BIOCHEMIST – ALL IN ONE ARTICLE

Bringing ease-of-use to microscopy

From the Philosopher's Stone to the Researcher's Dream

Although naturally occurring luminescence has been observed for many centuries, the first study on it was not completed until the golden age of alchemy by Vincenzo Casciarolo, who discovered 'Bolognian Phosphorous' often referred to as 'Bolgnian Stone' or '*Litheophosphorous*'. Much was debated and written on this supposed philosopher's stone, but the physico-chemical basis of its absorption and emission of light remained poorly illuminated. Through seminal work by many researchers, including Sir George Stokes, our knowledge of such properties has clearly increased, and we have now a deep knowledge about specific excitation and emission wavelengths of an enormous number of different fluorescent molecules. As a result, fluorescence (and to a lesser extent luminescence) are used across many life science research protocols to track and analyse molecules within many different paradigms. Although a number of molecules posses intrinsic fluorescence (or even luminescence), the majority of applications involve the use of exogenous fluorescence dyes or recombinant proteins to tag a molecule of interest. Arguably, the investigative tool that has benefitted the most from the application of this fluorescence knowledge, and in turn contributed greatly to the advancement of science, is microscopy. But fluorescence microscopy and its derivations are far from simple and involve a large amount of user knowledge. A new concept though, is set to provide advanced

microscopy that bypasses the need for in-depth optical know-how – a researcher's dream.

Applying fluorescence to microscopy

By carefully labelling molecules of interest with fluorescent dyes they can be seen clearly down a microscope by illuminating the sample with the required excitatory wavelength. Combining the resultant image with the same field of view captured using a standard contrast technique, such as differential interference contrast (DIC), enables the cellular location and distribution to be visualised more clearly. By tagging more than one molecule with different fluorophores it is possible to get a real feel for their proximity or even co-localisation. Importantly, a number of dyes have been discovered or developed that accumulate in key areas such as the nucleus (e.g. DAPI) or the mitochondria (MitoTracker), which provide very clear organelle level resolution. It is clear therefore that fluorescent dyes are very powerful additions to the researcher's microscopy tools, but also that an experiment can quickly become complex with multiple dyes.

And then there is the three-dimensional nature of cells to consider; this is a major source of image blur, as the fluorescence emission from molecules above and below the focal plane of the image lead to a diffuse glow as they are still captured by the microscope optics. From the microscope side, the increasing use of fluorescence has also required concomitant changes in microscope design and the materials used. Although it is not possible to cover this in its entirety within this article, it is important to highlight a few key considerations.

The illuminating light must be supplied at the right wavelengths to excite the various fluorophores in use. This light can either be directed through the microscope optics and onto the sample via the objective (reflected

fluorescence) or via a separate optics system via a condenser (transmitted fluorescence). Either way, the illuminating light must first be filtered to provide only the correct excitation wavelength, and its intensity must be controllable, a process that is usually achieved through the use of different neutral density filters. The emitted light must then be collected, magnified and focused by the microscope optics in such a way that resolution increases with magnification. The glass used in the optical components, such as the lenses and prisms, needs to be capable of transmitting as much of the fluorescence as possible across the entire spectrum. The microscope must also prevent harmful wavelengths of light from reaching the researcher's eyes, whilst filtering the emitted light for the desired wavelength. There are also many optical artefacts that affect microscope imaging, such as spherical and chromatic aberrations, all of which must be corrected for to achieve clarity.

It is clear from this, that even basic fluorescence microscopy involves a large number of components and a decent amount of user know-how. Adding experimental factors, such as Z-stacks (a series of images taken at various depths through the sample), multiple fluorescence dyes, multiple regions of interest and time lapses for example, increases this complexity, making successful microscopy much more involved for the user.

Confocal intricacy

A further level of intricacy is introduced with advanced techniques such as confocal microscopy. There are a number of different ways of achieving confocality in microscopy, but the most common is referred to the confocal laser scanning microscopy (cLSM). For this, a finely tuned and focused laser beam is scanned across the sample at high speed, illuminating the fluorophores as it goes. The emitted light is collected as per normal, but as it is focussed by the microscope it passes through a 'pinhole' which

essentially eliminates any light that has originated from out of focus parts of the image. As a result, the image created is highly resolved in the X, Y and Z axes. The extent of the confocal effect can generally be controlled by changing the diameter of the pinhole, and other important features, such as the scan speed and scan pattern are also fully controllable. This clearly adds a large number of other considerations for the user and makes acquiring great images even more difficult.

Taming the beast

As such pivotal techniques, a broad variety of advanced microscopy systems have been successfully developed for the imaging of fixed, as well as live, cell and tissue samples. With uses in both medical and biological sciences, these microscope systems are all too often inaccessible to less experienced users, due to the knowledge required to 'drive' them properly. Many institutes will therefore directly, or sometimes indirectly, appoint a microscope imaging expert to provide the on-site know-how to generate the stunning and informative images possible with such systems. Fluorescence and confocal microscopy though, are becoming more and more popular and microscope imaging specialists are therefore overwhelmed with imaging requests. In response to this clear need, microscope manufacturers, such as Olympus, have taken all the complexity of fluorescence and cLSM systems, and packaged them into compact, easy-to-use systems which enable simple, step-by-step, operation via clear on-screen instructions. With one-click operation, these self-contained microscopes have the ability to automatically locate the best-possible settings, removing the need for the user to adjust any complicated optical set-ups. Highly advanced imaging is therefore made accessible in-lab, to researchers of all experience levels.

The usability dividend

By making these complex techniques more easily accessible to the everyday researcher, the workload of the experienced microscopists can be re-assigned to performing and developing real cutting-edge microscopy techniques. What is more, scientists can be in complete control over every aspect of their research and may even broaden the spectrum of the study they undertake. Furthermore, if the complete process was able to take place conveniently in-lab, procedures could be effectively streamlined for an exceptionally smooth workflow. Simple and convenient access to complex microscopy techniques can be made available to a much wider audience, without any compromise on the quality of the image obtained.

Microscopy in a box

The novel idea of unifying all of the components required to perform either confocal or fluorescence microscopy into a single, compact 'box' removes some of the complexities, and subsequently increases the usability, of such microscopy systems. In order to meet this need, Olympus has recently introduced its all-in-one microscope series: the FSX100 fluorescence system and the FluoView FV10i cLSM system.

Ease-of-use without compromise

All the necessary components for high-end imaging are contained within the main body of the units. For example, the cLSM instrument includes a scanning unit, built-in vibration insulators, with an incubator and automatic water supply added for the water-immersion model. Furthermore, the accompanying software is user-orientated to facilitate easy operation. Once the sample has been placed on the stage and the lid closed, the microscope automates the rest of the process, pausing for user input at vital stages. Time-consuming tasks, such as focusing and setting the correct exposure levels are automated, enabling the production of an

optimised image without the need for any additional user input. In the fluorescence system, the user can select the desired illumination wavelength on-screen and the correct filter is automatically used. The cLSM model automatically optimises detection bandwidths for a broad range of fluorophores. Both systems will automatically set themselves to image at optimal conditions, however these settings can be further adjusted, as required. The microscopes then automatically compile an overview of the sample that, not only helps the user to identify regions of interest quickly, but also provides a constant top-level navigation aid during imaging.

Combining a fully-automated system with user-friendly software and advanced optics ensures that, while the usability of the systems is simplified, the end-result remains of a high standard. Using superior, high resolution apochromatic objectives ensures that any aberrations are fully-compensated for. This, in combination with versatile imaging systems, enables fluorescence, phase contrast and brightfield images to be obtained with ease. Furthermore, these compact systems can capture snapshot images, z-stacks, time-lapses, multi-position experiments as well perform multiple image alignment. Fluorescent channels can also be directly overlaid in live mode for highly-resolved, real-time observation. In addition, they are able to combine the above processes to produce very advanced time-lapsed, multi-channel, Z-stack images at multi-positions.

Flexibility

The ability to accept standard slides as well as a range of culture dishes further increases the versatility and usability of such systems. As such, dry, as well as water- and oil-immersion slides are all fully-compatible. Both water and oil interfaces can be used to increase the resolution by enabling the use of objectives with numerical apertures (NAs) above 1.0. The cLSM

water-immersion model, even completely automates water dispensing, removing the need for any further user input.

In-lab imaging

Self-contained, independent and mobile microscope systems are a highly advantageous addition to any laboratory due to their space-saving design, making them easily portable between different laboratories. Such systems can therefore be kept on a trolley to be easily transported to various locations, if required. The sealed, light-proof design of a self-contained microscopy system provides an ideal imaging compartment for maximising the sensitivity of such systems. This eliminates the need to transport samples to a fixed microscopy system in a darkened room for analysis and enables detailed examination of samples to occur in daylight at the point of discovery. Furthermore, the additional incorporation of an incubator with the water immersion cLSM makes it possible to perform time-lapse imaging of live cells without lengthy equipment set-ups. Constant sample temperatures can be maintained, along with stable humidity and specified CO_2 levels.

Conclusion

Both fluorescence and confocal imaging techniques are widely acknowledged as being pivotal to a broad range of applications. Performing such complex microscopy has previously been tasked only to experienced and knowledgeable microscopists. As a result, such personnel may be overloaded with requests from researchers that do not have the in-depth knowledge of fluorescence or confocal microscopy to image samples themselves. These complex instruments provide extremely high-quality images, but require extensive training to operate efficiently. As a result, technology has been developed to simplify the interaction with such complex microscopy to make it accessible to a wider audience. Self-

contained, fully-automated systems have been combined with user-friendly software to guide the researcher through the complete process, without compromising on image quality.

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