Label-free microscopy of mitotic chromosomes using the polarization Orthogonality Breaking technique

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The vast majority of the microscopy methods currently available to study biological samples require staining prior to imaging. Nevertheless, the ability to reveal specific cell structures or organelles in a label-free manner remains desirable in different contexts. Polarization microscopy has long been considered as an interesting alternative to fluorescence-based methods in order to gain specificity on the imaged biological samples. In this work, we show how an original polarization imaging technique, implementing micro-wave photonics and referred to as orthogonality-breaking (OB) microscopy, can provide informative polarization images from a single scan of the cell sample in a fast and sensitive way. For OB imaging, the sample is probed with a laser setup simultaneously generating two orthogonal polarizations shifted in frequency by a few tens of MHz. If the imaged samples display some polarimetric properties, the orthogonality between the two polarizations is broken, leading to a beatnote interference signal that can be detected with a fast detector. The comparison of the images of various cell lines at different cell-cycle stages obtained by OB polarization microscopy and fluorescence confocal images shows that an endogenous polarimetric contrast arises on compacted chromosomes during cell division. This technique paves the way to label-free real-time polarization imaging of mitotic chromosomes with further potential applications in histology and cancer diagnosis.

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INTRODUCTION

Over the last decades, the relentless development of novel optical imaging techniques addressing specific issues of the biology or biomedical community has given rise to a huge number of unconventional imaging techniques. Several of these were granted with worldwide commercial and scientific success such as Optical Coherence Tomography, advanced confocal fluorescence microscopy, non-linear microscopy, phase imaging, etc. However, a number of interesting challenges still remain open in this interdisciplinary research field. For some of these challenges, polarimetry can inspire novel solutions to overcome some of the remaining bottlenecks. For instance, the study of cellular biological mechanisms is overwhelmingly performed with confocal laser scanning fluorescence microscopy, which allows the structures of interest to be imaged at high spatio-temporal resolution. Nevertheless, as for any fluorescence-based method, confocal microscopy requires the labeling of the sample using either fluorescent compounds displaying specific localizations within the cells, dyes coupled to antibodies or recombinant chimeric constructs composed of a fluorescent protein fused to a protein of interest. Although this labeling step has proven to be usable in multiple contexts, it also suffers some limitations. First, per se, it requires an additional, potentially lengthy, step in the sample preparation, thus limiting the throughput of the experiments. Second, due to the crosstalk between the emission spectra of the fluorescent dyes, it is often difficult to image simultaneously more than four different structures within the same sample. Finally, in the context of the observation of living samples, the labeling procedure may induce cytotoxicity and interfere with the tracked biological mechanisms. These different drawbacks justify the need for the development of label-free microscopy techniques. Among the techniques that are currently investigated (non-linear microscopy, quantitative phase microscopy, diffraction tomography...), polarimetric approaches can provide multi-dimensional physical information on the samples, which could thus convey also some selectivity in order to identify cell constituents [1–5], investigate the internal organization of biological structures [6–9] or discriminate unhealthy tissues [10–13] without the need for labeling. Most of the time however, polarimetric imaging is not given preference, due to the poor sensitivity of most systems and to the complex calibration and lengthy acquisition procedures of standard polarimetric techniques based on the sequential acquisition of several images [6, 14, 15].

In this context, a direct, sensitive and fast polarimetric imaging technique, referred to as “orthogonality-breaking” (OB) polarimetric sensing has been proposed a few years ago [16–18]. It is based on a microwave photonics approach, and allows different polarimetric properties (birefringence, dichroism, and depolarization) to be identified from a single sample scan using the appropriate detection modality [19]. In this paper, we report how such a technique has been implemented on a commercial fluorescence confocal microscope set up. We demonstrate that this new imaging modality can be used to observe mitotic chromosomes at different stages of the cell division with high contrast and at the spatial resolution allowed by the microscope setup, i.e., approximately $\approx 150$ nm within the focal plane. Being able to monitor such cellular structures can prove useful to quickly identify proliferating cells within a biological sample or to identify cells displaying altered architecture of their genomic material, a feature that is shared by multiple tumor cells [20].

MATERIALS AND METHODS

Theory

Principle of orthogonality-breaking polarimetric sensing

OB polarimetric imaging is based on the use of a specific dual-frequency dual-polarization (DFDP) laser illumination to probe the sample. The frequency difference imposed between the two polarization components of equal intensities must lie in the radiofrequency (RF) range, typically 10's to 100's of MHz, in order to match the typical bandwidth of common photodetectors (PD) (photodiodes, avalanche photodiodes (APD)...), and avoid chromatic dispersion effects during light propagation in the setup and the sample [16]. It has been shown that the illumination polarization states should preferably be left/right circular [18], in which case the fast temporal evolution of the electric field produced corresponds to a linear polarization state rotating at RF frequency. Due to the imposed orthogonality between the two components oscillating at distinct frequencies, the intensity of such beam has a constant value, even when measured with a fast PD.

When such a beam interacts with a sample, the intensity detected upon interaction with it remains constant, unless some polarization “orthogonality-breaking” takes place. In this configuration initially proposed in [16], such OB only occurs when light interacts with a dichroic sample (absorption anisotropy): the detected intensity shows a fast RF temporal modulation (beatnote), due to the interference of the two frequency components of the probe beam [18, 21]. Interestingly, we showed previously that the normalized amplitude of the beatnote (ratio of the beatnote amplitude
(AC) by the average intensity value (DC)), which we shall refer to in the following as orthogonality-breaking contrast (OBC) is a direct measure of the diattenuation rate of the dichroic sample [18, 21]. Moreover, the estimated phase of the detected beatnote is directly linked to the direction of the optical anisotropy (here, absorption anisotropy) which is responsible for the OB phenomenon [18, 21].

The main interest of such OB polarimetric modality is that the polarimetric information is retrieved from a single acquisition/scan of the image, hence ensuring easy and fast implementation on existing imaging setups such as microscopes. However, the polarimetric information is retrieved from the analysis of a RF-modulated optical signal, which requires fast PDs and dedicated demodulation electronics (lock-in detection or quadrature demodulation board) to measure the in-phase (I) and in-quadrature (Q) signal components. The OBC and phase informations can finally be retrieved using the following relations:

\[ OBC = \sqrt{I^2 + Q^2} \text{ DC}, \quad \text{and,} \quad \varphi = \arctan \frac{Q}{I}. \]  

(1)

The requirement of high-speed detection/demodulation hence hinders the use of wide-field cameras, and the technique so far has been implemented in a laser scanning imaging configuration, even for remote measurement applications [22]. For this reason, OB polarimetric sensing is well suited to be implemented on a confocal laser scanning microscope setup, as will be shown in the following.

Dichroism/birefringence polarimetric imaging with induced-OB modalities

The standard OB imaging modality described above has a strong specificity since OBC contrast can only appear if the sample shows absorption anisotropy (diattenuation). This can be interesting for a number of applications. However, in biology where the samples of interest are rather transparent, dichroism is not the most likely to occur among other polarimetric effects. To broaden the scope of application of this unconventional approach, it has recently been shown that it is possible to gain sensitivity on other polarimetric effects, such as pure depolarization and birefringence, by slightly modifying the detection setup [19].

These complementary modalities have been referred to as “induced” OB as the RF beatnote carrying the polarimetric information is generated on an analyzing element placed ahead of the detector, after light has interacted with the sample. Two interesting modalities have been identified. The first one, called linear-induced OB (LI-OB), consists in using a circular left/right DFDP illumination and a linear polarizer in front of the detector, and allows depolarization contrasts to be revealed on purely depolarizing samples [19]. The second modality, referred to as circular-induced OB (CI-OB) uses also a circular DFDP illumination, but requires a circular analyzer in front of the detector. This is commonly obtained by combining a quarter-wave-plate (QWP) and a linear polarizer with eigenaxes oriented at 45° from each other. This last modality has the strong potential to reveal interesting OBC contrast, not only on dichroic samples, but also on birefringent samples [19]. In the remainder of this article, these three OB modalities will be used and compared in terms of efficiency for polarimetric imaging of cell samples.

Microscopy setup description

In this section, we describe how a standard laser scanning microscope (Leica TCS-SP2 inverted microscope setup) has been modified in order to handle OB polarimetric imaging in transmission, while maintaining the ability to perform confocal fluorescence imaging. This constraint was necessary to be able to overlay OB and fluorescence images and thus identify the cellular structures giving rise to the observed polarimetric contrasts. A sketch of the whole setup is given in Fig. 1.a.

Dual-frequency dual-polarization illumination

To perform polarimetric cell imaging in the visible range, we use a polarization-sensitive Mach-Zehnder based free-space architecture comprising an acousto-optic shift of 80 MHz in one of the two arms (acousto-optic modulator, MT80-A1-VIS, AA OPTOELECTRONICS). This setup is represented on the 3-D sketch of Fig. 1.b allows us to obtain a linearly-polarized DFDP beam from a 40 mW commercial blue laser (PC14584, NEWPORT, λ = 488 nm). The DFDP source has previously been extensively described in [17].

In order to control the optical power deposited on the sample, a mechanically controlled optical valve has been designed using the association of a half-wave plate (HWP) (WPHSM05-488, THORLABS) and a Glan polarizer
FIG. 1. (a) Sketch of the OB polarimetric microscope setup implemented on a standard fluorescence scanning microscope (Pol: polarizer, QWP: quarter-wave plate at 488 nm, APD: avalanche photodiode, BT: bias-tee, DFS: dual-frequency source, DM: dichroic mirror, PH: pinhole, PM: photomultiplier). (b) 3D-sketch of the dual-frequency dual-polarization laser source at $\lambda=488$ nm designed for the experiment (HWP: half-wave plate, PBS: polarization beam splitter, AOM: acousto-optic modulator, GP: Glan polarizer). (c) Example of test images recorded from the fluorescence confocal microscope (Fluo), and from the polarimetric acquisition setup: the DC image provides the transillumination image, the I and Q channels are further processed to build the OBC and phase images displayed in next figures.

inserted between the laser output and the Mach-Zehnder-like setup. The orientation of the Glan polarizer is adjusted to ensure that the intensity of the two components of the DFDP beam share equal intensities. The direction of the HWP is controlled by a rotating mount (URS50 + SMC100 controller, NEWPORT), allowing remote tuning of the optical power from the LabView (NATIONAL INSTRUMENTS) program used to control the whole setup.

In order to convey the DFDP beam into the microscope scanning head, we use a single mode polarization-maintaining optical fiber (P3-488PM-FC-2, THORLABS) whose eigenaxes have been aligned with the polarization directions of the DFDP beam. A mechanical shutter controlled by the LabView software is placed ahead of the fiber injection and avoids illumination of the sample before an acquisition is launched, so as to reduce fluorescence bleaching.

Microscope setup

The DFDP beam conveyed by the fiber is coupled into the confocal microscope (TCS-SP2, LEICA) through the original infrared port using a dichroic mirror (ZT488rdc, CHROMA, cut-off wavelength 498 nm), as sketched in Fig. 1.a. This makes it possible to perform fluorescence and polarimetric imaging simultaneously, as the excitation laser lines available in the confocal head (514 nm and 635 nm) can be used to illuminate the sample and the fluorescence emitted in the backward direction can be detected through the confocal pinhole. The filter wheel of the inverted microscope stand has been equipped with a removable QWP at 488 nm (WPQSM05-488, THORLABS), allowing the linear DFDP beam to be converted into a circular left/right DFDP illumination at the sample plane. Finally, the light
is focused onto the sample by the microscope objective. The images displayed in the following have been recorded with one of the two following objectives: a 10× objective for test images and polarimetric measurements on synthetic samples (Fig. 2), whereas the images of cells have been obtained with an oil-immersion 63× objective.

Upon interaction with the sample, light is detected on the one hand in the classical confocal backscattering mode for the fluorescence emission, and the image reconstruction is handled by the TCS-SP2 system. On the other hand, the polarization information is measured in transmission by detecting the OB beatnote at 80 MHz on an avalanche photodiode (APD) (Silicium, 400 MHz bandwidth, APD430A, THORLABS), through a custom optical arrangement comprising the original microscope condenser lens (f′=28 mm), a switchable plane mirror, and an additional focusing lens (f′=17 mm) (See Fig. 1.b). This configuration enables full operation of the LEICA microscope in its original mode, but hinders the ability of true simultaneous imaging since a slight tuning of the microscope focus must be ensured when switching between confocal fluorescence imaging and polarimetric OB imaging.

Finally, in order to implement the three OB modalities described above, removable polarization analysis elements have been inserted after the sample, between the condenser lens and the last focusing lens. A linear polarizer allows LI-OB to be performed, while CI-OB requires an additional QWP to be inserted before the polarizer, with eigenaxes oriented at 45° from the polarizer direction.

**Polarimetric data acquisition and image reconstruction**

As described in the theoretical section, OB polarimetric imaging requires a specific detection/demodulation chain to recover the polarimetric information from the estimated amplitude and phase of a RF beatnote, at 80 MHz in the present case. For that purpose, a first homemade “bias-tee” electronic circuit separates the 80 MHz AC component of the detect photocurrent from its continuous-wave DC component. As sketched in Fig. 1.a, the latter is directly sampled and digitized on the analog-to-digital conversion (ADC) module of a input/output board (NI-USB 6356, NATIONAL INSTRUMENTS, 16 bits, 1.25 MS/s per channel).

As for the AC component, it can be demodulated either with a lock-in amplifier [17], or using a custom-made I/Q demodulation circuit at the dedicated 80 MHz frequency. Here, we use the I/Q demodulation approach which has been extensively described in previous work [22]. On our microscope setup, the RF reference (local oscillator) used to demodulated the AC photocurrent is given by the 80 MHz RF signal that drives the acousto-optic modulator, with appropriate amplification to match the nominal operating point of the RF mixers (+7 dBm) involved in the demodulation circuit. At the output of the I/Q demodulation circuit, the two quadratures (I and Q) are low-pass filtered (1 MHz cutoff frequency) and further amplified using two identical switchable gain voltage amplifiers (from ×3 to ×34) so as to optimally match the input range of the ADC board which eventually samples and digitizes the I and Q signals along with the DC component.

The three digitized signals are then processed with a LabView program on a computer to build the three raw polarimetric images (DC, I, Q), from which the OBC amplitude and phase maps are computed using the relations of Eq. (1), taking into account the relative gain factors and input ranges of the three channels. Data acquisition and reconstruction of the images are triggered by the “frame” and “line” trigger signals from the LEICA bench which are also digitized on the ADC board. In addition, to avoid photodamage or bleaching of the sample and to minimize aging of the AOM, a +5V signal is output from the I/O board only when the acquisition is started, in order to open the mechanical shutter of the DFDP source and to enable the RF high-voltage supply of the AOM.

Using a 200 Hz scanning speed on the LEICA system allows polarimetric images of 256×256 pixels to be recorded within 1.5 s. In order to cope with the acceleration/deceleration phases of the galvanometric mirrors and avoid spatial distortion in the obtained images, the simplest way consists in acquiring an image format of 512×256 pixels and remove by software the 128 first and last pixels from each line, ending up with distortion-free 256×256 pixels images. An example of raw DC, I and Q images recorded with a ×10 objective on a stage micrometer is displayed in Fig. 1.c, along with the corresponding fluorescence image.

For the sake of accuracy of the estimated OBC amplitude and phase maps, the I and Q images displayed in the following figures and used to compute the OBC amplitude and phase values have been corrected by software from slow phase drifts due to unwanted optical path changes in the DFDP source. For that purpose, we estimate the slow drift on the quadrature images by evaluating the local average values of the I and Q signals at four locations around the cell (typically, the four image corners), and by using a linear regression to calculate the slow linear trend of the I/Q signal across the image. This slow trend is finally removed to provide the I and Q images as those displayed in Fig. 3.c. Such post-processing steps could be avoided in future implementation of the system by removing the unwanted optical phase drifts in the system.
Sample cells preparation and fluorescence imaging

HeLa and U2OS cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL penicillin and 100 U/mL streptomycin and maintained at 37°C in a 5% CO₂ incubator. Cells were seeded on coverslips 24 h prior to fixation. For DRAQ5 staining (U2OS cells), media was removed and cells were washed once with phosphate buffered saline (PBS) for 3 min at room temperature. Cells were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature and washed twice with PBS. Cells were stained with DRAQ5 (0.5 mM in PBS) for 15 minutes and washed three times with PBS before coverslips were mounted on slides using ProLong® Gold (THERMO FISHER SCIENTIFIC). For microtubule staining (HeLa cells), media was removed and cells were washed once with PBS for 3 min, fixed with 4% PFA for 15 min and washed twice with PBS, all performed at room temperature. Cells were permeablised with 0.2% Triton X-100 in PBS for 5 min, washed twice with PBS and placed in blocking buffer (5% BSA, 0.05% Tween-20 in PBS) for 60 min at room temperature. Cells were then incubated with anti-tubulin antibody (0.2 µg/mL, sc #62204, INVITROGEN) in blocking buffer overnight at 4°C. Cells were washed three times with 0.1% Triton X-100 in PBS before incubation with Alexa Fluor 488 anti-mouse IgG (2 µg/mL, A11001, INVITROGEN) diluted in blocking buffer at room temperature for 1 h in the dark. Cells were washed twice with 0.1% Triton X-100 in PBS and counterstained with Hoechst (1 µg/mL in PBS) for 10 minutes. Cells were washed three times with PBS before coverslips were mounted on slides using ProLong® Gold (Thermo Fisher Scientific). Fluorescence excitation of Alexa Fluor 488 and DRAQ5 were excited using 514 nm and 633 nm lasers respectively. For fluorescence detection, we used bandpass filters adapted to the fluorophore emission spectra.

RESULTS AND DISCUSSION

Validation on synthetic samples

Before applying the OB polarimetric imaging to cell samples, we validated the method and the setup on synthetic test samples. These samples were selected for their known anisotropic optical behaviour, such as dichroism and birefringence, and the obtained images displayed in Fig. 2 are in good agreement with the theory presented above and in Ref. [19]. In this figure, the left column shows the transillumination image obtained with a ×10 objective from the measured DC signal of the APD, while the center and right column respectively display the OBC amplitude and phase maps obtained from Eq. (1). The phase value being irrelevant when the AC amplitude is very low (i.e., low OBC), the phase map has been thresholded and the irrelevant phase values have been represented in gray color.

The images in the first two rows have been obtained on a dichroic sample, namely, the cut edges of a polaroid sheet deposited on the stage micrometer surface, and exhibiting a rather shredded structure. The standard OB imaging modality has been used in Fig. 2.a, whereas images of Fig. 2.b have been obtained with the circular-induced modality (CI-OB). As expected, the in-focus parts of the dichroic sample show strong OB contrast with the two OB modalities. Theoretically, the estimated phase provides an indication about the relative orientation of the absorption anisotropies in the observed sample. The phase maps obtained here showing different values on the polarizer shreds are in fair agreement with the expected behaviour.

The second synthetic sample tested corresponds to a piece of plastic (birefringent) tape sticked on the stage micrometer. The piece of tape is clearly visible in the transillumination image of Figs. 2.c and 2.d. It has been imaged here with the standard OB (Fig. 2.c) and the CI-OB modalities (Fig. 2.d). Again, as expected based on the theory, such a birefringent sample does not break the polarimetric orthogonality, hence resulting in a very low OBC amplitude in standard OB. However, the birefringent nature of the sample clearly appears through the significant OBC amplitude obtained on the birefringent sample with the CI-OB modality. As for the phase maps, the constant value of the phase estimated on the piece of tape is in good agreement with the expected uniform orientation of the phase anisotropy of such sample.

These first imaging results on test synthetic samples thus validate the correct operation of the polarimetric OB microscope. It can be noted however here that the amplitude values of OBC (sometimes above 100%) lack quantitative precision. This would require added complexity of the system calibration and data processing [19, 23], but does not prevent qualitative evaluation of the imaging data gathered on biological samples and presented below.

OB polarimetric observations in cells

We imaged human osteosarcoma U2OS cells using the confocal fluorescence modality of the TCS-SP2 Leica system, and compared the fluorescence images with the transillumination (DC) and polarimetric images (OBC amplitude and
FIG. 2. Examples of OB polarimetric images recorded with a 10× objective on synthetic samples. Left column: DC-transillumination image; Center: OBC amplitude map; Right: OBC phase map. (a): standard OB modality on the edge of a dichroic sample (tears of polaroid sheets); (b): Circular-induced OB modality on a similar sample to (a); (c): standard OB modality on cuts of a birefringent sample (plastic tape); (d): Circular-induced OB modality on the same sample as in (c).

phase maps) acquired using the method and system described above. An interesting outcome of these experiments is the observation of an intrinsic polarimetric OB contrast in mitotic cells. An example of such acquisition is displayed in Fig. 3, where a U2OS cell, stained with DRAQ5 for DNA fluorescence labeling, has been imaged with the confocal fluorescence microscope (Fig. 3.b), and with the three OB modalities (Fig. 3.c). The spatial distribution of the chromosomes within this cell as seen on the fluorescence image suggests that it is at an early mitotic stage, most probably prometaphase. The analysis of the raw polarimetric images (I and Q) shows that a moderate OB contrast can be observed in standard OB imaging modality (first raw of Fig. 3.c). Such OB contrast seems to arise at compacted chromatin, as shown by the fact that the spatial distribution of the OBC signal (right column in Fig. 3.c) resembles the fluorescence image. The linear-induced OB modality (LI-OB) is inefficient to reveal an interesting contrast in this structure (middle row), as could be expected from such a thin and transparent sample for which light depolarization induced by multiple scattering events during light propagation is unlikely. In contrast, the circular-induced modality (CI-OB), which is able to reveal contrast on birefringent samples, displays the strongest I and Q quadrature signal contributions, and makes it possible to retrieve a clear OBC amplitude map (lower row, right) with high signal-to-noise ratio. For this reason, we shall restrict ourselves in the following to this CI-OB modality, which seems best adapted to the imaging of compacted chromatin in mitotic cells.
FIG. 3. Fluorescence and OB polarimetric images of a U2OS cell whose DNA has been labeled with DRAQ5. Images were obtained with a 63× oil-immersion objective. (a) Transillumination image; (b) Fluorescence image; (c) OB polarimetric images with I/Q channels and OBC amplitude maps for standard OB modality (upper row); linear-induced OB (middle row) and circular-induced OB (lower row).

Label-free polarimetric contrast of mitotic chromosomes

The clear CI-OB contrast obtained in a dividing U2OS cell such as presented in Fig. 3 is very encouraging towards the possibility to identify mitotic chromosomes from an endogenous polarimetric contrast, without the need for fluorescence labeling. It was however important to verify that such contrast was not specific to this cell line and not due to the DRAQ5 labeling of the DNA. Moreover, a more thorough analysis was required to confirm that the polarimetric contrast colocalizes with the mitotic chromosomes.

For this purpose, we performed some additional acquisitions in two types of cells: U2OS cells, described in the previous section, and HeLa cells, a widely used human cancer cell line derived from cervix. These cells were labeled either with DRAQ5, to highlight the DNA (U2OS cells), or with an anti-tubulin antibody, to display the microtubule network (HeLa cells). We imaged cells undergoing mitosis in both OBC and fluorescence modalities and the two images were overlapped to be able to assess the colocalization between the two signals. The results are displayed in Fig. 4. It can be readily observed in Fig. 4.g that the OBC amplitude map overlaps very well with the strong fluorescence signal emitted by the DRAQ5 dye labeling the chromatin. When imaging a dividing HeLa cell which microtubules were tagged by immunofluorescence, we observed in Fig. 4.g the overlay between the OBC amplitude (in green) and the fluorescence (in red) signals, a characteristic metaphase spindle composed of microtubules (seen in fluorescence) handling mitotics chromosomes (seen in the polarimetric channel). This last result demonstrates that...
FIG. 4. Comparison of transillumination (a-b), CI-OBC images (c-d) and fluorescence (e-f) acquired on U2OS cells labeled with DRAQ5 to highlight the DNA in fluorescence (upper row), and on HeLa cells with microtubules labeled by immunofluorescence (lower row). In each case, the superimposition of the fluorescence and OBC amplitude images (g-h) shows clear colocalization between the OBC contrast and the compacted chromatin in mitotic cells. The two insets (i-j) show two examples of phase maps extracted from the OB images revealing additional morphological contrasts on the compacted chromatin.

the polarimetric contrast observed for the mitotic chromosomes is not specific to a single cell type and is not due to DNA labeling by DRAQ5, hence paving the way to label-free imaging of mitotic chromosomes in dividing cells.

In addition to the OBC maps whose amplitude seems to be related to chromatin compaction, the OB polarimetric techniques provide additional information by analyzing the estimated phase of the OB signal at each pixel of the image [19]. Two examples of phase maps (estimated only on pixels showing significant OBC amplitude) are displayed for a U2OS cell (Fig. 4.i) and a HeLa cell (Fig. 4.j). Interestingly, these two phase images reveal additional morphological information in the compacted chromatin that can be observed neither in the OBC amplitude maps nor in the fluorescence images. Further investigation is required to possibly relate such observations with morphological/organizational structures in compacted chromosomes [24].

As a last experiment to confirm the interest of such endogenous polarimetric contrast in condensed chromatin, we provide in Fig. 5 a comparison of the transillumination (upper row), OBC amplitude (middle row) and fluorescence (lower row) images of U2OS cells at different cell stages. From left to right, the analysis of fluorescence images allows us to identify cells in interphase (a), late prophase (b), prometaphase (c), late prometaphase (d), metaphase (e), and finally late telophase (f). This figure shows that the polarimetric contrast seems to only arise at compacted chromatin in cells undergoing mitosis, while no clear OB contrast could be observed in interphase cells (Fig. 5.a). On a more quantitative basis, the magnitude of the OB polarimetric contrast seems to follow the chromatin compaction during the mitotic process, suggesting that OB imaging could be used to monitor the chromatin compaction state. This is confirmed by the graph displayed at the bottom of Fig. 5. We plotted the ratio of the OBC amplitude averaged over the regions of interest (ROIs) highlighted in blue, by its mean value in the surrounding background. The selected ROIs correspond to the regions where labeled DNA can be identified in the fluorescence images. With this definition of a “contrast” ratio, the minimum value of 1 corresponds to the absence of any contrast with respect to the background.
CONCLUSION

In this article, we have shown that polarized microscopy using “orthogonality breaking” approaches could provide valuable label-free information in biological samples. More specifically, it has been shown that an endogenous polarimetric “circular-induced” OB contrast could be clearly obtained at mitotic chromosomes during cell division, which has been confirmed by colocalization with fluorescence images recorded on the same samples. As the polarimetric modality used in this study is very fast, only requiring a single scan of the sample, this technique has the potential to allow real-time live cell imaging to monitor chromosome dynamics during mitosis. In this context, modifying the microscope setup to enable imaging in a reflection configuration would ensure strict simultaneous fluorescence and polarization live-cell imaging.

Applying this label-free technique to study the chromatin compaction state could open promising perspective for histology studies, such as the identification of abnormal chromatin compaction arising in some cancers cells [25, 26]. Further investigation is being conducted to assess the interest of this approach for imaging other cellular samples such as embryos [27, 28], or biological tissues. This next experimental work will also address the thorough and rigorous calibration of the setup in order to provide more reliable quantitative assessments of the estimated OBC amplitude and phase values, which can be of great interest to investigate the interesting morphological structures revealed by these two complementary contrasts.

AUTHORS CONTRIBUTIONS

M.T., M.A., S.H. and J.F. designed the research. M.A. and J.F. designed the laser source as well as the I/Q demodulation RF-setup. M.T., S.D., E.G., G.L.M. and J.F. designed and assembled the microscope setup. G.L.M., E.G., P.R. and J.F. designed the experiment control & data acquisition program. R.D., R.S., G.L.M. and S.H. prepared
the cell cultures and samples. R.D., R.S., G.L.M. and J.F. performed the experiments. J.F. and S.H. performed the data analysis and interpretation. J.F. and S.H. wrote the paper. All the authors revised the manuscript.

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