

# Problems with time lapse microscopy

## Temperature induced focus/sample drift

Upholding the environmental conditions around the cells conducive to preservation of cellular structure and function is critical to both the biology of live cell imaging and the preservation of object focus. The maintenance of temperature is controlled by several sub-systems to create the overall effect, namely heating of the space around the sample, sample heating and the objective lens, whilst maintaining a steady state around the microscope as a whole.

The heating of the objective lens is critical in live cell systems and can be overlooked when investigating biological function with live cell imaging. When the objective lens is not heated a thermal gradient results which draws heat down from the sample at precisely the point at which you wish to image. Thus even though the temperature around the stage and of the slide maybe controlled, the bit you are looking at will be a few degrees cooler. Furthermore the temperature gradient also heats the objective lens and the microscope body. To lessen the effect of the temperature gradient an isolation collar is also required which will resist the effects of the heat gradient from the objective lens to the microscope body. The heated sample holder is a vital part of the temperature maintenance procedure but can cause focusing inconsistencies when imaging. Part of the temperature control mechanism of the sample holders requires fine electronic control of the heat output. When a sample heater is initially powered there is often an overshoot so that a sample can be subject to temperature a degree above what is required, sample heating by water is more gentle and less prone to flux such as the system available by Okolab. Another associated problem with heated sample holders is that the very nature of reacting to the temperature probe and altering the temperature of the holder can alter the focus. Some hot plate methods of sample heating can have up to five temperature sensing routines a second which all can affect the focus. It is now possible with some software to temporarily stop the temperature adjustment routine whilst the sample is being visualised.

The environment outside the microscope/environmental chamber must also be controlled. For the capture of images with a x100 objective lens under widefield illumination to capture all of the data in the axial dimension 0.2 - 0.3 micron steps are often used to record the data. If temperature eddies occur around the microscope due to draughts caused by over zealous air conditioning, focus can be lost by the equipment expanding or contracting thus moving the sample a couple of microns. When imaging microbes the microscope should be placed on a full isolation/anti-vibration table and the equipment placed in its own room ideally with low impact temperature control, where the fans from such a device are directed away from the microscope. If the baseport is being accessed for camera and emission filter wheels draughts can flow through the hole in the anti-vibration table, it is often advisable to pad the hole with bubble plastic. If possible draughts can be minimised in small microscope rooms by ensuring that entry into the room is via a sliding door with a curtain pulled across the door way to reduce the effect of cooling air currents from the outside corridor.

## Temperature induced deterioration of objectives

It is thought beneficial if all lenses are kept in a suitable incubator (only £150) which is maintained permanently at the experimental temperature. It is possible that repeated heating and cooling of lenses could damage the glues/mountings used to hold the optical components of objective lens in place hence by keeping a range of objective lenses at the required temperature the life time of the objective lenses may be extended. Within an environmental chamber that contains the microscope there is a range of temperatures and the controlling temperature probe should be as close to the sample as possible, thus leaving objective lenses in the environmental chamber is not a substitute for an objective incubator.

## Temperature independent focus/sample drift

Once temperature is controlled it is still possible that focus can be lost due to sample movement. Focus can be maintained by additional routines such as software autofocus or by a focusing IR lasers. Autofocus via software is suitable when time frames between sets of images allows the movement of the axial drive however this may cause a stress response due to the increase in illuminating light required (Lindqvist *et al.*, 2004) or photo bleaching of the fluorescent protein. It is possible to autofocus using a less critical label e.g. use a fluorescent nuclear label for focusing and then switch to another filter set and image the fluorescent protein, but this will still increase the amount of harmful UV damage to the sample. A hardware method for autofocus via an infra-red laser, such systems as the Nikon TE2000-PFS perfect focus system and the Applied Scientific Instrumentation CRIF, are emerging as solutions to compensate for focus drift caused by mechanical or sample drift without using UV derived light for autofocus. The technique employed monitors the distance between the coverslip and the objective lens and provides feedback to the focusing mechanism.

## Detection

The detection of light emanating from the biological data over a time lapse investigation can be a challenge where low light detection is required. To minimise bleaching and stress, it is vital that the minimum amount of light be used to excite a fluorescent protein/fluorophore thus every component in the light path must be considered, light source (even filed illumination with high life time such as the 1000 hour Xenon light sources) objective lens (high numerical aperture, high light throughput), neutral density filters, fluorescent filter sets and camera sensitivity. When setting up a multi-labelled investigation increasing neutral density filters should be utilised for each set of filters on set-up of the experiment until salt and pepper (shot) noise is evident, then the neutral density filter should be decreased until the noise is reduced to an acceptable level.

## Reference

Lindqvist, A. & others (2004). Characterisation of Cdc25B localisation and nuclear export during the cell cycle and in response to stress. *J Cell Sci* 117, 4979-4990.