Flow-Driven Loading of Living Cells with Impermeable Fluorescent Molecules

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BIOGRAPHY

Jolanta Paltauf-Doburzynska obtained her MSc in maths and physics from the Nicolaus Copernicus University in Torun, Poland, and her PhD in biophysics from the University of Graz. For the past two years she has been head of the fluorescence microscopy and biophysics division at ZMF Graz. Her research interests are the investigation of biophysical properties of living cells, ion regulation, and fluorescence microscopy of intracellular compartments.

ABSTRACT

We describe a new flow-driven technique for the fluorescent labelling of living cells with phallotoxins. For labelling we used two dyes: Oregon-Green phalloidin and rhodamine phalloidin. Cells were treated in a shaker with 350 rotations per minute for 7 or 24 hours. Control cells were loaded with phallotoxins using a standard protocol for permeabilized and fixed cells. In both flow-treated and control cells we could detect fluorescent actin filaments. Control cells incubated with phallotoxins without flow loading did not show stained actin filaments. The mechanism of phalloidin loading into the cell is unknown but we suppose that during the flow procedure the fluid cell membrane and its protein components are disrupted allowing small dye molecules to enter the cell. This method provides a new avenue for loading of living cells with membrane impermeable molecules and could therefore find numerous applications in molecular biology, gene technology and fluorescence microscopy.

KEYWORDS

light microscopy, confocal, fluorescence, phalloidin, actin, cytoskeleton, live cells

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INTRODUCTION

Actin filaments are one of the major components of the cytoskeleton and participate in various cellular dynamic processes [1-3]. They may be efficiently labelled using fluorescently-conjugated phallotoxins isolated from the deadly mushroom *Amanita phalloides*. The most commonly used member of this family, phalloidin, binds to filamentous actin (F-actin) from a wide range of species including plant, animal, and fungal cells and does not discriminate between different actin isoforms. Phalloidin can be conjugated to a wide variety of fluorescent dyes. Indeed the whole family of phallotoxins is used for labelling, identifying and quantifying F-actin in formaldehyde-fixed and permeabilized tissue sections, cell cultures or cell-free experiments.

Phallotoxins induce a variety of specific changes in myofibrillar functioning and are therefore a potentially valuable tool, for example in muscle research. By binding to actin in thin filaments, phallotoxins can change active tension, the spectrum of mechanical relaxation times, ATPase activity and Ca^{2+} sensitivity [4].

However, cell membranes are impermeable to phalloidin. In the standard protocol for the labelling of actin filaments cells are usually fixed, permeabilized and then incubated at room temperature with phallotoxins such as rhodamine-phalloidin, Oregon Green-phalloidin, etc. [5,6]. Phallotoxins have not been used extensively with living cells. However, living animal cells have been labelled by using a relatively mild lysolecithin permeabilization procedure facilitated by the small molecular size of the label [7]. Some groups have microinjected rhodamine actin in living cultured smooth muscle cells [8-10]. Alessa et al. used vital staining of F-actin with Rhodamine Phalloidin using saponin [11].

In different studies the localization of actin filaments has been demonstrated on the mitotic spindles by using rhodamine-phalloidin staining [6,12]. Rhodamine phalloidin can be used for the quantification of F-actin because of the linear dependence of its binding on F-actin concentrations [13]. Fluorescence microscope observation of myofibrils incubated with rhodamine-phalloidin and coumarine-phallacidin showed an initial appearance of fluorescence bands at the Z-lines [14]. Benkoel et. al. have shown the labelling of F-actin in human hepatocytes using rhodamine-phalloidin. F-actin was distributed along the plasma membranes and at the bile canaliculi [15]. Nagayama et al. have measured the tensile properties and actin filament distribution in rat aortic smooth muscle fixed and stained with rhodamine-phalloidin [16].

Oregon Green-phalloidin is another frequently used dye from the phallotoxins family for the visualisation and determination of filamentous actin in fixed cells [17, 18]. Oregon Green-phalloidin has been used for the co-localization of actin and syntaxins 2 and 3 in actin- rich areas at the plasma membrane [19].

In this article we describe a method for the non-invasive labelling of actin *in vivo*. With the help of this technique it is possible to investigate the unchanged and non-disrupted actin cytoskeleton and its dynamics.

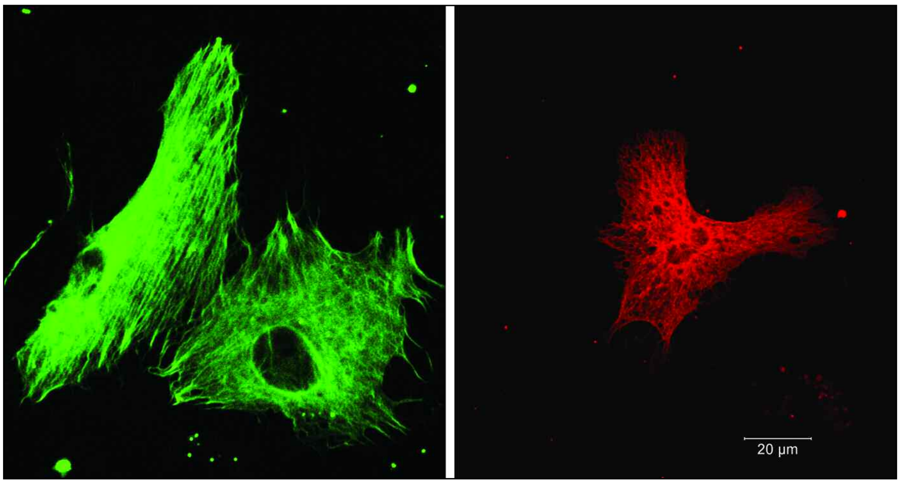

Figure 1:
Actin fibers in living endothelial cells labelled with Oregon Green-phalloidin (left) or rhodamine-phalloidin (right).

MATERIALS AND METHODS

The endothelial cell line EA.hy926 was cultured in dishes for 3 days. Confluent cells were then incubated and shaken with 350 rotations per minute for 7 or 24 hours at room temperature with HBSS medium (with Ca^{++} and Mg^{++}, without phenol red; Gibco) containing 165 nM of the fluorescent dyes rhodamine-phalloidin or Oregon Green-phalloidin (both from Invitrogen). The cells were then washed with phosphate-buffered saline (PBS, pH 7.3; Gibco) and placed in HBSS to let them rest. Before the experiments the cells were placed in fresh PBS medium for 2 hours.

Control cells were also cultured for 3 days. Then they were fixed and permeabilized. Cells were washed twice with pre-warmed PBS and then fixed in 4% formalin solution (Sigma) for 30 minutes at RT. The cells were then washed three times with PBS and permeabilized in 0.05% Triton X-100 (VWR) in PBS for 6 minutes. After washing with PBS the cells were labelled with rhodamine-phalloidin or Oregon Green-phalloidin. After 20 minutes cells were washed with PBS three times. For long-term storage the cells should be air dried and then mounted in a permanent mounting. A second control was incubated only with phallotoxins without flow loading, washed with phosphate-buffered saline and placed in fresh PBS medium.

All cells were examined on a Zeiss Meta LSM510 confocal laser scanning microscope using the single track mode and a 40× Neofluar objective. We used 543 nm laser wavelengths for excitation of rhodamine-phalloidin and 488 nm for Oregon Green. Fluorescence emission was detected at 573 nm and 520 nm, respectively.

RESULTS AND DISCUSSION

Fluorescently labelled actin is an important tool for the investigation of the dynamics of the cytoskeleton. In most studies the standard protocol for fixed cells is used. In our study we investigated the possibility of staining living cells with fluorescent cell-impermeable phallotoxins such as rhodamine phalloidin and Oregon Green phalloidin. Living endothelial cells were shaken in a medium containing fluorescence dye. After 3 hours we could already detect actin fibres in a few cells (Figure 1). After 7 and 24 hours the amount of fluorescently labelled cells was 40 % and 60% respectively. We also investigated living cells that were not flow loaded, but incubated with both phallotoxins for 7 or 24 hours. After incubation cells were washed with PBS and then put into fresh medium. We could see dye molecules only in the cell membrane (Figure 2). Figure 3 shows control fixed cells stained with the standard protocol as described in the materials and methods.

Our protocol is a new method in fluorescence techniques. Labelled cells are neither permeabilized nor fixed. Therefore the intracellular network is not disrupted. Also the cell membrane remains unchanged.

Phallotoxin conjugates are small molecules with an approximate diameter of 12–15 Å. It is possible that during the flow-loading procedure these molecules enter the cell membrane, which is often described as a two-dimensional fluid mosaic dotted or embedded with proteins like channels, transporters and receptors. This model was first proposed by G.L. Nicolson [20]. As a lipid bilayer, the cell membrane is semipermeable. This means that only some molecules can pass unhindered in or out of the cell. These molecules are either small or lipophilic. Other molecules can pass in or out of the cell if there are specific transport molecules. There are two possible transport mechanisms: passive or active transport. In our model we suppose the first one. Unlike active transport, passive transport does not involve any chemical energy (from ATP). Passive transport means moving of different chemical substances across membranes through diffusion of hydrophobic and small polar molecules. Another possibility is the facilitated diffusion of polar and ionic molecules [21]. We suppose that the flow-mechanism facilitates entry of dye molecules due to a diffusion-like phenomenon.

We also tested the stability of fluorescence during proliferation (Figure 4). Endothelial cells were loaded with Oregon Green or rhodamine phallotoxins using the flow–driven procedure. Detection was done once each hour for a total of 30 hours using a Cell Observer (Zeiss). With this device it was possible to collect several phases of cell growth under similar conditions to standard cell culture. Figure 4 shows cells loaded with Oregon Green after the start of the experiment (left) and after 30 hours (right). Fluorescence intensities were stable and had similar values in the beginning and in the end of the experiment (i.e. there was minimal bleaching).

A primary application of the flow technique may be the study of actin filaments in living cells. For example we investigated cells from the human endothelial cell line EA.hy926 under normoglycaemic and hyperglycaemic conditions. The exposure of endothelial cells to a hyperglycaemic medium (DMEM with 30 mM or 44 mM glucose) caused changes in the structure of the intracellular compartments such as mitochondria [22]. We could observe changes in the actin filament architecture; they were reorganized, 'collapsed', and more concentrated (Figure 5). The actin staining in

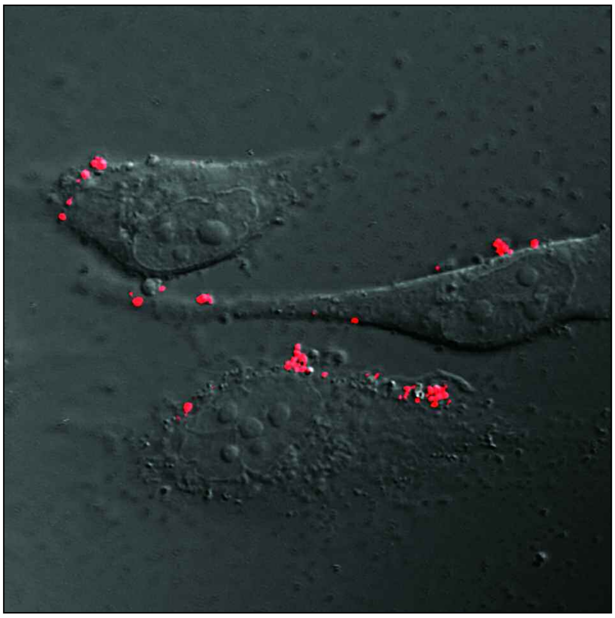

Figure 2:
Living endothelial cells incubated with rhodamine-phalloidin for many hours. Dye molecules were detectable only in the cell membrane. Cells are displayed using an overlay of brightfield and fluorescence images.

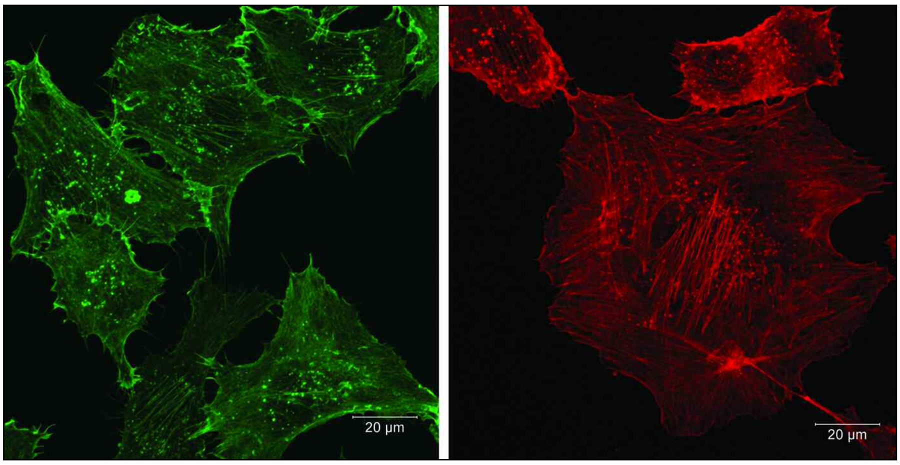

Figure 3:
Actin fibres labelled with Oregon Green-phalloidin (left) and rhodamine-phalloidin (right) after fixation and cell permeabilization.

control cells was more regularly distributed; actin clusters were not observed (Figure 6).

The great advantage of this technique is that it could find more applications in molecular biology and fluorescence techniques such as the transport of vectors to improve transfection efficiency (experiments are in progress). Established methods of molecule delivery are either of a biological nature such as viral vectors, of a chemical nature such as liposome fusion, or of a physical nature such as micropipettes, electroporation, biolistics or opto-injection at ultraviolet and visible wavelengths.

Our method could be a tool for the delivery of drugs, genes and other membrane impermeable bioactive molecules as well as cell membrane impermeable fluorescence dyes, crystals or nanoparticles. We suppose that this method could also be used in the future for gene transfection or gene therapy.

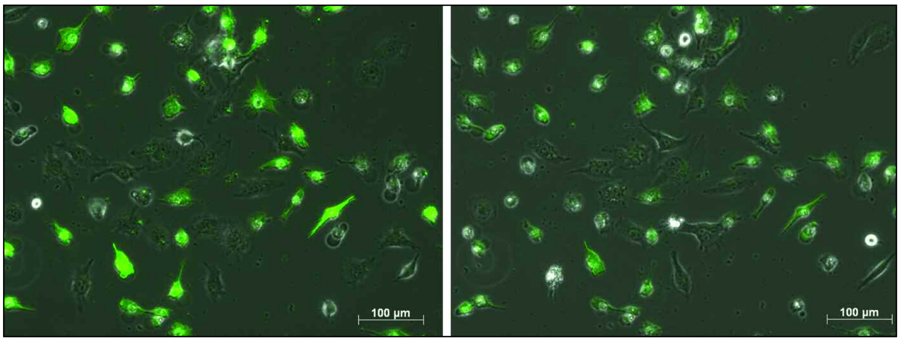

Figure 4:
Fluorescence stability of Oregon Green-phalloidin. Living endothelial cells were loaded with Oregon Green using the flow procedure and imaged in a cell observer each hour for 30 hours. Cells after start of experiment (left) and after 30 hours (right).

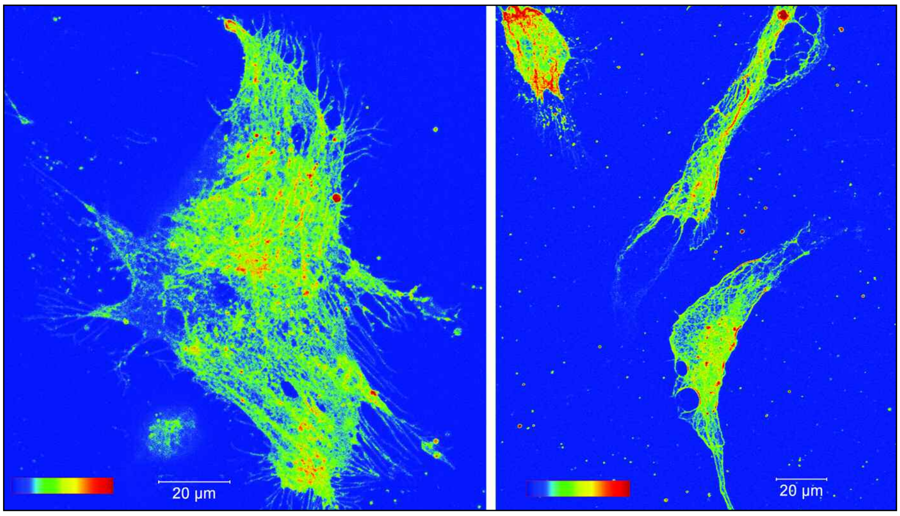

Figure 5:
Actin filaments under hyperglycaemic conditions. Endothelial cells were loaded with a high glucose concentration for 24 h, and then shaken for 24 h in medium with Oregon Green as described in the Methods. Fluorescence was detected after a rest time of 2 hours. Colour intensity scale bar shows lowest (blue, background) to highest (red) fluorescence intensities.

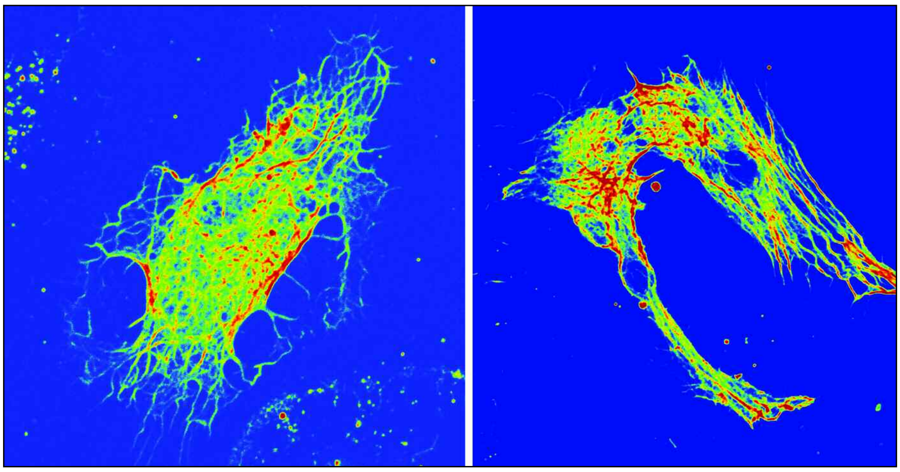

Figure 6:
Actin filaments in normo-glycaemic cells without treatment with high glucose. Cells were shaken in medium with Oregon Green for 24 h as described in the Methods. Fluorescence was detected after 2 hours. Colour intensity scale bars and magnification scale bars as in Figure 5.

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