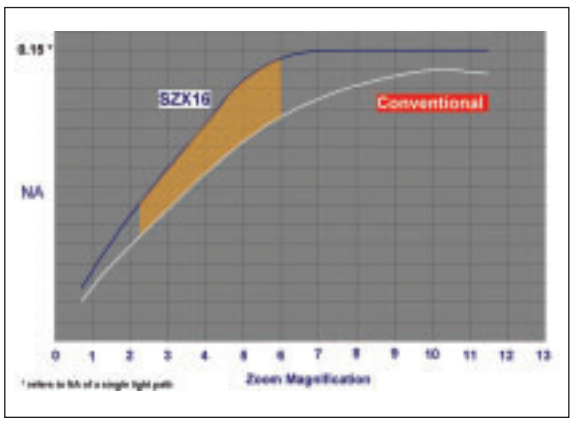

Figure 3: The optimised NA / magnification ratio of the SZX16 research stereo microscope (blue line) compared to a conventional stereo microscope (red line)

The SZX16 uses the parallel light path principle of a Galilean (or telescope) optical system. This provides excellent flexibility since more optical features (such as fluorescent illumination) can be placed into the parallel light paths. In order to support the larger lenses than conventional stereo microscopes, the SZX16 uses specially adapted optics to give a greater distance between light paths. This enables significantly increased numerical apertures (NA) to provide excellent light signal collection. Consequently, the SZX16 provides the highest possible resolution throughout its magnification range (*Figure 3*) and has a maximum numerical aperture of 0.3, giving an unprecedented resolution of 900 line-pairs per millimetre.

FROM WHOLE ORGANISM TO CELLULAR DETAIL

In addition to enabling improved image clarity, the specially adapted optics of the SZX16 have also extended the stereo zoom range to provide far greater flexibility. Its world leading zoom ratio of 16.4:1 means that its parfocal objectives can zoom from 3.5x to 230x in one smooth movement, with the two position nosepiece attached. Such a large magnification ratio is ideal for research areas such as developmental biology. For example, an overview of the whole organism enables the identification of regions of interest which can be zoomed in on very easily and quickly. Furthermore, by using parfocal objectives there is no need to refocus when switching objectives.

Due to the unprecedented resolution and sensitivity achieved by the SZX16, even faint fluorescent signals can be detected easily from anywhere within the sample. For any advanced fluorescence instrument, the illumination source should be as close to vertical as possible and

Figure 2: The Olympus MVX10 macro zoom microscope

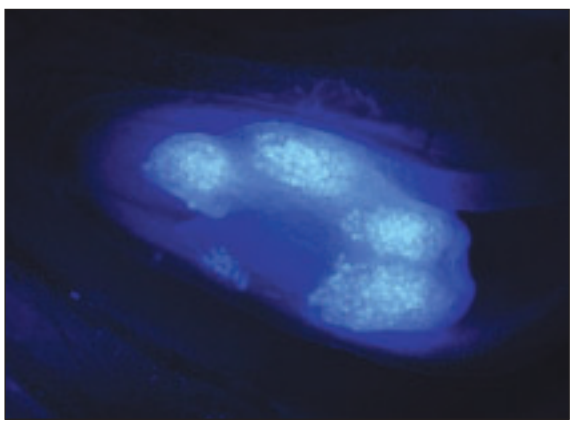

Figure 4: A plant stamen illuminated using UV light, with pollen showing autofluorescence. Taken using an SZX16 with a DP71 Camera.

completely integrated to provide even and controllable fluorescence. The SZX16 utilises a perpendicular fluorescence illumination pathway and, as a result, avoids the artefacts generated when using alternative pathways. Using high quality light sources and filter components means that they all combine to produce the perfect fluorescence stereo system for the user and application where depth perception and accurate micro-manipulations are also required (*Figure 4*).

It is often important to use a stereo microscope in tandem with compound microscopes when a procedure requires the highest magnification and resolution possible. In these situations, e.g. fluorescence pre-screening, it is advantageous to have an advanced stereo microscope to provide increased efficiency and clarity. It therefore needs to offer the best resolution and brightest, clearest images possible within its usual working zoom range.

A NEW VIEWPOINT ON MACROFLUORESCENCE IMAGING

To effectively eliminate this need to switch between a stereomicroscope and a compound microscope during fluorescence screening procedures, Olympus has introduced the MVX10 MacroView.

This is a completely unique instrument for both micro and macro fluoresence observation. It combines maximum detection sensitivity at the lowest magnifications with a high magnification zoom for fine detail resolution within small organisms, organs, tissues and even cells. This enables researchers to clearly see fluorescent images of whole organisms right down to the cellular level with unprecedented brightness, resolution and precision (*Figure 5*).

The MVX10 is a mono-zoom microscope that uses a single, large-diameter optical path optimised to collect the weak light generated by fluorescence at all magnifications. Together with the extremely high NA this results in exceptionally good signal-to-noise (S/N) ratios, ensuring excellent contrast for the observation of even faint fluorescence signals.

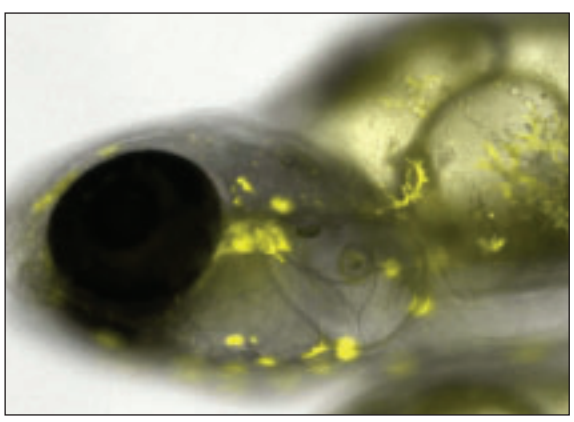

Figure 5. A young Medaka one day after hatching with macrophages labelled with membrane YFP driven by a macrophage specific promoter. Courtesy of Dr. Adam Cliffe, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany.

From low magnification confirmation to detailed observation, the MVX10 is ideal for a wide range of fluorescence techniques. Furthermore, its smooth, stepless 10x zooming and a maximum magnification of 252x produces superb brightness and resolution.

Together, these advances also mean that specimens can be exposed to fluorescent light for shorter periods, thereby minimising the risk of photo bleaching, or even cell damage.

This is also true at near-infrared wavelengths where the MVX10 has superior transmission properties effectively increasing the range of useable fluorophores. Another unique design introduction in the MVX10 is a pupil division mechanism in its light path that can mimic the effect of stereomicroscopy when a certain amount of image depth is required.

ACCURATE RECORDS

The growing need for fluorescence observation of organisms at low magnification has also increased the need to record fluorescence images at high magnification using high performance digital cameras. The imaging device is one of the most critical components in fluorescence microscopy analysis. This is because it determines at what level specimen fluorescence may be detected, the relevant structures resolved and/or the dynamics of a process visualised and recorded. Numerous properties are required for effective fluorescence microscopy imaging, including: high resolution, extreme sensitivity, cooling, variable exposure times and an external trigger function. All fluorescence microscopy cameras should at least offer high signal sensitivity, low noise and the ability to quantify intensity of intensity distribution.

The advanced new Olympus DP71 camera, produces perfect colour representation for brightfield images, as well as highly sensitive fluorescence detection. The camera uses the same colour reproduction technology as high definition television (HDTV) and therefore, full-frame (1360 x 1024) live images can also be displayed at 15 frames per second. Further to this the camera produces a maximum digital image resolution of 12.5 mega pixels for storage. Consequently, the SZX16 and MVX10 are designed for both optical and digital use to create extremely versatile macro-to-micro imaging systems.

IN CONCLUSION

With life being such a complex blend of known and unknown interactions, it is important to take a step back and look at the bigger picture. To do this properly though, requires the correct tools. Advances in the technology behind microscopes, such as the new Olympus SZX16 stereo and the MVX10 mono-zoom microscopes, are certainly addressing these needs. Such technological developments have now made it possible to apply advanced techniques, such as fluorescence, when using 'zooming' microscopes and gain completely clear 3D views from whole organism right down to fine details.