SPECIAL ARTICLE

CYTOSOLIC CALCIUM IMAGING BY CONFOCAL LASER SCANNING MICROSCOPY: APPLICATIONS IN MEDICINE

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ABSTRACT

Confocal microscopy is a valuable tool for analysing cell and tissue structure. Compared to conventional microscopic techniques, it has the advantages of increased image resolution and the capability for 3-D reconstruction. The introduction of ion-sensitive fluorescent probes has enabled second messenger systems to be studied in relation to cell physiology and function. The confocal laser scanning microscope (CLSM) is best suited for this purpose. Cytosolic calcium signalling with the CLSM has enhanced our understanding of calcium-mediated signal transduction pathways. Given that calcium signalling plays a central role in cell in the mediation of cell functions, any aberration may induce pathological states.

Keywords: *confocal microscopy, calcium signalling, free cytosolic calcium, calcium-sensitive Fluo-3 dye*

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INTRODUCTION

The advent of microscopy has aided biological research, especially in the analysis of cellular structure and function. Conventional light and electron microscopy are routine tools in basic and applied research. In recent years, confocal microscopy is increasingly used although the principles were first described by Minsky some 40 years ago[1]. It offers several advantages over the other two forms of microscopy, viz., (a) increased resolution and detection sensitivity, (b) elimination of out-of-focus images, (c) optical sectioning of samples, thereby eliminating artifacts seen in physically sectioned specimens, (d) visualisation of living and fixed cells with greater clarity, (e) generation of three-dimensional reconstructions, and (f) visualisation and quantification of ionic fluctuations in living cells[2-6].

ANATOMY OF A CONFOCAL LASER SCANNING MICROSCOPE

There are essentially three types of confocal microscopes: (a) stage scanning microscope, (b) beam scanning confocal microscope, and (c) spinning disk confocal microscope. The confocal laser scanning microscope (CLSM) belongs to the beam scanning class of microscopes. The CLSM employs rapidly scanning mirrors to scan a laser beam (usually either argon ion laser or a krypton-argon ion laser) across the specimen[5]. The lasers are able to excite many fluorochromes and thus enable cellular structures tagged with dyes to be visualised[7]. UV beams for visualising dyes excited by shorter wavelengths (340-360 nm) are also commercially available. Of the three types of confocal microscopes, the CLSM is the best instrument for analysing fluorescent probes.

The basic components of the CLSM are: a laser source, an excitation filter, a dichroic mirror, a series of highly reflective mirrors, oscillating galvanometer mirrors, a high numerical aperture objective lens for focusing the light as a scanning spot and a photodetector. The microscope used could be either an upright or an inverted microscope. The CLSM uses software for acquisition of images and subsequent processing.

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Table I – Pathological states associated with deranged Ca^{2+} signalling

Cardiovascular disorders
Hypertension[14]
Atherosclerosis[15]
Ischaemic heart disease[13]
Arrhythmias[16]
Cerebrovascular disease[13]
Psychiatric disorders
Manic depression[17]
Malignant hyperthermia[18]
Sepsis[19]
Cancer[13,20]

Fig 1 – Phosphoinositide signal transduction pathway. Binding of ligand to G-protein coupled receptors initiates hydrolysis of phosphatidylinositol 4,5 biphosphate (PIP_2) via activation of Phospholipase C ß (PLC ß). PIP_2 is hydrolysed into inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates release of free cytosolic Ca^{2+} via IP_3 receptors (IP_3R). DAG stimulates calcium dependent protein kinase C (PKC).

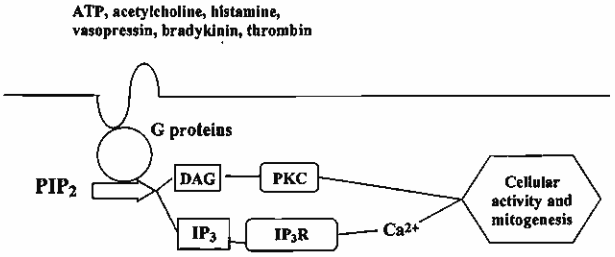
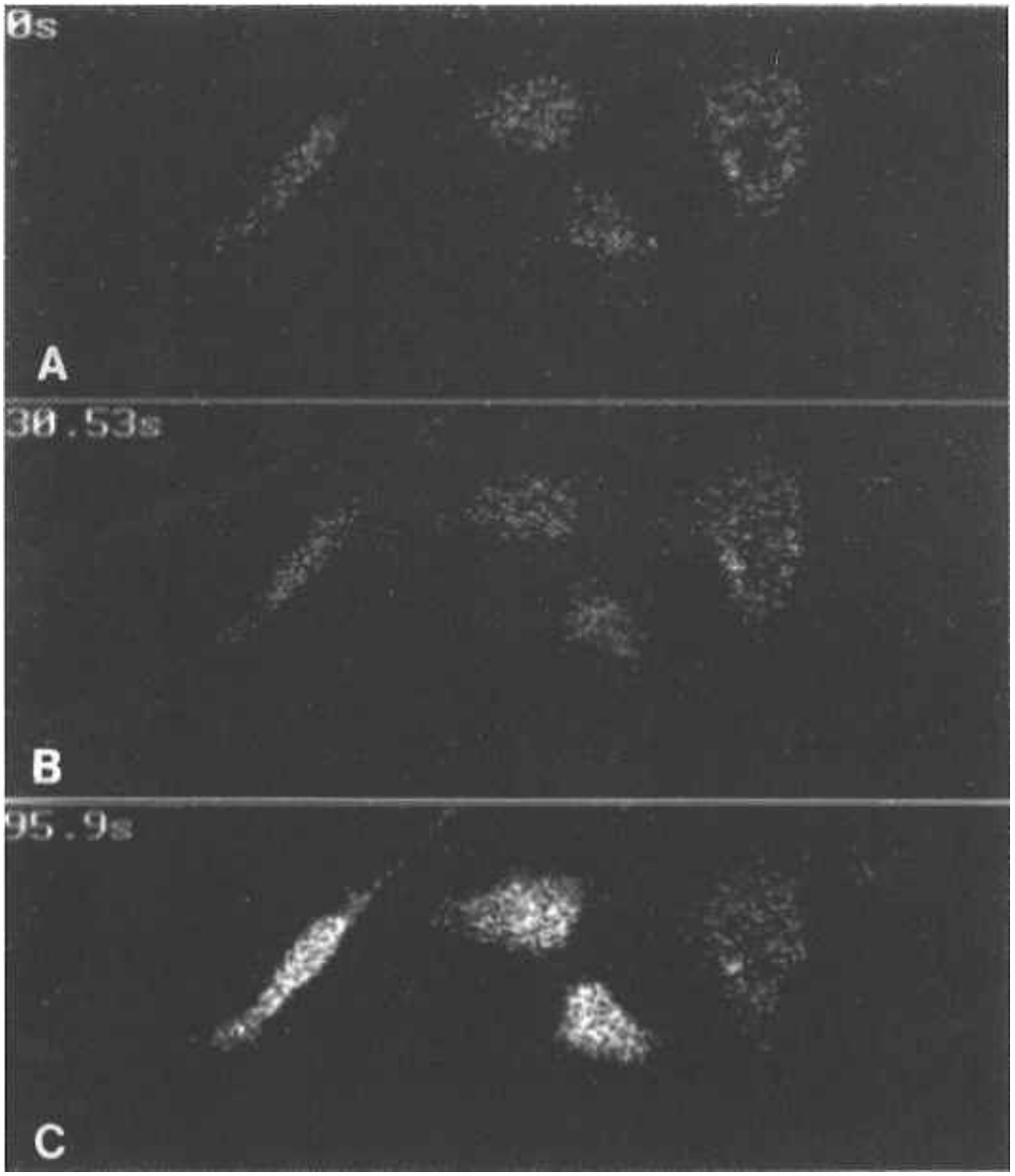


Fig 2 – Real time cytosolic calcium imaging of oral KB carcinoma cells treated with 1 mM ATP. Frame A represents start of experiment. 1 mM ATP is added to the incubating medium just before Frame B. Frame C represents the time point of maximal cytosolic calcium release.



SOME APPLICATIONS OF CONFOCAL MICROSCOPY IN MEDICINE

In the field of medical applications, amongst others, CLSM has been used as a tool in:

(1) diagnostic histopathology. 3-D impressions would aid in arriving at certain diagnosis by providing additional information on volume, shape and DNA content of individual nuclei[8].
(2) functional and structural studies of the nervous system[9,10].
(3) structural and physiological studies of embryogenesis such as lineage tracing and physiological imaging[11].
(4) investigating calcium signalling which will be the topic of focus here.

CALCIUM SIGNALLING AND MEDICINE

Free cytosolic calcium is the key coupling step in the mediation of almost if not all, cellular functions[12]. Calcium signalling appears to play a dual role in that it is required for cellular function on the one hand and yet could be potentially toxic. Deranged calcium signalling has been associated with pathological conditions (an excellent review is given by Berridge[13]) as seen in Table I.

CALCIUM-MEDIATED SIGNAL TRANSDUCTION AND CANCER

Transmembrane cell signalling mediated via cell surface receptors include the following: G-protein catalyst intermediates, protein phosphorylation or dephosphorylation and ion channel activity[20]. In these signal transduction pathways, calcium may act as the direct second messenger or it may produce secondary or tertiary messengers. An example of G-protein coupled phosphoinositide signal transduction is shown in Fig 1.

Calcium-mediated signalling has been observed in malignant transformation[21,22]. Transformation of NIH 3T3 cells were observed when cells were transfected with either the serotonin 1c receptor[21] or the α1-adrenergic receptor[22] which is known to activate inositol trisphosphate. EGF receptor activation is

known to increase intracellular Ca^{2+} through phosphorYlation and activation of phospholipase C-γ providing the linkage between phosphorylation and cancer[23].

In a similar vein, calcium signal transduction pathways have also been implicated in tumour growth, invasion and metastasis. Release of cells from anchorage to the substratum, the first step in the metastatic cascade, has been linked with phosphoinositol hydrolysis[24,25]. Calcium has also been reported to regulate metastasis via G-protein-mediated motility signalling[26] and directly through modulation of the cytoskeletal elements[27].

Thus, manipulation of calcium-mediated signal transduction pathways is an attractive target for the design of cancer chemotherapy. One such drug that has been used in clinical trials is carboxyamido-triazole (CAI)[20]. CAI is known to have selective effects against receptor mediated Ca^{2+} influx and reported to inhibit tumour growth and metastasis[20,28].

CYTOSOLIC CALCIUM IMAGING BY CONFOCAL MICROSCOPY

There are several Ca^{2+}-sensitive fluorophores (Fluo-3, Fura 2, Rhod 2, Indo 1, Quin 2 and Fura Red amongst others) that have been used for investigating the distribution and kinetics of cytosolic calcium[29,30]. Fluo-3, a long wavelength Ca^{2+} indicator[31] is particularly suitable for use with long wavelength excitation sources such as the argon laser. The dye has very weak fluorescence unless bound to free cytosolic calcium as illustrated in Fig 2. The methodology for this experiment is described below.

Oral cavity epidermoid carcinoma cells (ATCC CCL 17), purchased from the American Type Culture Collection were grown in Nunc coverglass chambers. Cultures were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10 μM Fluo-3, AM (acetoxymethyl ester) purchased from Molecular Probes, at 25°C for 30 minutes. Cultures were washed twice with bicarbonate saline buffer and incubated in the same buffer on a heated stage before the addition of 1 mM ATP[33] to the incubating medium. Image acquisitions were performed with the Carl Zeiss LSM 410 Inverted Confocal Microscope equipped with an 10 mW argon ion laser (485/514 nm) and coupled with a Carl Zeiss LSM 4 base programme on a 1 Gbyte internal hard disk of a personal workstation as previously described[34].

CONCLUSION

Confocal microscopy has been labelled as a product of the "renaissance" in light microscopy that has taken place over the last decade[35] and the "ultimate" microscopic technique for 3-D imaging of living or fixed specimens[36]. The CLSM has contributed significantly to the study of Ca^{2+} associated signal transduction mechanisms. However, just like all sophisticated microscopic instruments, correct usage by the operator is crucial. The fact that excitation of fluorophores in living specimens may produce cytotoxicity and thus affect interpretation of results, has also to be borne in mind.

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