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LETTER

Multimodal biophotonic workstation for live cell analysis

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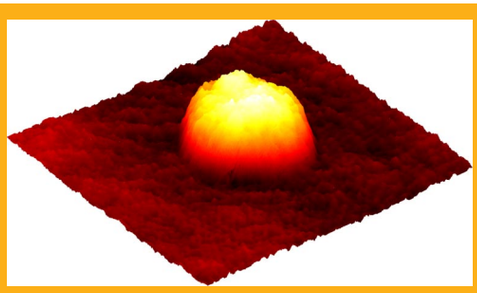
A reliable description and quantification of the complex physiology and reactions of living cells requires a multimodal analysis with various measurement techniques. We have investigated the integration of different techniques into a biophotonic workstation that can provide biological researchers with these capabilities. The combination of a micromanipulation tool with three different imaging principles is accomplished in a single inverted microscope which makes the results from all the techniques directly comparable. Chinese Hamster Ovary (CHO) cells were manipulated by optical tweezers while the feedback was directly analyzed by fluorescence lifetime imaging, digital holographic microscopy and dynamic phase-contrast microscopy.



False color coded pseudo 3D representation of the quantitative phase image of a living CHO cell.

1. Introduction

When the microscope was invented in the 17th century, people were not aware of the vast amount of life that could be found in a single drop of seemingly clear water. The invention of Frits Zernike's phase contrast method [1] allowed researchers to visualize cells and other phase objects in the microscopic world. Since then the observation of organisms in vivo has substantially advanced, including Nomarski's differential interference contrast [2] leading towards interferometry and digital holography [3, 4]. The advantage of all of these methods is that they are non-invasive and thus enable long-term incubation and observation to determine biophysical properties of organisms. Together with the measurement of biochemical properties, for which fluorescent microscopy, in combination with fluorescent staining or tagged fluorescent proteins, is arguably the gold-standard [5], this information can yield valuable in-

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sight into cellular activity. In this letter, we present a multimodal approach to the measurement of both biophysical and biochemical properties to determine the influence of the micromanipulation of cells. The setup includes four different techniques combined in a single biophotonic workstation integrated in a commercially available microscope. Holographic optical tweezers (HOT) are used for micromanipulation, while dynamic phase-contrast microscopy (DynPCM) provides real-time monitoring of cell changes and movement. Digital holographic microscopy (DHM) interferograms recorded during the manipulation of cells provide a high-resolution detection of deformations and refractive index properties of the sample, whilst fluorescence lifetime imaging (FLIM) monitors the fluorescence lifetimes of dyes deposited in the membrane of cells. In order to test our system in a real biological experiment, Chinese Hamster Ovary (CHO) cells, which have been treated to contain a membrane-localized dye, are stretched using optical tweezers and observed by all three imaging methods (DynPCM, DHM, FLIM) simultaneously.

2. Workstation technologies

Since their invention in 1986 [6], optical tweezers have been used in the life sciences for a wide range of experimentation, and have been combined with a multitude of imaging modalities including confocal laser scanning microscopy, epi-fluorescence, multiphoton microscopy, and Raman spectroscopy [7]. Their functionality has been greatly increased with the advent of multiple dynamically addressable tweezers, made possible with acousto-optic deflectors or spatial light modulators [8]. One exciting application of optical forces is that of optical stretching. Here, a cell is pulled in order to characterise its plasticity, allowing the discrimination between highly elastic cancer cells and less compliant healthy cell phenotypes [9].

Digital holographic microscopy enables label-free, quantitative phase contrast imaging for high resolution technical inspection and minimal invasive live cell analysis and provides information about the intracellular refractive index ([10] and references therein). Compared to other phase-contrast methods, interferometry-based techniques, and optical coherence tomography or optical coherence microscopy, DHM provides quantitative phase-contrast with subsequent numerical focus correction (multifocus imaging) from a single recorded hologram. In combination with algorithms for the quantification of the image sharpness, numerical autofocusing without mechanical focus realignment is possible [11].

Dynamic phase-contrast microscopy consists of the combination of an all-optical novelty filter with a commercial microscope. The setup that is incorporated here uses two beam-coupling in a photorefractive barium titanate crystal to achieve background suppression and quantitative phase-measurements in a mapping area of $0 \leq \varphi \leq \pi$ [12]. Due to these working characteristics, DynPCM provides a high Signal-to-Noise-Ratio, even for pure phase-objects [13]. In this experiment, the focus is on the novelty filtering capability of the setup. It complements DHM in the sense that it does not need any image reconstruction. Hence, it enables real-time observation of dynamic processes at camera-dependent frame rates. This allows researchers to instantly detect movement in a biological sample or a microflow [14].

Fluorescence Lifetime Imaging is an attractive method for contrasting different molecular dyes and their environment [15]. Compared to fluorescence intensity imaging, FLIM is independent of fluorophore concentration and excitation intensity and can provide quantitative readouts of local fluorophore environment [16]. Therefore, this technique provides complementary information on local physical and chemical molecular parameters. FLIM is usually implemented using a pulsed or externally modulated laser for the excitation, however, it has recently been shown that inexpensive light emitting diodes (LED) can also be an effective excitation source for FLIM [17]. In this project, we use FLIM to read out the fluorescence lifetime of the membrane-localized dye, di-4-ANEPPDHQ [18], as a reporter of membrane lipid order in order to track the effect of trapping on the cell membrane over the course of the experiment.

3. Technical assembly of workstation

The novel biophotonics workstation was built on a Nikon TE2000 platform (Figure 1). For the holographic optical tweezers, a Coherent MIRA900 laser (800 nm CW) pumped by a Verdi-V5 was coupled into the upper turret of the microscope. A spatial light modulator (SLM, Hamamatsu X8267-13) was relayed to the back focal plane of a 60X 1.4 NA Nikon microscope objective (MO). Multiple tweezers were created by a superposition of lens and prisms elements on the SLM, allowing full 3D control of arbitrary numbers of objects. In the experiment, a double-trap was used to stretch the cell. The lateral distance between the traps was increased in steps of 100 nm at a rate of approx. 100 nm/s.

For the combination of DHM and DynPCM, the respective components were designed to operate at the same wavelength of 532 nm to simplify illumina-

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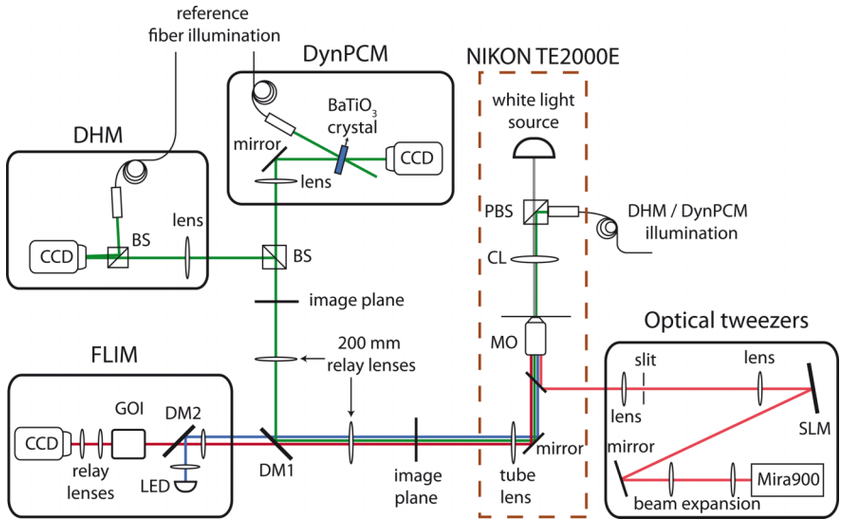

Figure 1 (online color at: www.biophotonics-journal.org) Concept for the workstation based on a Nikon TE2000 microscope; see text for details.

tion. A fiber coupling box (not shown in Figure 1) with three fiber outputs as well as an illumination adapter were developed in close collaboration between the Institute of Applied Physics and the CeBOP in Münster. A frequency-doubled Nd : YAG laser (Coherent Verdi) was split up in the fiber coupler using two beamsplitters. For the connection of the coupler box and the actual setup of DHM and DynPCM, polarization-maintaining fibers (Thorlabs 488 PM) were used. The illumination adapter fits onto the microscope condenser mount and introduced the laser light into the illumination path by a polarizing beamsplitter (PBS). In this configuration, simultaneous illumination at all desired wavelengths as well as white light observation was possible. A dichroic mirror (DM1) separated the 532 nm from the fluorescence light for FLIM, a 50/50 beamsplitter (BS) divided the image information for DHM and DynPCM. For DHM, the intermediate image plane was imaged onto a CCD (The Imaging Source DMK 41BF02, Bremen, Germany), where off-axis holograms were recorded. The numerical reconstruction of the quantitative DHM phase contrast images from the digital holograms was performed by spatial phase shifting-based reconstruction as reported previously ([10] and references therein). In case of unfocused imaging numerical auto-focusing was applied as described in [19]. The refractive index determination of suspended cells was performed as described in [19]. For DynPCM, signal and reference beam interfered within a photorefractive barium titanate crystal that was placed shortly after the Fourier plane of the imaging lens [14]. Due to the fact that DynPCM requires long-time stability for the grating formation in the photorefractive crystal, the imaging path from the microscope to the DynPCM setup was encapsulated with protective tubing.

In the FLIM setup, which was configured to operate in the wide-field frequency domain, an inexpensive blue Light Emitting Diode (LED) (AcuLED DYO 455 nm) was used as the excitation source and was driven electrically at $f = 80$ MHz with a DC bias [18]. A dichroic mirror set (DM2) with 450–480 nm excitation filter and >515 nm emission filter was used to direct the excitation light into the microscope and separate the fluorescence emission onto a gated optical image intensifier (GOI). The GOI is a high trigger rate gated intensifier (Kentech Instruments Ltd. model HRI) that was sinusoidally modulated at the same frequency as the LED drive source with its output phosphor screen being imaged onto a CCD camera (Hamamatsu Orca ER). For each FLIM image, five separate images were recorded from the CCD for five different relative phases of the HRI and LED drive signals that were temporally separated by 2.5 ns. A fluorescent plastic slide (CHROMA Red) was used as a known standard in order to provide calibration for the system. The phase lifetime was obtained from the phase $\Delta\varphi$ of the signal in the measured fluorescence images relative to the excitation using the calculation $\tau = (\Delta\varphi/2\pi f)$.

4. Sample preparation

Chinese Hamster Ovary (CHO) cells were routinely cultured in a humidified atmosphere of 5% CO2/ 95% air at 37 °C in 25 cm^2 Nuncleon culture flasks (Fischer, UK) containing 5 ml of modified Eaglés medium with 10% foetal calf serum (Sera Labs, UK), 20 μg/ml streptomycin, and 20 μg/ml penicillin. Immediately prior to experimentation, cells were

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trypsinised, treated with 5 μM di-4-ANEPPDHQ (Invitrogen, UK), centrifuged for 10 minutes at 200 rcf, re-suspended in Hanks balanced salt solution (−Mg2−Ca2+) and plated onto 170 μm thick glass bottomed dishes (WORLD PRECISION INSTRUMENTS) which were previously coated with poly-2-hydroxyethyl-methacrylate (20 mg/ml in ethanol) to prevent adhesion. All solutions were obtained from Sigma, UK unless otherwise stated.

5. Cell analysis

As proof-of-concept experiment to demonstrate the workstation's capability to combine optical micromanipulation with all three imaging methods simultaneously, cell compartments of a suspended CHO cell, treated with a membrane-staining fluorescent dye (di-4-ANEPPDHQ), are trapped with HOT and moved to apply stress to the cell as described above. The membrane staining dye is reported to change its fluorescence lifetime according to the degree of order in the lipid bilayer [18]. Stretching cells has been demonstrated theoretically to reduce the order of lipid tails [20], which produces weaker lipid packing and higher diffusion rates [21] such that the translational freedom, or lateral mobility, of liquid ordered membranes is 2–3 times lower than that of liquid disordered membranes [22]. Thus a decrease in fluorescent lifetime of di-4-ANEPPDHQ might be expected following a decrease in lipid order generated by the process of stretching. Figure 2 summarizes the observations from the first experiment.

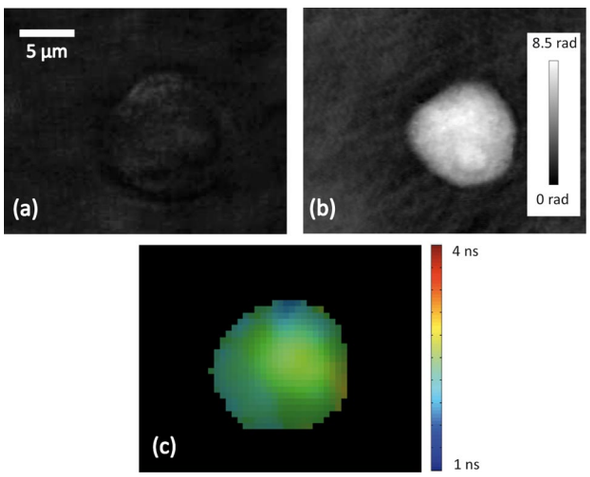

Figure 2 (online color at: www.biophotonics-journal.org) Simultaneous multimodal imaging of suspended CHO during tweezer stretching: (**a**) DynPCM, (**b**) DHM, (**c**) FLIM image showing the phase lifetime calculated from five phase-delayed acquisitions.

DynPCM highlights the parts of the cell under motion, e.g. due to the optical forces, for real-time inspection of the movement and refractive index changes. In this experiment, only the cell membrane (Figure 2(a)) is visible since it is the only part that moves significantly during the stretching process. The rest of the cell is suppressed due to the stable position in the optical trap. The evaluated DHM image maps the quantitative phase retardation of the light inside the cell (Figure 2(b)). Within the measurement accuracy, no variation of the optical thickness during the stretching process could be found. This observation is supported by the fact that most parts of the cell stay suppressed in phase sensitive DynPCM imaging. Figure 2(c) displays the map of phase lifetimes obtained by FLIM as described above. In our experiment, the average fluorescence lifetime was 2.27 ns (+/−0.33 ns). While the observed lifetime did vary over the course of the experiment, there was no systematic correlation between the optical stretching of the cell and the fluorescence lifetime of the membrane dye for the observed cells. We further note that under these degrees of optical stretching, the phase-sensitive techniques DynPCM and DHM did not show significant changes of morphology and integral cellular refractive index properties.

6. Conclusion

We have successfully demonstrated the assembly of a biophotonic workstation, which combines for the first time four different biophotonic techniques that have so far only been used in separate setups. Due to the fact that the wavelengths were carefully separated, the imaging modes could all be used at the same time. The feasibility of this combined approach was demonstrated with a multimodal observation of a CHO cell, where DynPCM provided real-time monitoring of a cell, while FLIM and DHM reconstruction was used to investigate changes in refractive index and membrane lipid order due to stretching with HOT. Our simple proof-of-principle experiment shows the potential of the biophotonic workstation, and we believe that the combined availability of these complementary techniques will enable biologists to develop new experiments, for example spatially probing the biophysical or biochemical properties with HOT actuated microprobes [23], for which our biophotonic setup offers all capabilities.

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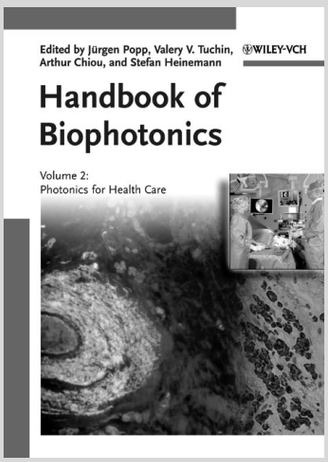

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