PLAYING WITH TIME

– digital imaging comes of age

Seeing dynamic intra- and extra-cellular events as they happen is central to revealing the mechanisms of disease, identifying key targets for drug intervention and assessing the impact of drug treatment on living cells. Interpreting these events through the microscope can sometimes be challenging because they take place on widely different timescales.

By Dr Pam Pickering

A signalling event may take place in a fraction of a second, for example, a timescale so fast that the human eye may not be able to register important information. Others may take place over several days where it becomes difficult to detect any dynamic change at all. These challenges can be overcome by exploiting developments in digital time-lapse imaging, which allow researchers to 'play with time' so that images provide the most meaningful information possible.

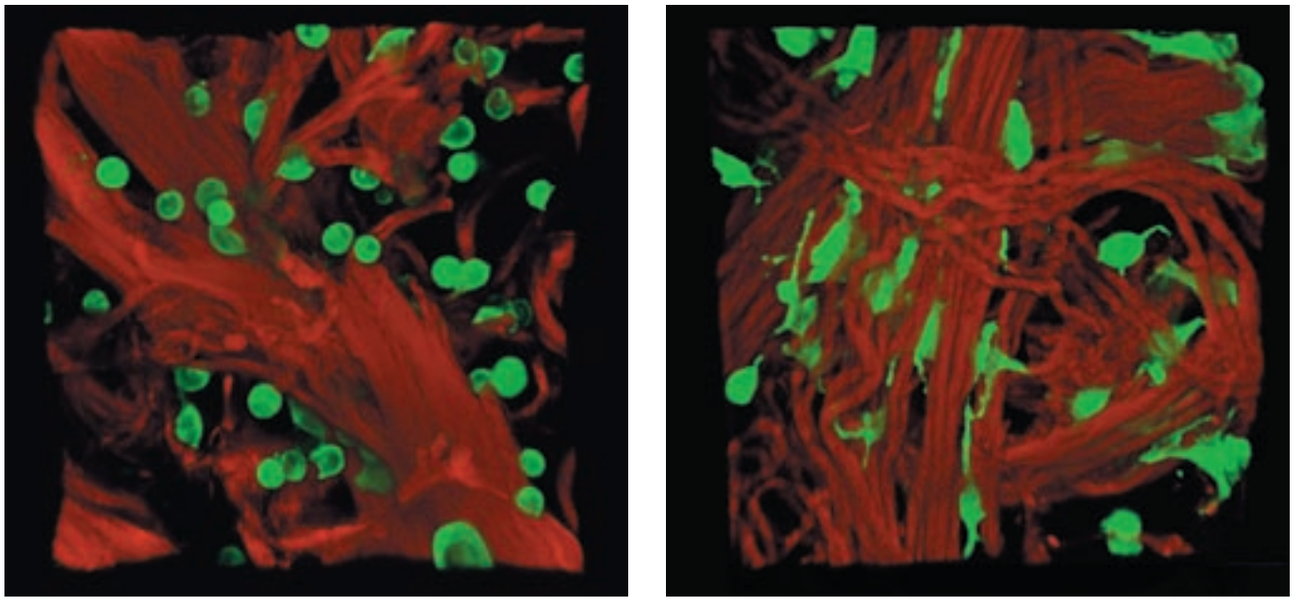
Time-lapse imaging

Manipulating time through time-lapse imaging involves capturing images at pre-determined time intervals of milliseconds, minutes, hours or even days and then replaying them at rates that reveal the most about each event.

For relatively slow processes taking several hours or days, such as embryonic development, what has been recorded may need to be accelerated to allow researchers to identify change more easily. Images can be captured at 30-minute intervals, for example, and then replayed at video rates (25 frames per second).

Making sense of very fast events such as calcium signalling can be more demanding as extremely high temporal resolution is required in image capture. Imaging rates of 100 frames per second or more are now available allowing rapid changes to be captured continuously (depending on data acquisition capabilities) or, for example, at millisecond time intervals. Images can then be replayed at slower rates for easier interpretation. High-speed image capture demands high sensitivity to capture enough signal to create an image within a very short timeframe. Cooled CCD cameras are generally used as they limit background noise so that weak signals such as low-level fluorescence can be captured more easily.

Time-lapse imaging is an extremely powerful technique in drug discovery as it offers researchers true controls – the possibility of measuring key cellular parameters before drug treatment, for example, and the response of those same cells to the



Still shots taken from 3-D time-lapse movies showing GFP-expressing human melanoma cells (green) migrating through three dimensional collagen scaffolds (red), before (left) and after (right) the addition of Y27632, a compound which inhibits downstream signalling from Rho through ROCK. A distinct change in cell morphology is evident. The scale bar at the foot of Image 1 is 100µm. Images were taken using a Nikon C1 confocal imaging system with Nikon TE2000 inverted microscope with incubator jacket and using a Nikon PlanFluor ELWD x40 objective

drug over time. Time-lapse imaging is applicable to any microscopy technique suitable for imaging cell dynamics such as TIRF, FRET, FLIP and FRAP but additionally provides researchers with greater flexibility in image capture and interpretation. Confocal time-lapse imaging, furthermore, allows events to be recreated in 3-D so that dynamic events can be viewed from any angle.

Addressing challenges in time-lapse

Long-term time-lapse imaging can present challenges especially in maintaining cell viability and in ensuring that images are not compromised by vibration, mechanical and thermal drift in the microscope set-up. Similarly, it is important to be aware that cells of interest may 'round-up' or migrate to move out of the plane of focus during the imaging period.

Stability problems can largely be overcome with the use of structurally rigid equipment that is resistant to vibration, temperature and drift. It is also useful to have microscope control in the X, Y and Z directions so that the dimensions of the imaging frame can be managed precisely to accommodate cell movement.

Cell viability is the main obstacle to long-term imaging. While environmental chambers offer substantial protection in maintaining constant temperature, pH and humidity, cells can still be damaged by strong light sources used during the imaging process. This is particularly important in laser scanning confocal microscopy (LSCM) where photodamage from bright laser sources used to generate fluorescence emissions can accumulate over multiple scans. Many fluorophores generate damaging free radicals on photobleaching although many of the recently developed probes, such as the GFP proteins, offer enhanced photostability and greater brightness. Antioxidants, such as ascorbic acid, can be added to the medium to help reduce oxidative damage.

Phototoxicity can be minimised by separating images widely in time, by limiting light exposure with high NA objectives, and by the use of high sensitivity photon detectors, which can capture weak signals and reduce the need for strong excitation sources. Additionally, the pinhole aperture in LSCM systems can be opened wider to accelerate image acquisition rates. While this may compromise image resolution, post-imaging deconvolution can help restore image quality.

Multiple labels

Time-lapse imaging can be a lengthy process and it makes sense to capture as much information as possible from one set-up. Multiple fluorescent labels allow several cellular components to be measured simultaneously as long as their emission wavelengths can be distinguished. This allows, for example, molecular interactions, compartmentalisation and cell structure studies to be carried out in one experiment.

When using multiple fluorescent labels it is possible to use a device that allows two or more colours to be detected simultaneously on the same CCD. Alternatively, it is possible to use a high-

speed filter wheel or monochromator to change the excitation wavelength with the camera capturing the data sequentially. The ultimate solution for resolving multiple emission spectra is to use a spectral detector, which can accurately detect closely spaced emission wavelengths

Into the fourth dimension

Cancer can spread through the body by a process known as metastasis where cells detach from a tumour and migrate to other organs and tissues. If researchers could identify the biochemical mechanisms determining when, where and how tumour cells move, they may be able to identify key targets for pharmaceutical intervention to inhibit metastasis and control disease.

Dr Hugh Paterson and the Oncogene Team at the Institute of Cancer Research, Chester Beatty Laboratories, London, are currently establishing a robust 3-D *in vitro* model for the study of cell migration using collagen matrices or scaffolds. "Tumour cells tend to move in two distinct ways – either with an elongated, polarised motion or with a rounded, blebbing movement," said Dr Paterson. "Cells in the elongated mode secrete proteases which break down collagen allowing cells to 'snip' their way through the collagen mesh. Blebbing cells move by squeezing their way through existing gaps in the matrix.

"At one time it was hoped that inhibitors to proteases released by elongated cells might prevent metastasis. Potent protease inhibitors were developed to this end but have proved to be disappointing in clinical trials. The most likely reason is that cells in the elongated mode can flip to the blebbing movement, which is protease-independent," Dr Paterson continued. "The signal to flip between the two types of movement seems to depend on both intracellular and environmental signals."

Elongated or blebbing – which way to go?

Work at the Chester Beatty Laboratory has shown that there are biochemical pathways controlling actin/myosin contractility specific to the two types of movement. The Rho protein and its effector ROCK is a requirement for blebbing movement while another small GTPase called CDC42 and its effector MRCK seems to be more strongly associated with the elongated type of movement. "Whichever type of movement predominates seems to depend on the balance between Rho and CDC42 signalling," said Dr Paterson. "Environmental factors may also be involved. We know, for example, that stromal fibroblasts close to tumour cells can be influenced by the tumour to produce growth factors. Some of these growth factors, in turn, have been implicated in influencing movement in the tumour cells."

Dr Paterson's team have produced two colour 3-D models where fibroblasts and tumour cells are fluorescently labelled so that their interactions can be monitored over time within the collagen matrix using time-lapse imaging. Using Nikon's C1 confocal microscope, 3D images can be created using the in-built software making it possible to see events within the matrix from any angle.

"What separates our research from that of most other groups is that we are creating images in four dimensions – 3-D imaging over time – to study cell movement in a reproducible and controlled environment," Dr Paterson explained. "Once a biochemical pathway has been implicated in a particular movement pattern, it is possible to exploit inhibitors to block that pathway and see what effect this has on cell behaviour. It is also possible to block multiple pathways. Another approach is to over express certain genes in cells to stimulate a particular pathway and then monitor effects on the system."

The challenges of 4-D imaging

The major challenge in this kind of imaging is to keep cells alive. "We are imaging from periods of just a few hours to 10 days or more. Cells are enclosed in an environmental chamber but the cells also need to be fluorescently labelled so that they can be easily identified in the collagen matrix," said Dr Paterson. "We had originally planned to use vital stains – but these produce cell-damaging free radicals when exposed to laser light. We now use green fluorescent proteins (GFPs), which are less cytotoxic. With plasma membrane targeted GFPs it is possible to generate fluorescence close to the cell membrane. This can extend cell viability by several days as fluorescence is largely kept away from sensitive vital structures such as the mitochondria.

"Another important factor in long term imaging is the stability of the microscope set up. We have found that our Nikon system is totally stable over periods of several days. Focus isn't a problem as we take multiple thin sections in the Z dimension – so essentially we are imaging in a 3D cube several hundreds of microns deep – all of which is in-focus to accommodate the changing morphology of the cells," said Dr Paterson.

State-of-the-art

"Using the confocal microscope we have established a robust method of tracking cells in 4D. Using specialised software, we are able to measure the speed at which each individual cell travels, how

far it travels and in which direction. The pattern of movement can be described in numerical terms and these can be used to compare results when the system is treated with inhibitors or stimulants. We are still developing the technology and optimising reproducibility but it is a very promising system and we hope to get some important and interesting results in the next year," Dr Paterson concluded.

Time lapse to test dermatological formulations

Stiefel International R&D develops, manufactures and markets its own range of medically accepted products for the care and treatment of skin conditions such as acne, seborrhoea, atopic dermatitis, dry skin, psoriasis, warts and eczema. Its facility in Maidenhead, UK, specialises in topical formulation development, including ointments, creams, lotions and solutions.

"There is no one formulation that will suit every active ingredient," Stiefel's Senior Formulation Chemist explained. "Each time we have a new compound or have a new use for an existing compound, the formulation has to be custom-designed as it can play an important role in controlling the release of the active ingredient to the skin. Here, at Maidenhead, we are developing, testing and scaling-up formulation production to GMP standards to produce quantities suitable for clinical trials."

Microscopy is an important tool in this testing process. "While we have many technologies that enable us to quantify changes in a formulation, there is no substitute for actually being able to see

directly what is happening to a preparation. Microscopy is one of the most important tools for understanding the behaviour of a formulation under varying conditions."

One of the criteria tested is formulation stability under long-term storage or under different environmental conditions. Formulations are examined for any changes, such as crystal growth, that might indicate that the formulation is undergoing a process of degradation.

Crystals can be easily identified using polarised light microscopy. As well as providing information on absorption, colour, and crystal boundaries, polarised light microscopy can reveal detailed information about the structure and properties of crystals. Crystals can also be identified using melting points and this is achieved by heating samples on a hot microscope stage.

Time-lapse imaging is a useful tool in capturing changes in crystal structure as they enter and pass through a phase change. While it may take some-time to reach the melting temperature, when it occurs a phase change can occur very quickly. It can be useful to capture the images and replay the process at a slower speed so that melting behaviour can be studied in greater detail. Melting temperatures are highly diagnostic and time-lapse imaging used in this way can help to differentiate between very closely related compounds.

The laboratory's digital imaging software is calibrated to the graticule on the microscope stage so that the captured image corresponds exactly to what is seen through the microscope. This allows the formulation team to take direct measurements, for example, of crystals present in a sample. Annotation tools allow measurements and other comments to be recorded on to the images so creating 'report-ready' records of findings. The system includes a variety of image manipulation tools that enables the creation of composite images and overlays for easy image comparison.

Creating a record of findings is becoming increasingly important to the quality control process providing evidence of due diligence and lot-to-lot consistency in manufacture. "Microscopy and digital image capture are significant tools in our environment," Stiefel's Senior Formulation Chemist concluded, "and they are set to increase in value as digital records become routine." DDW

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