Microscopy & Microtechniques Focus

NOVEL LIVE-CELL DUAL EXCITATION/DUAL EMISSION IMAGING SYSTEM FOR THE SIMULTANEOUS VISUALISATION OF CYTOSOLIC Ca^{2+} SIGNAL TRANSDUCTION PROCESSES AND MITOCHONDRIAL DYNAMICS

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One of life science's primary challenges is to understand and define the role of a vast number of proteins in cellular processes associated with health and disease of living organisms. Revolutionising this research is the development of a wide range of fluorescent probes that can be used to non-invasively interrogate living cells, new microscope technologies and powerful computer software and hardware for digital image processing and analysis. These technical innovations in photonics, microscopy and IT have played a key role in the emergence of live-cell imaging as a viable laboratory tool, as well as a routine methodology that is practised in a wide range of biomedical research disciplines.

As a result there have been an increasing number of studies employing live-cell microscopy and imaging techniques at cellular and sub-cellular levels. Microscopy has progressed from the purely structural characterisation of fixed cells towards the investigation of processes in living cells. Static morphological observation can now be complemented by the characterisation of the three-dimensional (3-D) architecture of cellular structures. In turn, these innovations provide the ability to monitor dynamic cellular activities in living tissues with sub-micrometer resolution in real-time, and offer critical insights into the fundamental nature of cellular and tissue function, which are not possible using fixed cell techniques.

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A REVOLUTION IN RESOLUTION

Cellular processes are dynamic, and investigating how cells function allows for a better understanding of physiological and pathophysiological interactions. Such experiments have to be performed on living cells under relevant physiological conditions and have been significantly enhanced through recent developments in microscope technologies over the last 20 years. Since the invention of confocal microscopy and more recent advances such as confocal laser scanning spinning disk microscopy, multi-photon and deconvolution microscopy, today's live-cell imaging systems have dramatically improved the ability to detect biological specimens and offer the high spatial resolution required to image living cells without destroying them. Developments in specific fluorescent probes, such as dyes and fluorescent proteins, have further facilitated the study of complex cellular processes using fluorescent staining or labelling of various proteins, ions and lipids in living cells.

ILLUMINATION LIMITATIONS

The types and duration of cellular processes that can be imaged have been constrained by some microscopy systems. Monochromator-based illumination is limited with a single bandwidth excitation that cannot be compensated by fast switching the monochromators. Precision optical sections and digital image processing afforded by confocal and multi-photon microscopy can overcome this limitation, by allowing multi-wavelength illumination and they are widely used for a range of applications. However, live-cell imaging can be a complex task for microscopists unfamiliar with the techniques and tools. Moreover, by using living cells, the acquisition speeds are mostly limited by the low fluorescence of the specimen itself, thus resulting in slow scan rates and acquisition times. Consequently, due to the need for exposure to high light intensities from the focused laser beam, phototoxicity and photobleaching is a real issue when it comes to long-term experiments in living cells, such as cell proliferation and differentiation.

RESOLUTION SOLUTION

Imaging live cells in real time is fundamental to many applications of cell biology, and reliable application-specific imaging systems are essential to efficient, productive laboratory operation. Cell biologists are now demanding imaging systems that are increasingly multi-functional, have greater temporal and spatial sensitivity and can support the specific requirements of different applications. In response to these demands, sophisticated high-speed multi-fluorescence 3-D time-lapse imaging systems for live-cell microscopy have been developed to overcome the limitations of conventional imaging devices.

These advanced fluorescent modular imaging stations offer specialised all-in-one illumination systems with highly sensitive digital cameras and hyper-precision hardware control boards, and can be fully integrated with inverted and upright microscopes. These systems are optimised to image very fast processes in living cells and have been designed specifically to meet all experimental requirements. This allows researchers to design complex multi-parameter protocols for the simultaneous detection and interpretation of time-resolved imaging data.

These systems also offer many advantages over previous confocal and multi-photon microscopy instruments, such as ultra-fast wavelength switching and attenuation. In addition, dedicated real-time controllers coordinate all hardware and peripheral devices whilst functioning independently of the imaging computer. This enables precise synchronisation of sample illumination and real-time image acquisition, minimising phototoxicity and photobleaching.

EXPLORING THE POTENTIAL

To explore the potential of filter-based fluorescence imaging technology for live-cell microscopy, this article describes how specific benefits are offered with this technique compared with devices that employ monochromators as light sources, and how this technology permits the visualisation of signal transduction processes in cell biology research.

The use of fluorescent imaging has made it possible to gain insights into the role of mitochondria in intracellular calcium signalling. In addition, the development and application of sensitive fluorescent dyes for measuring the calcium concentration in the cytoplasm of living cells has led to a revolution in the understanding of how the specificity and versatility of the calcium cation (Ca^{2+}) as a communicator and regulator in signal transduction is accomplished, as well as the mechanisms controlling cellular calcium homeostasis.

MITOCHONDRIA AND CALCIUM HOMEOSTASIS

Mitochondria are essential membrane-enclosed organelles found in eukaryotic cells, and play a key role in processes such as oxidative phosphorylation, aerobic metabolism of glucose and fat, calcium signalling and apoptosis. Mitochondria are generally described as "cellular power plants" because they generate most of the cell's supply of adenosine 5'-triphosphate (ATP), used as a source of chemical energy [1].

Mitochondria also play an important role in the regulation of calcium homeostasis as they house targets for calcium signalling, and constantly accumulate and release significant amounts of calcium to and from the cytosol [2]. Calcium enters the cytoplasm from either outside the cell via calcium channels through the cell membrane or from internal calcium storages within the cell.

Cells are known to actively maintain extremely low cytosolic calcium concentrations $[Ca^{2+}]_C$ below 10^{-7} mol/L, and when stimulated with specific agonists, such as hormones, neurotransmitters and growth factors, mitochondria immediately sequester calcium [3]. These high concentrations of free calcium in the cell regulate an array of reactions by acting as intracellular mediators in a wide variety of crucial cellular responses.

As calcium is one of the most widespread secondary messengers used in signal transduction, its role in signalling is very comprehensive. The binding of agonists to specific receptors in the signal transduction pathway leads to the opening of calcium channels, which results in a massive flux of calcium ions from intracellular stores into the cytoplasm. This initial rise in concentration of calcium in the cytoplasm also stimulates the release of additional calcium ions [4].

This results in a rapid and significant increase of calcium that can bind to a number of effector proteins, resulting in a variety of specific cellular responses, including motility, secretion and enzyme activation. These in turn, coordinate many versatile cellular physiological processes, ranging from neurotransmitter release in nerve cells, the release of hormones in endocrine cells to smooth muscle contraction [3]. This clearly demonstrates that mitochondria not only act in a simple storage capacity, but also have the ability to specifically respond to this universal messenger and play a central role in the propagation of the calcium signal.

EFFECT OF Ca^{2+} ON MITOCHONDRIAL MOTILITY

It has been proposed that mitochondria need to be strategically localised at particular sub-cellular sites to enable them to participate in intracellular signalling as well as providing an energy supply.

A close association between sub-domains of the endoplasmic reticulum (ER) and the mitochondria surface appears to be necessary for the propagation of ER calcium release to the mitochondria, whereas close associations between the sub-domains of the plasma membrane and mitochondria appears to be important for the control of calcium entry [5].

The distribution of mitochondria to strategic sites has been shown to depend on a cytoskeleton-based transportation system, mainly along microtubules, and "docking proteins" have been identified on the mitochondria in various cell types [6]. These act as mitochondria-bound molecular motors and provide a means for the organelles to move along the cytoskeletal fibres.

Mitochondrial motility involves both long distance travel and complex local movements. These movements may also result in a change in the distribution of mitochondria in the cell, which in turn, results in the rearrangement of the spatial pattern of ATP production and calcium buffering [1].

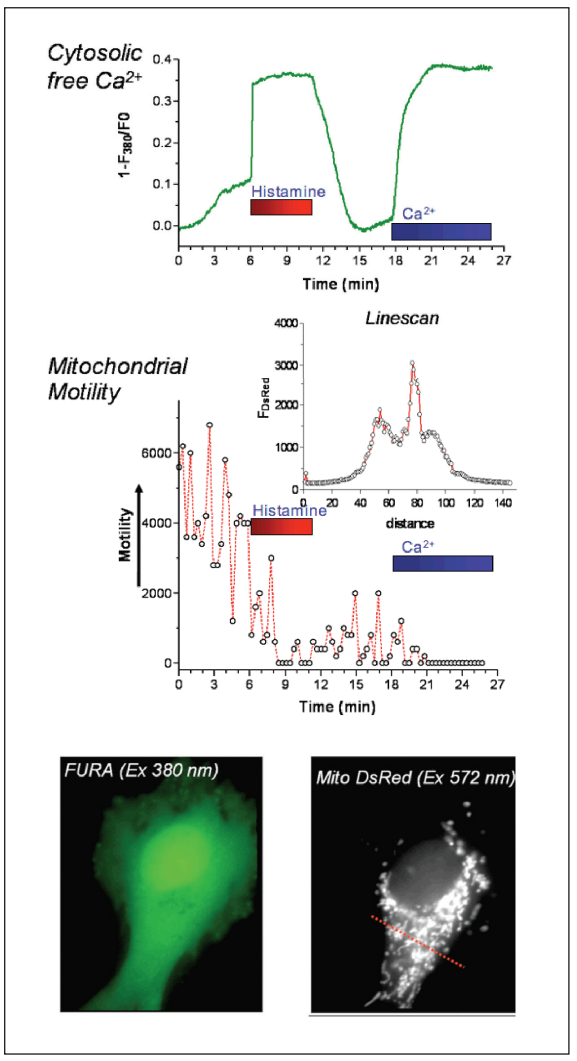

Figure 1. Simultaneous measurements of mitochondrial motility and $[Ca^{2+}]_C$ in human endothelial cells expressing mt-DsRed and loaded with Fura/2AM

In addition, mitochondrial movements may also increase the chance of dynamic interactions between other organelles and thus aid the transport of molecules between the cytoplasm and mitochondria. This means that mitochondrial motility and dynamic changes during motility may affect the signalling pathways and cell function [6].

From previous observations of living cells, mitochondrial motility is a well known phenomenon. Studies have also shown that mitochondrial calcium homeostasis and functions are influenced by the organelle's structural organisation, and that mitochondria motility is controlled by the cytosolic calcium messenger molecules that mediate the effect of agonists on cell function [6].

AIMS OF EXPERIMENTS

By employing a highly advanced dual excitation/dual emission fluorescence imaging system and exploiting specific fluorescent probes, we hope to establish a fluorescence imaging approach that allows the rapid multi-dimensional analysis of fluorescently-labeled cells, enabling us to monitor mitochondrial movement activity. Thus, the aim of these experiments was to make simultaneous measurements of cytosolic calcium levels in mitochondria, demonstrating its regulatory control of mitochondria motility. This, in turn, has allowed us to simultaneously explore the potential of filter-based fluorescence imaging technology for live-cell microscopy.

MATERIALS & METHODS

Equipment: These experiments were performed using the Olympus cell^R modular fluorescence imaging system. This high-speed multi-fluorescence 3-D time-lapse imaging system consists of an all-in-one MT20 illumination system, highly sensitive Olympus digital camera and a fully integrated Olympus IX2 series fluorescent inverted microscope, equipped with 40x and 60x oil emersion objectives.

The optimised epi-fluorescence illuminator was integrated with a 150 watt Xenon arc lamp, an 8-position excitation filter wheel that determined the illuminating wavelength and a 14 position attenuation shutter, which allowed switching excitation wavelengths and attenuation filters in 58 milliseconds. The excitation light was also controlled by rapid shutters, with on/off intervals of 1 ms, which reduced unwanted over-illumination and eliminated photobleaching, a fundamental pre-requisite for the imaging of living cells.

A dedicated Real-Time Controller running Linux, an integral part of the cell^R system, coordinated all hardware modules and peripheral devices independently from the Olympus imaging computer. This enabled precise synchronisation of sample illumination and image acquisition. Experimental execution and data evaluation was facilitated by the advanced Olympus cell^R software.

To enable fluorescent dyes FURA-2AM and DsRed2 to be imaged simultaneously, a novel system was used that employed the dual excitation and dual emission of both dyes. We used a dual band excitation filter, which excites at 380 nm and at 572 nm for free FURA-2AM and for DsRed2 respectively. As a sensitive black and white CCD camera was used, we imaged both the FURA-2AM and the DsRed2 simultaneously by employing an Optical Insights Dual-View™ Micro-Imager.

This is a beam splitter device which divides the camera chip into two chromatically different halves. This dual excitation/dual emission system is very well suited to imaging fast processes such as calcium changes combined with motility, and cannot be achieved by monochromator based imaging systems.

Experiments performed: A human endothelial cell line was transiently transfected with the red fluorescent protein marker DsRed2, which targets, and is retained by the matrix of intact mitochondria. Cells expressing mitochondria-targeted DsRed2 (mt-DsRed) were then loaded with Fura-2/acetoxymethyl ester (Fura-2/AM) according to our standard protocols. Fura-2/AM is a membrane-permeable derivative of Fura-2 and is a fluorescent probe that binds to free intracellular calcium.

It is widely used to measure cytosolic calcium concentrations as a result of calcium-induced fluorescence and is loaded into the cytosol to facilitate simultaneous imaging of many cells while preserving their cytosolic environments.

Histamine was used as an agonist to stimulate the cells, as it has been previously shown that histamine generates a transient rise in cytosolic calcium concentration, of approximately 1-2 µmol/L. All experiments were performed at room temperature.

RESULTS

The results obtained from mt-DsRed2-expressed and Fura/2AM-labelled mitochondria are shown. The data of simultaneously recorded cytosolic free calcium, expressed as $1\text{-}F_{380}/F_0$, and mitochondria motility obtained by the visualisation and calculation of red fluorescence crossing the line is shown. For measuring changes in free calcium the excitation of the FURA/2AM fluorescence at 380 nm was used in these experiments.

These unique experiments revealed a calcium-dependent rest in mitochondrial movements in human endothelial cells. Upon washout of the agonist and return of cytosolic free calcium concentration to resting levels, mitochondrial movements were re-established and disappeared upon addition of extracellular calcium. This yielded high cytosolic calcium levels due to still active calcium entry channels.

DISCUSSION

These experiments have shown that mitochondria are very mobile organelles and their motility is negatively correlated with cytosolic calcium levels. In particular, mitochondria motility is regulated by calcium ions with maximal movement at low resting cytosolic calcium levels $[Ca^{2+}]_C$, and a complete rest and suppression of motility occurs during agonist-induced $[Ca^{2+}]_C$, that is at higher levels of free calcium in the cytosol [6]. The molecular mechanism behind this calcium-triggered arresting of mitochondria is unknown, but may form the basis for a novel homeostatic mechanism in calcium signalling. This indicates that mitochondria may be recruited in a calcium-dependent manner to enhance local calcium buffering and/or ATP supply on distinct cellular demands [1]. In our laboratory we are working on proteins that are responsible for this phenomenon of which the physiological and pathological importance is still unknown.

By employing a highly advanced Olympus cell^R fluorescence imaging system and exploiting specific fluorescent probes, we have successfully established a fluorescence imaging approach that allows the rapid multi-dimensional analysis of fluorescently-labelled cells, enabling us to monitor mitochondrial movement activity. Simultaneous measurements of cytosolic calcium levels in mitochondria have been achieved, demonstrating its regulatory control of mitochondria motility.

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