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LETTER

Cell morphology and intracellular ionic homeostasis explored with a multimodal approach combining epifluorescence and digital holographic microscopy

Nicolas Pavillon[*,1], *Alexander Benke*[2], *Daniel Boss*[2], *Corinne Moratal*[2], *Jonas Kühn*[3], *Pascal Jourdain*[2], *Christian Depeursinge*[1], *Pierre J. Magistretti*[†,2,3], *and Pierre Marquet*[†,2,3]

[1] Advanced Photonics Laboratory (MVD), Ecole Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland
[2] Brain Mind Institute, Ecole Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland
[3] Département de Psychiatrie-CHUV, Site de Cery, 1008 Prilly, Lausanne, Switzerland
[†] Co-last authors

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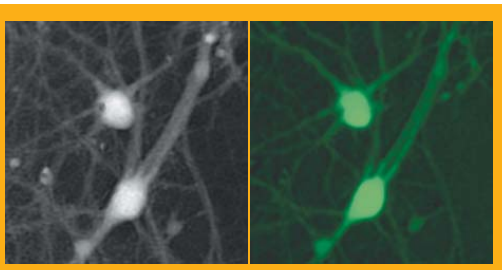
The authors have developed a live-cell multimodality microscope combining epifluorescence with digital holographic microscopy; it has been implemented with a decoupling procedure allowing to separately measure from the quantitative phase important cell parameters including absolute volume, shape and integral intracellular refractive index. In combination with the numerous different specific fluorescent cellular probes, this multimodality microscopy can address important issues in cell biology. This is demonstrated by the study of intracellular calcium homeostasis associated with the change in cell volume, which play a critical role in the excitotoxicity-induced neuronal death.



Comparison between quantitative phase (left) and fluorescence signal (right) on neuronal cellular bodies.

1. Introduction

A comprehensive understanding of the relationship between cell dynamics and physiological or pathological processes requires to be able to measure in real-time various cell parameters through the setting of a single examination.

Within this framework, we have developed a multi-modality imaging system combining digital holographic microscopy (DHM) with epifluorescence microscopy.

Fluorescence microscopy is a well established and widely used technique within the field of cell biology and has permitted in particular, in combination with the utilization of specific fluorescent cellular probes, the observation of dynamic processes in living cells ranging from vesicles fusion to the concentration changes of specific intracellular signaling molecules.

In contrast, DHM is an interferometric technique [1] making it possible to visualize in real time, from a single digital hologram, structure and dynamics of

* Corresponding author: e-mail: nicolas.pavillon@a3.epfl.ch

transparent specimens, including living cells, without the use of any staining and with a nanoscale axial sensitivity [2–7]. Concretely, such visualization results from the DHM ability to measure minute phase retardation or optical path difference (OPD) induced by the transparent specimen on the transmitted wavefront [8–10].

Practically, important biophysical cell parameters, including cell shape and volume, intracellular refractive index related to cell content as well as dry mass can be derived from this quantitative phase signal [11–13].

Some attempts to combine quantitative phase signal with fluorescence imaging have already been reported [14]. Here we present multimodality microscopy allowing to monitor simultaneously the dynamics of intracellular ionic homeostasis and the cell morphology changes derived from the DHM quantitative phase signal. This multimodal ability is illustrated by imaging the early stage of neuronal death induced by the excitotoxicitiy phenomenon, likely to be involved in several pathogenesis of brain damage including chronic neurodegenerative diseases [15]. Concretely, it has been possible to measure simultaneously the intracellular calcium dysregulation and the concomitant neuronal volume changes, occurring at the early stage of the glutamate-mediated excitotoxicity.

2. Experimental setup and measurement procedure

The measurements are performed on a transmission DHM setup (DHM T1000^{TM} from Lyncée Tec SA) specially modified to incorporate a second imaging module for fluorescence (cf. Figure 1). For the holographic measurement, the laser is divided into two beams; the object beam is illuminating the specimen and is combined with the reference beam on the measurement plane in the so-called off-axis configuration [16]. The interference pattern is measured by a charge-coupled device (CCD) camera, enabling recording of the hologram, and a further numerical reconstruction. The mirror reflecting the object beam onto the CCD camera is a dichroic mirror, making it possible to excite the specimen in epifluorescence. The excitation is performed with a monochromator (Till Photonics Polychrome V), allowing an easy choice of the excitation wavelength. The fluorescence signal is then detected after wavelength filtering with an electron multiplying CCD camera (Andor iXon DU887).

The study was performed on primary cultures of mouse cortical neurons cultured in Neurobasal medium supplemented with 2% B27 mix as described by

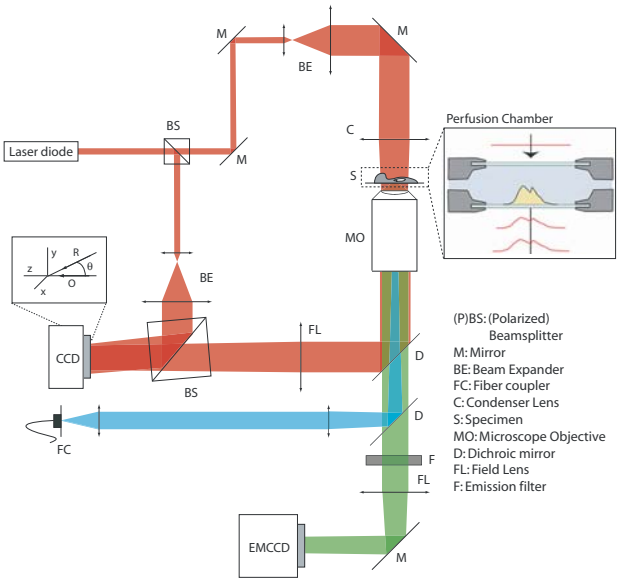


Figure 1 (online color at: www.biophotonics-journal.org) Principle scheme of combined epi-fluorescent and digital holographic microscope. The red beam shows the DHM laser beam path with object and reference arm, the blue beam is the fluorescence excitation in epi-illumination, and green is the fluorescence emission measured with the high-gain EMCCD.

Brewer et al. [17]. Cells were transferred in dishes containing glial cell monolayers, and then used after 21–35 days in vitro.

Before measurement, the cells were charged during 20 minutes at 37 °C with a solution containing 6 μM of Fluo-4 dye, which enables measurement of the intracellular calcium concentration $[Ca^{2+}]$ [18]. Neuronal cultures are then mounted in a closed perfusion chamber allowing a rapid washout and are perfused with a HEPES-buffered standard physiological medium at ambient temperature. The dye is excited at 490 nm, and has its peak emission at 515 nm, with acquisitions taken at 1 Hz. The cells are imaged with a 10× microscope objective (numerical aperture NA = 0.3), providing a field of view of approximately 500 × 500 μm.

3. Results and discussion

The excitotoxicity effect has been induced by prolonged applications of glutamate, the principal excitatory neuro-transmitter of the brain, on neurons in culture. The sequence of events leading to excitotoxicity starts in particular with the activation of ionotropic glutamate channel receptors, which allows the influx of Ca^{2+} and Na^{+} ions. The subsequent neuronal death, whose mechanisms are not yet com-

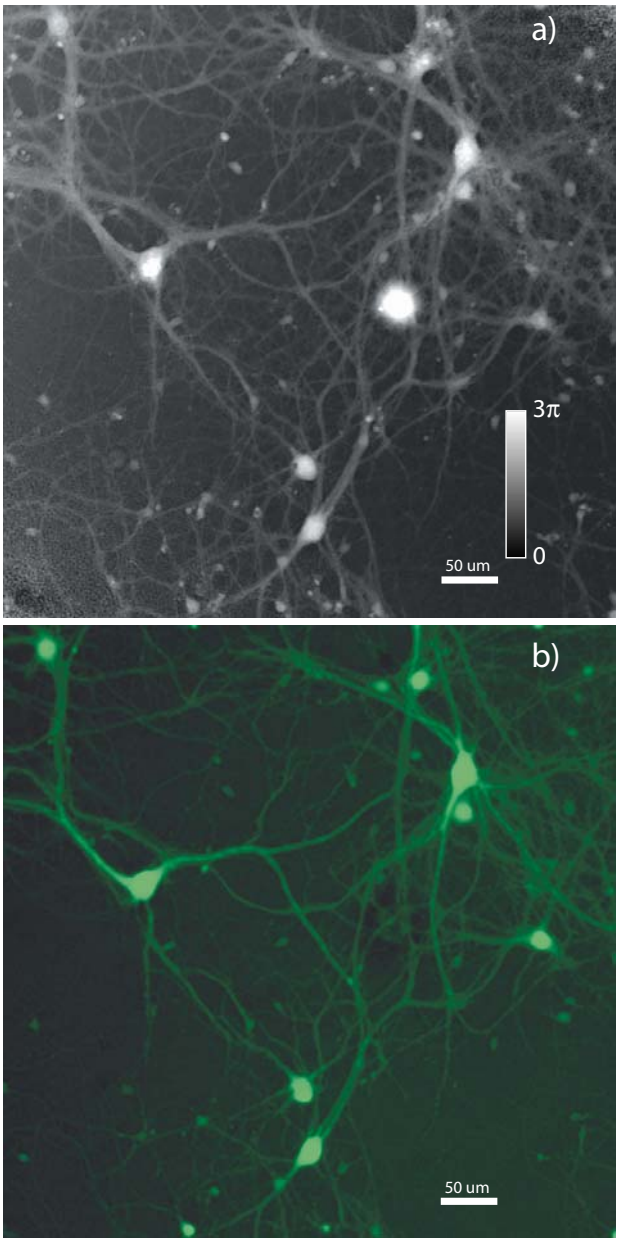


Figure 2 (online color at: www.biophotonics-journal.org) Image of living neurons measured with (**a**) digital holography providing quantitative phase (scale shown in radians) and (**b**) Fluo-4 dye fluorescence during high Ca^{2+} concentration.

pletely elucidated, is related to a dysregulation of the intracellular $[Ca^{2+}]$ homeostasis [19].

A typical image of measured cell culture is presented in Figure 2, with the quantitative phase provided by DHM (cf. Figure 2(a)) and Fluo-4 fluorescence (cf. Fig. 2(b)). The presented fluorescence image is shown during a glutamate application, thus providing a strong signal.

Typical cellular responses elicited by the application of glutamate pulses (30 μM, 30 s) are shown in Figure 3. Transient large increases in calcium concentration can be readily seen through the fluorescence intensity signal, while the quantitative phase signal shows concomitant reversible phase decreases. Otherwise, when the intracellular calcium does not recover because of a longer glutamate application (1 mn), i.e. when the intracellular calcium homeostasis is irreversibly altered, the phase signal also presents an irreversible decrease. Usually, typical fluorescence measurements imply a calibration step in order to get a relation between the fluorescence intensity signal and the effective ion concentration

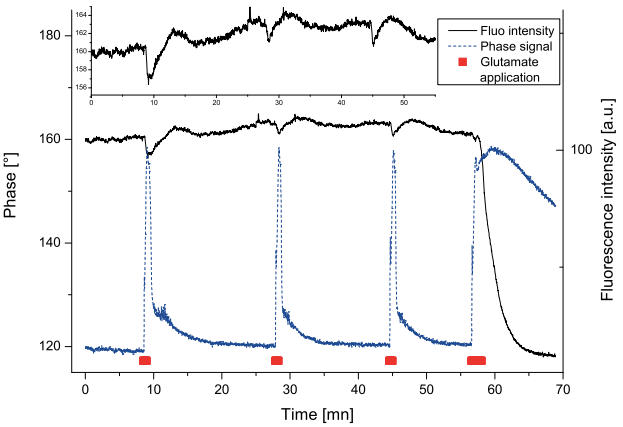


Figure 3 (online color at: www.biophotonics-journal.org) Temporal monitoring of quantitative phase and fluorescence intensity on a cellular body ongoing glutamate application (30 μM). One can readily identify the strong phase decrease simultaneous to $[Ca^{2+}]$ dysregulation in the cell. The inset on the top-left is a zoom on the phase signal.

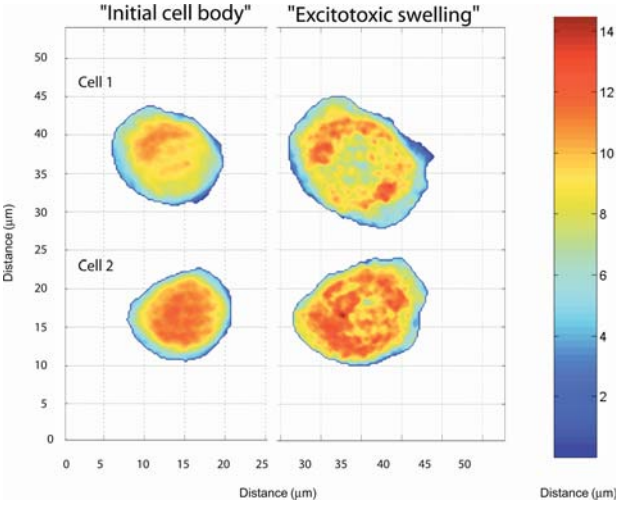


Figure 4 (online color at: www.biophotonics-journal.org) Morphometry of two neuronal bodies before ("Initial cell body") and after the onset ("Excitotoxic swelling") of the glutamate application inducing an irreversible phase decline of around 40 degrees. The *z*-axis (cellular thickness) is expressed in micrometers.

Table 1 Quantitative morphometric parameters (S_{cell}: cell surface; V_{cell}: cell volume) of the two neuronal bodies shown on Fig. 4.

		S_{cell} [μm^2]	V_{cell} [μm^3]	Mean Intracellular RI
Initial	cell 1	129 ± 20	1332 ± 230	1.365 ± 0.0003
	cell 2	162 ± 22	1412 ± 217	1.364 ± 0.0003
Excitotoxic	cell 1	210 ± 25	2106 ± 285	1.351 ± 0.0003
	cell 2	260 ± 28	2196 ± 280	1.353 ± 0.0003

$[Ca^{2+}]$, which was not performed in our experiments. However, typical signal shape is not modified by such a calibration [18].

In transmission, the phase signal φ corresponds to an OPD given by the following expression

$$\varphi = \frac{2\pi}{\lambda}\,\mathrm{OPD} = \frac{2\pi}{\lambda}(n_c - n_m)\,d \qquad (1)$$

where n_c is the integral refractive index (RI) of the cell, n_m the RI of the perfusion medium and d the thickness of the cell. In order to correctly interpret the glutamate-mediated phase signal decrease, a decoupling procedure allowing to separately retrieve the integral RI and cell morphology has been performed according to Rappaz et al. [11].

It results that the phase decrease corresponds to a neuronal swelling accompanied by a cellular surface enlargement as depicted in Figure 4. In addition, according to Eq. (1), we can observe that the glutamate-mediated phase decline results from the intracellular refractive index decrease as presented in Table 1. This decrease is consistent with a water influx accompanying for osmotic reasons the well-known Na^+ and Ca^{2+} ion entry through the glutamate ionotropic receptors; this results particularly in a dilution of the intracellular protein concentration, the cellular constituent, which largely determines the intracellular refractive index value. Consistently, the phase recovery accompanying the reversible phase decrease corresponds to a volume restoration.

A striking point is the temporal correspondence between the $[Ca^{2+}]$ peaks and the minimum of the phase signal which corresponds to the maximum of the neuronal swelling as well as the fact that the rapid restoration of the Ca^{2+} concentration is directly related to the volume recovery. In contrast, when a dysregulation of the intracellular $[Ca^{2+}]$ homeostasis persists, no more volume restoration occurred (cf. Figure 3). Otherwise, lactate dehydrogenase (LDH) release essay (which reflects cell lysis) showed that glutamate-mediated reversible phase signal does not induce neuronal death in contrast to the irreversible phase signal which definitively leads to a neuronal death (data not shown). These results are in good agreement with the recent studies which have stressed that the cell volume regulatory mechanisms are aimed above all to maintain ionic homeostasis, which plays a critical role in the activation/inactivation of the cell death [20].

4. Conclusion

This multimodality microscopy is an original live-cell imaging technique which has the ability to simultaneously explore some important biophysical cell parameters including the dynamics of cell morphology (volume, shape) and dry mass in particular, as well as a large variety of cellular processes thanks to different fluorescent probes used as specific contrasting agents. In this letter, the efficiency of such a multimodality approach has been illustrated by its ability to study the flux of calcium ions associated with the change in cell size, which plays a critical role in the regulation of the cell death mechanisms.

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