Multidimensional Contextual Analysis of Living Systems

a report by
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Scaling Biology

A wealth of new technologies for improving the drug discovery process have been introduced in recent years. Common among these is the scaling and automation of established laboratory techniques in an effort to enhance efficiency in the discovery process. These include the scaling of DNA sequencing to facilitate various genome projects, the implementation of transcriptional analysis using DNA microarrays and the use of new mass spectrometry techniques for proteomic analysis. A more recent addition to this scaling trend is the technology of fluorescence microscopy.

The power of the microscope lies in its ability to reveal both spatial and temporal events within living systems at very high resolution. Modern microscopes have the ability to view subcellular structures and to detect very subtle changes that occur within individual cells. Used in conjunction with a number of fluorescent reporter dyes and proteins, imaging has become more widely utilised in recent years.

Although the optical components of the microscope remain little changed over the years, the ability to make use of digital images for quantitative measurements has been improved dramatically by recent advances in camera technology and analytical software. Of particular note is the advancement of confocal technologies that allow acquisition of images at very high resolution, coupled with realtime image capture capabilities. With the new generation of charge-coupled device (CCD) cameras available, very low-light specimens or those possessing small numbers of molecules can be visualised within cells. Together with the tremendous computational power and software now available on the desktop, large volumes of digital image data can be manipulated, analysed and stored. Collectively, these technologies are providing the ability to look into cells in realtime to monitor cellular events within the context of the living organism.

Contextual Biology

The contextual view of biology – how events occurring within cells affect one another – represents a powerful new approach to our study of biology. Rather than dissecting cellular pathways to study single components *in vitro*, it is becoming possible to watch biochemical events unfold at the cellular and even organismal level.

Many assays traditionally used in the drug discovery and development process rely on single time-point measurements taken on populations of cells. While these approaches have afforded significant advances in terms of throughput in cell-based screening, they can miss important details lost through averaging the signal of interest into a background of non-responding cells (see *Figure 1*). In addition, since only a single time-point is being seen, events that occur before or after the signal is measured are missed – events that may be of biological significance.

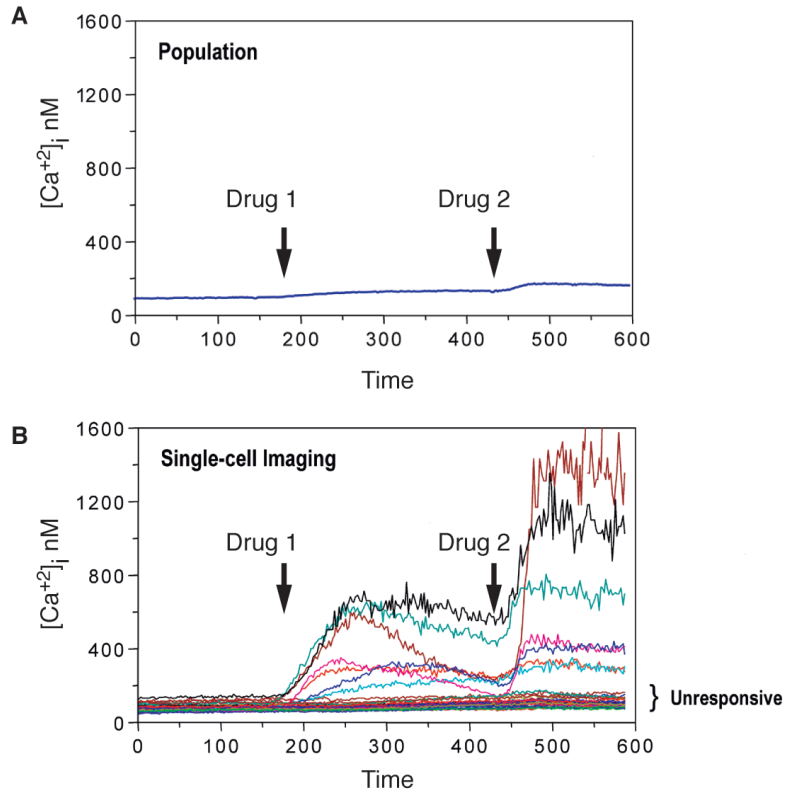
The ability to monitor individual living cells within a population at very high resolution can help us gain a better understanding of the biological relevance of a particular event by revealing heterogeneities within cell populations. As an example, if a 'hit' through an endpoint population screening procedure reveals a 20% response, it is unclear whether the result reflects a 20% response within all the cells, or that only 20% of the cells are responding at a 100% signal response. The technology that can provide this high-content information combines the spatial and temporal resolution of the microscope, the computational power of the modern PC and software algorithms to extract information from digital images.

The Confocal Microscope

In its simplest form, epifluorescence microscopy involves illuminating a sample with light, through an objective, at a wavelength determined by an excitation filter.[1,2] If a fluorophore within the sample is excited by a particular wavelength, it will emit

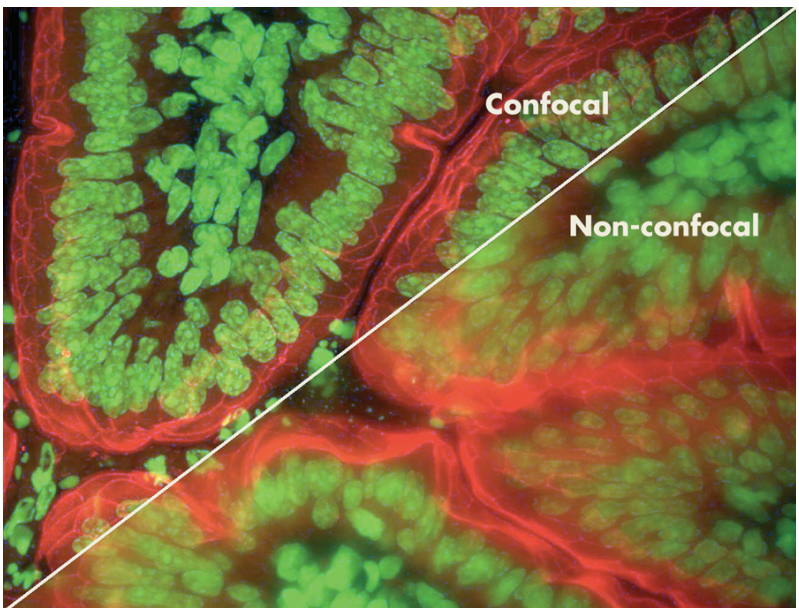
1. *M W Davidson and M Abramowitz (1998),* Optical Microscopy*, Olympus America, Inc., Melville, NY.*
2. *B Herman (1998),* Fluorescence Microscopy*, BIOS Scientific Publishers Ltd., 2nd edition, Oxford, UK.*

Figure 1: Single Cell Imaging



Kinetic traces of calcium flux in cell line expressing GFP fused GPCR receptor (A). Population-average kinetic trace fails to reveal significant Fura2 ratio change upon addition of known GPCR agonist (Drug 1) or control compound (Drug 2) (B). Single cell imaging reveals a subpopulation that is responding to agonists in a background of non-responders, as well as a significant heterogeneity of response within responding cells. Cell line courtesy of Aptus Genomics.

Figure 2: Confocal and Non-confocal Image of a FluoCell™, 16µm Cryostat Section of Mouse Intestine Stained with Alexa Fluor 568 Phalloidin and SYTOX Green Nucleic Acid Stain



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light at a longer wavelength, which, in turn, is filtered by an emission filter to remove any light outside of the filter's bandwidth. The incident light can penetrate to different levels within the sample and cause fluorescence excitation throughout the sample. All the emitted light is visible to the detector, typically a CCD camera or photomultiplier tube (PMT). With thick specimens, the non-confocal image can appear fuzzy due to the detection of fluorescence emissions above and below the plane of focus (see *Figure 2*).

The objective of confocal microscopy is the elimination of out-of-focus light, thus producing a high z-resolution image.[3] Confocal fluorescence microscopes achieve this via two principal mechanisms. First, incident light is focused through the objective to a particular point within the specimen after passing the light through a very small aperture such as a pinhole or slit. The focusing helps to limit the excitation of fluorophores above and below the plane of focus. Second, any emission that is above or below the plane of focus is blocked from reaching the detector by passing it again through a pinhole.

Atto Bioscience invented a mercury arc lamp-based spinning disk confocal (CARV) that allows high-resolution imaging without the spectral and mechanical limitations of lasers.[4] The disk contains multiple sets of spirally arranged pinholes placed in the image plane of the objective lens. By moving the specimen perpendicular to the focal plane in a step-wise manner, and capturing images at each step, it is possible to develop a stack of images that can be recombined computationally to form a three-dimensional (3-D) image. Whereas 3-D imaging is a key feature of confocal microscopes, the ability to image a single focal plane also has the benefit of being able to improve signal to noise, reduce photobleaching and isolate redistribution events to a single plane, allowing better subcellular localisation and thereby improving evaluation of biomolecular movements.[5]

The Pathway HT

The Pathway HT combines a high-resolution spinning disk confocal unit, as described previously, with software that enables individual cells to be monitored, measured and analysed. The unit (see *Figure 3A*) includes a unique optical path that allows imaging to take place during a compound addition step, thereby making kinetic analysis possible. The

3. *C J R Sheppard and D M Shotton (1997),* Confocal Laser Scanning Microscopy, *BIOS Scientific Publishers Ltd., Oxford, UK.*
4. *P G Vanek, B Somasundaram, J Brooker and G Brooker (2002),* Real Time, Full-spectrum Confocal Microscopy, *American Biotechnology Laboratory, pp. 10–12.*
5. *J B Pawley (Ed.) (1995),* Handbook of Biological Confocal Microscopy (The Language of Science), *Plenum Pub Corp, 2nd edition.*

robotics-accessible stage has controllable temperature and CO_2, allowing longer running experiments to be performed. The system employs two mercury arc lamps, each with an eight-position filter wheel and one shared five-position dichroic mirror on the excitation side. For emission wavelengths, there is a second five-position dichroic and an eight-position emission filter. Detection is accomplished using a high-quantum-efficiency CCD camera, or alternatively the samples can be viewed using a binocular port. The confocal unit is mounted on a slider such that it can be placed into the light path for high-resolution imaging, or can be moved out for applications requiring wide-field imaging (see *Figure 3B*).

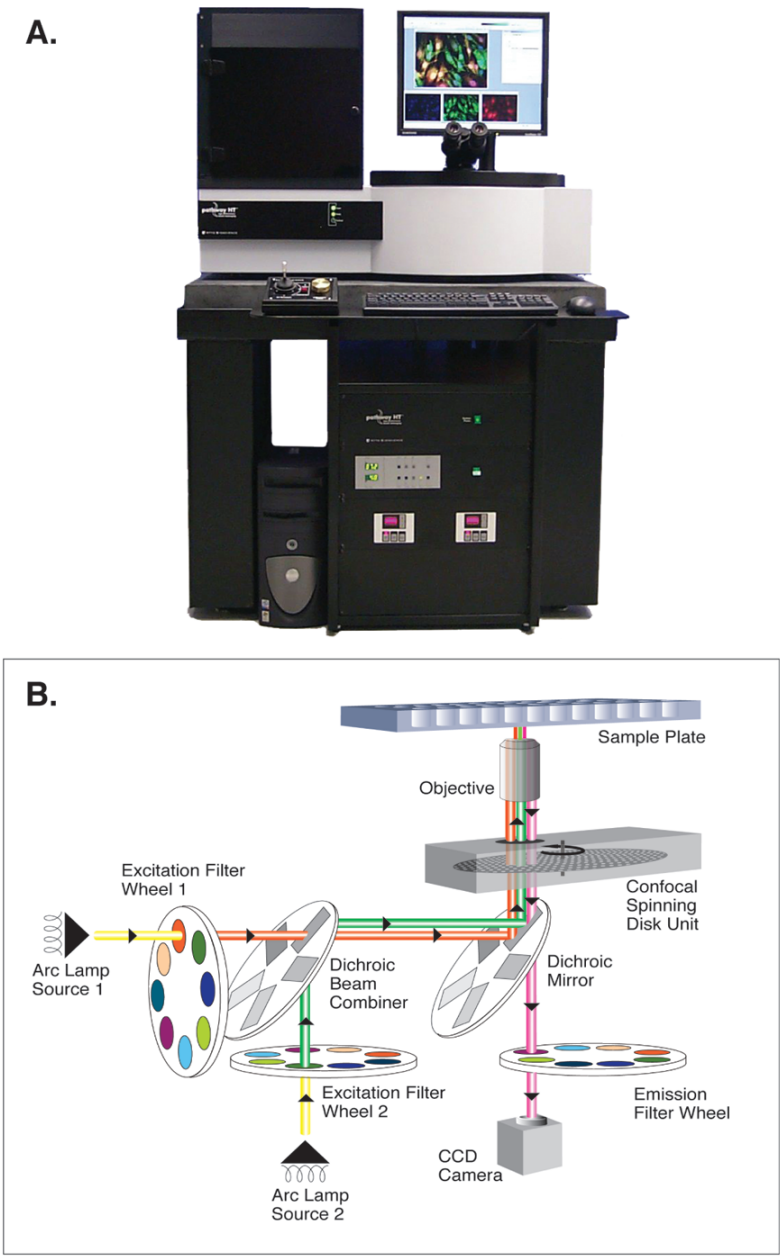
Software

At the heart of the system, the Windows-based software not only handles the mechanical operations of the system, but also provides flexible tools for data acquisition and analysis. A key specification for our software development was to build in flexibility to drive a broad range of endpoint and kinetic assays. The image acquisition software provides full control of the camera exposure, filters, liquid addition steps and timing steps for the plates being imaged. The software also allows the user to specify the resolution of the images, how many images to take per well and a variety of other parameters for imaging. Captured images can be stored in an easily navigable hierarchical folder format for future analysis. The software can also perform many types of analysis in realtime without the need to store images.

On the analysis side, the software has to be capable of interpreting the information contained within the images. Changes can be classified by measuring two fundamental parameters: fluorescence intensity (pixel intensity) and fluorescence location (pixel position within field of view). By measuring pixel intensity changes and/or pixel location changes, it is possible to categorise biological responses and quantitate those changes. The software needs to be able to identify and quantitate the fluorescence changes taking place and, in kinetic mode, be able to track these changes over time.

The first step in measuring and quantitating fluorescence is to identify and segment individual cells such that fluorescence changes can be assigned to a particular cell or region of interest (ROI) within a cell. A number of computational algorithms can be applied to provide automated segmentation without user intervention, and these have been shown to be very effective for a broad variety of cell types and morphologies. The second step is to define how fluorescence intensity within each ROI is to be handled.

Figure 3: A) Pathway HT Confocal High Content Imaging Platform. B) Schematic Representation of the Pathway HT Light Path With a Dual-excitation, Single-emission, Ratio Dye



The Pathway HT system uses dual high-intensity mercury arc lamps (Arc Lamp Source 1 and 2). Light from these sources is passed sequentially through one of two eight-position excitation filters (Excitation Filter Wheel 1 and 2), for a total of 16 possible excitation wavelengths. The excitation light is reflected/passed through two selectable dichroic filter wheels and projected through a broad-spectrum spinning disk confocal device to the objective and onto the specimen. Fluorescence emissions from the sample are collected by a high numerical aperture objective, passed once again through the same spinning disk confocal unit, through the dichroic mirror and then through one of eight selectable emission filters to a high-quantum-efficiency CCD camera. Note: the binocular viewing port is not shown.

Once ROIs are identified, a variety of quantitative measurements can be made. The simplest involves measuring fluorescence increases or decreases within each ROI and graphing, in realtime, the intensity for each ROI as a function of time. If the region of interest corresponds specifically to a cell, one can readily identify which cells are responding, which are not, and response differences within the cell population. The software can be configured to classify groups of cells into any number of categories, for example cells that respond over a particular

fluorescence intensity threshold and those below. Each cell is then colour-coded and readily seen as fitting one of the user-defined categories. Raw data can also be exported for statistical analysis or presentation in third-party software programs such as Excel or Spotfire.

Analysis of fluorescence redistribution is more complicated, and different software-based analysis approaches can be applied. For certain redistributions, for example cytoplasmic to nuclear translocations, the nucleus can be defined as the region of interest, and fluorescence intensity increases can be monitored within each ROI as a function of time.

Redistributions, wherein fluorescence moves from a less well-defined region of the cell to another, are more difficult to quantitate using the ROI approaches outlined previously. One approach to this type of redistribution is to apply algorithms originally developed by the defence industry for satellite imaging. In this approach, multiple features of the cell are measured generically, and features that are being affected by the biology are identified. These features are then used to define a cellular-event-dependent feature set, and images are scored for their relative similarity to a particular feature set. In this way, images can be placed into categories of 'most-like' images that are then correlated to a particular biological event.

Conclusion

The power of imaging to address biological experimentation lies in its combination of spatial and temporal resolution. This combination of features enables very high-resolution images to be captured over time, each with a high content of biological information. A critical capability of the emerging high-content screening technologies is the ability to handle both fluorescence intensity data within discrete cell areas, as well as the ability to apply pattern recognition algorithms to the evaluation and quantitation of biological events. Ultimately, high-content contextual cellular analysis promises to reveal a tremendous amount of functional biological information. ■